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Cell-Derived Nanovesicle Systems: AComparative Study of Loading Capacity, Encapsulation Efficiency, Stability and Applications

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Abstract—All eukaryotic cells release nanosized extracellular vesicles, or nEV, into the extracellular spaces. They have been found in various bodily fluids and are critical as intercellular communication mediators. nEV transport proteins, lipids, metabolites, and nucleic acids from donor cells to destination cells nearby or at a distance, altering the recipient cells' biological characteristics. Because of this specific characteristic, EVs are an excellent drug carrier for medicinal purposes. Compared to manufactured nanoparticles, natural encapsulation (nEV) has several benefits, such as biocompatibility, natural targeting capability, and long-term safety. Additionally, nEV can be swiftly loaded with the preferred medication, separated from a variety of biological sources, and altered to increase their effectiveness as specific drug delivery vehicles. Here, we review these facets of nEV and discuss the factors to consider when selecting the drug loading technique, nEV source, and surface modification tactics. We also discuss the obstacles that need to be removed for nEV-based drug delivery technologies to reach their full potential in clinical settings.

Keywords: Nanovesiclesystems, nEV, Cell-Derived, Encapsulation,

1. Introduction

Eukaryotic cells discharge lipid bilayer membrane particles known as extracellular vesicles (EVs) into the extracellular environment. Either membrane budding or the endocytic route can be the source of EV. When EVs develop through the endocytic pathway, they are referred to as "exosomes," whereas "microvesicles" are discharged through direct membrane budding. The normal sizes of exosomes and microvesicles are 30 to 100 nm and 100–1000 nm, respectively. Exosomes and microvesicles have a non-strict size distribution that varies based on the physiological state.

Similarly, there is also some latitude in surface marker protein expression. Consequently, unless there is concrete proof of their biogenesis, the International Society for Extracellular Vesicles (ISEV) advises against this terminology[1,2]. Most bodily fluids, including blood, saliva, and urine, have been found to contain EV. Even though EVs were first believed to be nothing more

than waste bags, cells expelled to eliminate undesirable biomaterial, they are now widely recognized as intercellular communicators with essential roles in cellular biology. EV operates in localized and distant locations, acting in paracrine and autocrine[3,4]. EVs' cargo, consisting of proteins, metabolites, lipids, and nucleic acids, gives them biological activity.

The donor cell type affects the EV's composition and physicochemical characteristics, which might shift under pathological situations and result in altered biological functions[1,5]. These characteristics and their widespread presence in bodily fluids have prompted much research into their clinical value in developing biomarkers. Furthermore, much attention has been paid to their potential application in medicine administration due to their inherent ability to carry bio information.It is still difficult to transport the medication to the target spot efficiently. Most therapeutic medications have poor efficacy because they lack quick systemic clearance and tailored distribution.

Consequently, patients experience unintended short- or long-term harm and cannot gain as much as they should. Synthetic delivery technologies, including carbon nanotubes, liposomes, and metal nanoparticles, have been created to overcome these restrictions, but their stability, biocompatibility, and long-term safety are still clinical issues. That's why there's much optimism for nanosized electric vehicles (nEVs) as a new medicine delivery mechanism. nEV has various attractive properties and can be isolated from multiple sources. Furthermore, because of the unique makeup of their membrane, they may selectively homing and have an extraordinary ability to interface with the destination cells. The following sections review many nEV-related topics and significant advancements in their creation as effective medicine delivery systems.

2. Sources of Extracellular Nanovesicles

While practically every kind of cell may create nEV, not all nEV produced by cells are suitable for use in human medicinal applications. When utilized as drug carriers, nEVs should be easily accessible and have an appropriate surface protein composition to avoid an adverse immune response. Human tumor cells, red blood cells, dendritic cells, mesenchymal stem cells, bovine milk, and plant juices or extracts have all been used to isolate nEV for pharmaceutical loading and delivery[1,6–8] (Figure 1). The following is a discussion of the benefits and drawbacks of these sources:

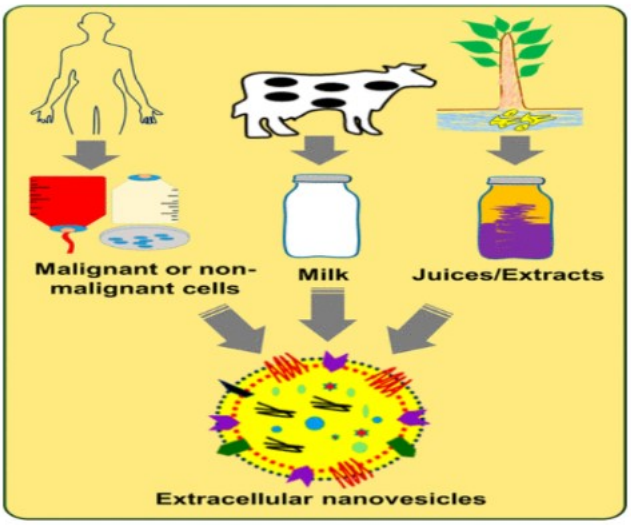

Figure 1: Extracellular nanovesicles can be isolated from human (malignant cells, nonmalignant cells, red blood cells, immature dendritic cells, mesenchymal stem cells, etc.), animal (bovine milk), or plant (extracts and juices) sources.

2.1. Tumor Cells

Because tumor-targeting surface proteins are present in the nEV produced by tumor cells, these cells are desirable for nEV isolation for drug delivery applications. It has been demonstrated that nEV generated from ovarian cancer cells and loaded with cisplatin limits the growth of the tumor in the ovarian cancer mouse model[9]. Their usage has been opposed because tumor-promoting cargo and surface protein composition of tumor cell-derived nEV have been demonstrated to amplify malignant behavior and induce immune suppression. Significantly, they can block T cell effector activity because tumor cell-derived nEVs include PD-L1 on their surface, which binds to PD-1 expressed on T cells.

2.2. Red Blood Cells

The "O" group of human red blood cells (RBCs) is being investigated as a potential source of nanoEV for medicinal applications. Large amounts of RBCs are available from the blood bank. Moreover, RBCs can be extracted from the same patient to prevent any unintended immunogenic reaction brought on by cell contamination. RBCs may be treated with calcium ionophore, phorbol-12-myristate-13-acetate, or lysophosphatidic acid to increase EV secretion. Adding ascorbic acid to murine RBCs in storage has been shown to improve post-transfusion recovery and minimize nEV generation and release[10].

2.3. Dendritic Cells

Dendritic cells (DCs) are the immune system's most efficient antigen-presenters. The creation of immature dendritic cells (imDCs)-derived neural epithelial cells (nEV; imDC-nEV) from healthy donors' peripheral blood mononuclear cells (PBMCs) has been studied for medicine delivery. ImDC-nEV has low surface markers such as MHC-I, MHC-II, CD86, and CD40, which may reduce the immune response and protect it from systemic clearance. It has been postulated that nEVs produced by imDCs (large vs. little) may play different roles in the T helper cell response. Smaller nEVs secrete Th1 cytokines, suggesting that their cargo contents and MHC distribution on the nEV surface vary from larger nEVs that secrete Th2 cytokines[11].

2.4. Mesenchymal Stem Cells

Since mesenchymal stem cells (MSCs) may be effectively extracted from various tissues, including bone marrow, adipose tissues, and umbilical cords, they have become an increasingly popular option. MSCs are a plentiful source of nEV because they can also grow in vitro. Since MSC-derived nEVs are nonimmunogenic, they have no negative effects[7]. Conversely, MSC-derived nEV has been demonstrated to promote recipient cell proliferation in specific situations[12]. There are also worries that MSC-nEV may encourage the vascularization of tumors. On the other hand, some research claims that nEV generated from MSCs has antitumorigenic properties. Since MSC-derived nEVs are still being investigated as drug delivery systems, it is evident that further research is required to comprehend these distinct behaviors fully.

2.5. Milk

One abundant, easily accessible, and reasonably priced source of nEV that has gained popularity is bovine milk. Because milk-derived nEVis cross-species tolerant and resistant to digestive fluids, they make oral delivery medication carriers appealing. These nEV load medicines that are hydrophilic or hydrophobic with equal efficiency. According to a study on mice, nEV from bovine milk that had been fluorescently labeled remained stable in the bloodstream for up to six days[8].Further thorough research is necessary to rule out any possible toxicity or prion contamination while using milk nEV as a medicinal vehicle.

2.6. Plant

A significant amount of nEV can be obtained from edible plant extracts, and nEV derived from plants is safe to use in oral formulations. They can act as natural therapeutic agents in various clinical diseases and are stable over a wide pH range. It's interesting to note that multiplenEV produced from plants are selective for particular recipient cells. For example, grape-derived nEVs preferentially enter into intestinal stem cells, preventing mice from developing colitis caused by dextran sulfate sodium[13].

3. Methods for Drug Encapsulation

There are two methods for encapsulating the medication in the nEV: passive and active. Natural processes are used in passive drug loading. In contrast, external physical (sonication, extrusion, freeze-thaw cycle, and electroporation) or chemical (saponin application and click chemistry) approaches are utilized in active loading. Active cargo loading is far more successful than passive loading because it briefly breaks or extends the nEV membrane, allowing the medication to enter[14].

3.1. Passive Loading

Passive loading is achieved by incubating the drug with either the donor cell or the nEV (Figure 2A). On the one hand, the hydrophobic interaction between the drug and the lipid layer of nEV accelerates the slow process of drug direct diffusion into nEV. In contrast, during incubation, the donor cell may actively absorb the drug via endogenous cellular mechanisms before being packed into nEV and released into the culture media. A plasmid overexpressing the therapeutic component may be introduced into donor cells, allowing therapeutic miRNA or peptide to be packed into micro EVs. After expressing the drug, donor cells wrap it in nEVs and release it into the culture medium (Figure 2B). Depending on the kind of donor cell, drug-loaded nEVs absorb, package, and release differently. It was shown that SR4987 mesenchymal stromal cells treated with low paclitaxel (PTX) generated PTX-loaded nEV with antiproliferative properties against pancreatic cancer. Passive loading has been employed for nEV-based targeted delivery of oncolytic viruses (OVs) other than chemotherapeutics. OVs were encapsulated by nEVs derived from ligand-expressing liver cancer cells, which were subsequently efficiently delivered to the tumor site and protected against systemic clearance. However, the downside of these procedures is that they are time-consuming. Furthermore, endogenous drug ejection mechanisms and hydrophobicity may inhibit drug uptake and release by donor cells[6].

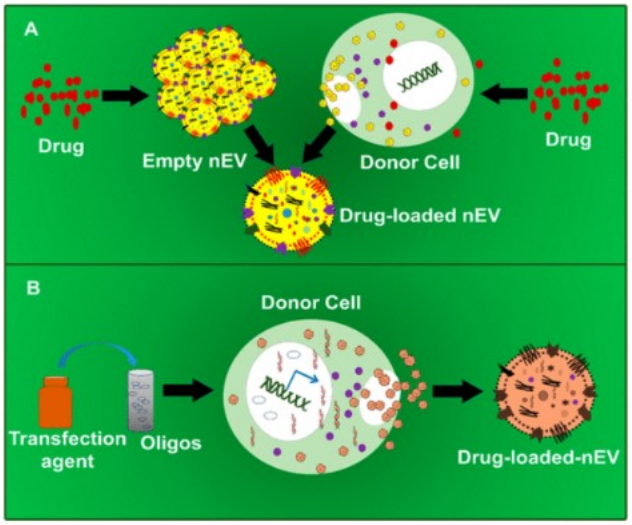

Figure 2: Passive loading of the drug into nEV. (A) The drug and nEV mixture is kept at room temperature to allow drug loading through the diffusion process. Alternatively, donor cells are incubated with the drug at 37 °C, where they take up the medicine, package it into nEV, and release them in the culture medium. (B) For therapeutic miRNA or peptide packaging into nEV, donor cells can be transfected with a plasmid overexpressing the therapeutic entity. The donor cells then express the therapeutic, package it into nEV, and release it into the culture medium.

3.2. Active Loading

Active medication loading into nEVs is accomplished by providing an external physical push or completing a chemical reaction (Figure 3). Several techniques have been investigated for active drug loading, as detailed below.

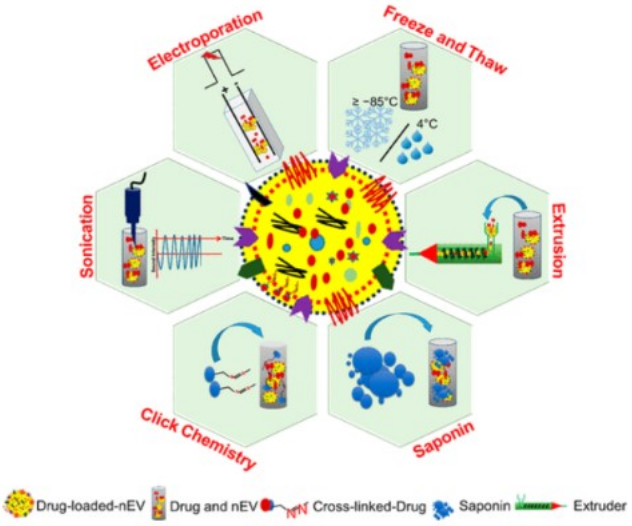

Figure 3: Active loading of the drug. Drug molecules can be actively loaded inside nEV by applying an external physical force or through a chemical reaction. In the sonication process, sound waves are used to transiently disrupt or permeabilize the membrane of nEV to allow drug entry via diffusion. Similarly, electroporation permeabilizes the nEV membrane by applying electric pulses. Subjecting the nEV to repeated freezing and thawing cycles also creates membrane pores, allowing the drug entry. In the extrusion process, the drug and nEV mixture are passed through tiny pores under high pressure to facilitate the drug's forced diffusion. Saponin treatment also creates membrane pores and allows drug loading into nEV. In the "click chemistry" reaction, drugs are anchored to the external membrane surfaces of the nEV.

3.2.1. Sonication

The technique of stirring up particles in a solution using sound waves, typically those with ultrasonic frequencies (>20 kHz), is known as "sonication." Light sonication may momentarily disrupt the membrane, allowing substances such as pharmaceuticals to be released and absorbed

from the suspension medium. Different cycle frequencies and sonication periods are required for optimal loading depending on the kind of medication being loaded. The maximum loading of honokiol into nEV was recently reported after six cycles of sonication with a 30-second pulse and 30-second break, and it was accomplished without compromising integrity, desirable polydispersity index, or size. Another research found a similar result using sonication to load PTX into nEV. Sonication may lead medicine to accidentally stick to the exterior of nEV in addition to internal encapsulation. Following the fast release of the medication connected to the outside, the medicine stored inside the nEV progressively releases[7,15].

3.2.2. Extrusion

A lipid syringe extruder with 100–400 nm porous membranes is filled with medicament and nEV and pushed through with force. The procedure is carried out at a regulated temperature. The medicine can be taken up because of a brief disruption of the exosome membrane during the procedure. On the other hand, strong mechanical force can permanently damage the nEV membrane and reduce the effectiveness of drug loading. There's a chance that unfavorable changes will also occur to other membrane characteristics. One study that used nEV generated from cultivated breast cancer cells revealed that thorough extrusion has an unfavorable effect on the nEV's integrity and zeta potential[14]. However, without sacrificing integrity, a gentler extrusion of nEV generated from RAW264.7 macrophages led to the best catalase loading. More research is necessary because it may be argued that the sources of nEV and loading entities differed between the two trials.

3.2.3. Freeze and Thaw Cycles

This straightforward procedure does not need complex equipment or much technical knowledge. Medication and EV are briefly incubated at ambient temperature before frozen in liquid nitrogen or at -80°C. To ensure proper loading, the liquid is frozen at room temperature again, and the process is repeated. The drug-nEV ratio, incubation time, and number of freeze-thaw cycles must all be modified for different drug-nEV combinations. To create exosome-mimetic nanoparticles, phospholipid-based liposomes and nEV membranes were bonded using the freeze-thaw process. Exosome-mimetic particle production is more scalable and less labor-intensive in preclinical and clinical contexts. Additionally, the fusion process is more straightforward to control. On the negative side, repeated freezing and thawing cycles might promote nEV aggregation and increase the polydispersity index of the drug-loaded nEV with a broad size distribution, reducing drug absorption efficiency. Furthermore, the extrusion or sonication techniques are believed to have a better drug-loading yield than the freeze-thaw approach[16].

3.2.4. Electroporation

Electroporation has historically transported drugs, DNA, and RNA into cells. Still, it has recently been used to insert siRNA and miRNA into nanoparticles. SiRNA and miRNA are charged and larger than several hydrophobic drugs, like PTX; therefore, they cannot be diffused passively

inside the nEV. The lipid bilayer membrane of the nEV spontaneously holes due to the electric current generated during the electroporation procedure, enabling the medicine or siRNA/miRNA to travel inside. Following loading, the EV membrane's integrity is quickly recovered. The electroporation method's millisecond exposure periods cause a small temperature increase (1 °C each pulse), minimizing thermal damage to the nEV. According to studies, during electroporation, the size of the nEV increases in direct proportion to the applied electric fields. This size growth may be regulated using an appropriate buffer to ensure nEV integrity while preventing aggregation[17].

3.2.5. Saponin Treatment

A surfactant molecule called saponin combines with membrane cholesterol to produce complexes. As a result, it creates pores in the EV membrane during incubation with nEV, increasing permeability. Comparing saponin treatment to other methods like electroporation and incubation, it is demonstrated to be a feasible strategy for drug encapsulation into the nEV. High loading efficiency is achieved using the saponin approach, and the size of the nEV is not significantly affected. It reached a substantially greater drug loading of hydrophilic porphyrins than passive approaches. Nonetheless, this technique has specific issues, such as the worry about saponin-induced hole creation in the recipient cell membrane and in vivo hemolytic activity. Consequently, employing a low saponin concentration and eliminating them from the solution is crucial before delivering the nEV[14].

3.2.6. Click Chemistry

The molecules are directly covalently bonded to the surface of nEV via the copper-catalyzed azide-alkyne cycloaddition (click chemistry) procedure. The click chemistry technique is compatible with aqueous buffers, has quicker reaction times, and gives you greater control over the conjugation site. (17) nEV are cross-linked with alkyne groups using carbodiimide-catalyzed coupling. The surface of 4T1 cells that produced nEV was efficiently covered with azide-fluor 545, a fluorescent molecule, by crosslinking with the carboxyl group of 4-pentanoic acid. This cross-linking was facilitated by amine groups on the nEV membrane. Crucially, the combination of azide-fluor 545 and nEVdid not affect the growth or absorption of recipient cells. Furthermore, copper-free click chemistry was employed to attach the azide group-containing nEV to the targeting peptide, improving their distribution to cancer cells[15].

4. Modification of Extracellular Nanovesicles for Targeted Delivery

Intravenous injection in mice demonstrated that nEV, like other medications and drug carriers, is subject to systemic clearance via the liver and spleen. Immune cells such as macrophages and the complement system, an essential component of the innate immune system, are also involved in removing circulating nEV. The destiny of nanoEVs in circulation is determined by their surface makeup, which may include particular antigen proteins. Changes to the nEV's surface are required to overcome these limitations. Furthermore, surface modification may reduce

medication-induced undesired off-target systemic side effects while improving focused drug delivery for the optimum therapeutic result. Several surface modification procedures have been investigated, as described below[18].

4.1. Manipulation in the nEV Donor Cells

Manipulation of the donor cell is an indirect method for changing the surface of the released nanoEV. Tian et al. created mouse imDCs to express Lamp2b, a lysosome-associated membrane glycoprotein coupled to an iRGD peptide unique to αv integrin. This peptide was found on the surface of the modified imDCs' EVs. Later, doxorubicin (DOX) was loaded onto these nEVs and administered intravenously to mice. nEV delivered DOX to αv integrin-positive breast cancer cells and suppressed their growth without causing obvious injury. (18) In another study, the transmembrane domain of the platelet-derived growth factor receptor (PDGF-R) coupled to the high-affinity EGFR ligand GE11 peptide was engineered to express in HEK293 cells. (19) This peptide was found on the surface of released nanoEVs, with a higher affinity for breast cancer cells expressing EGFR. Furthermore, EGFR-expressing breast tumor xenografts were targeted for i.v. administration of these nEV in RAG2-/- mice, which also contained let-7a miRNA cargo. A significant disadvantage of this donor cell manipulation technique is the difficulty in achieving optimum and consistent transfection efficiency, which is dependent on the kind of donor cell, the transfection agent, and the plasmid vector size[19].

4.2. Direct Surface Modification of Extracellular Nanovesicles

4.2.1. Pegylation

The most popular method for extending the duration of a nanomaterial's blood circulation and boosting its bioavailability is pegylation or surface modification of nanomaterials using poly(ethylene glycol) (PEG). Pegylation prevents nEV from being cleared from circulation by creating a hydration layer on its surface, making it harder for nEV to be recognized as a foreign substance. In one study, PEG-coated nEV generated from mouse neuroblastoma cells were injected intravenously into animals[20]. NonpegylatednEV were quickly removed within 10 minutes. However, PEG-coated nEV was detectable in circulation for more than 60 minutes. (20) In a different study, nEV generated from RAW264.7 macrophages were fused with pegylated liposomes to form a PEG coating, which raised the nEV's bioavailability[21].

4.2.2. pH-Responsive Modification

By adding pH-sensitive functional groups to the nEV surface, the acidic tumor microenvironment (TME) surrounding cancer cells canselectively deliver drugs to specific tumor locations. The benefits of pH-responsive nEV produced by coating mouse macrophage RAW 264.7 macrophage-derived nEV with 3-(diethylamino)propylamine (HDEA) were illustrated by Lee et al. Moreover, the anticancer medication DOX was loaded onto HDEA-conjugated nEV. The HCT-116 tumor cells' CD44 receptors were actively bound by these surface-modified nEV, effectively responding to low pH and stopping tumor cells' development in vivo and in vitro[22].

In further work, a pH-labile imine bond disintegrated in an acidic environment was used to conjugate DOX with nEVs produced from bovine milk. The modified nEV decreased the formation of squamous cell carcinomas in mice and demonstrated regulated release of DOX in vitro under acidic conditions[23].

4.2.3. Glycan Modification

It has been proposed that charge-based interaction, pattern recognition, or both aid in nEV targeting and absorption in endogenous surface molecules such as glycans. Royo et al. used neuraminidase to remove sialic acid from mouse lung cell-derived nEV surfaces. After being supplied intravenously to mice, sialic acid elimination reduced the negative charge of nEV, increasing their interaction with lung cells and resulting in higher accumulation in the lungs[24]. In another study, α-d-mannose was used to modify the surface of bovine serum-derived nEVs. Following intradermal injection, mice's lymph nodes contained more mannose-modified nEV. These results support the study of glycan modification on the nEV surface to deliver targeted medications[25].

5. Clinical Trials

Many preclinical experiments have shown remarkable results, prompting the start of various clinical trials to examine the viability, security, and effectiveness of nEV as a medicine delivery method. Clinical experiments are being conducted on EVs from plants, milk, and human sources (www.ClinicalTrials.gov). Pancreatic ductal adenocarcinoma (PDAC) frequently has KRAS mutation, which is linked to worse patient survival and ongoing tumor growth. The MD Anderson Cancer Center is financing a phase I clinical trial investigation (NCT03608631) to determine the efficacy of nEV generated from MSCs loaded with minor interference RNA (siRNA) against KrasG12D in patients with metastatic PDAC who have this mutation. A previous study on the efficiency of nEV derived from the mouse hepatocarcinoma tumor cell line H22 for methotrexate (MTX) administration in a murine hepatocarcinoma ascites model revealed considerable tumor growth reduction without the usual adverse effects. (8) These findings paved the way for an ongoing clinical study (NCT02657460) to assess the efficacy of nEV loaded with MTX derived from autologous tumor cells for treating malignant pleural effusion in patients with advanced lung cancer. Curcumin's hydrophobicity limits its practical use as a treatment despite indications that it slows the growth of colon cancer. A phase I clinical experiment (NCT01294072) evaluates plant-derived nEV's ability to deliver curcumin to colon cancer cells. It has been proven that nEVs derived from grapes initiate tissue remodeling when pathological damage occurs. (12) In light of these findings, a phase I clinical study (NCT01668849) is underway to investigate the potential of grape-derived nEV in reducing oral mucositis associated with head and neck cancer treatment with chemotherapy and radiation.

6. Conclusion and Future Perspective

Nature-derived nEV are gaining popularity as next-generation medication delivery systems for treating various disorders because of their excellent biocompatibility (Figure 4). Preclinical models and, more recently, clinical studies are being utilized to assess the efficacy of formulations that include nEV obtained from diverse biological sources. However, there are certain limitations to employing nEV as well. For example, collecting exosomes in sufficient quantities from an autologous source is typically tricky. Furthermore, due to their inherent bioactivity, autologous nEV—such as those derived from the patient's tumor cells—may promote immunosuppression and chemoresistance.

Furthermore, since autologous nEVs are not readily accessible, clinical studies cannot determine their safety and usefulness. The quality and purity of the nEV may also influence how well the drug is put into it. Find a trustworthy source of nEV and develop an isolation technology capable of producing pure, undamaged nEV on a large scale. Future research must createmany highly loading-capable, well-characterized nEV from reliable allogeneic sources. Developing techniques to increase nEV homing abilities and ensure the planned intracellular destination of the encapsulated drug should also be the primary goal of the work. In the following years, there should be significant progress in EV and its applications in illness detection and therapy. Consistent advancement in biology, chemistry, and nanotechnology will help us overcome the current obstacles. More specific disease models that might provide preclinical data on the efficacy and safety of nEV medicine delivery devices are being developed. Top-line findings from recent clinical studies using nEV as a drug carrier in cancer patients are expected. These attractive drug delivery vehicles may soon be deployed in clinical settings, thanks to more rigorous in vivo research and continuous advances in nEV separation, drug loading, and engineering.

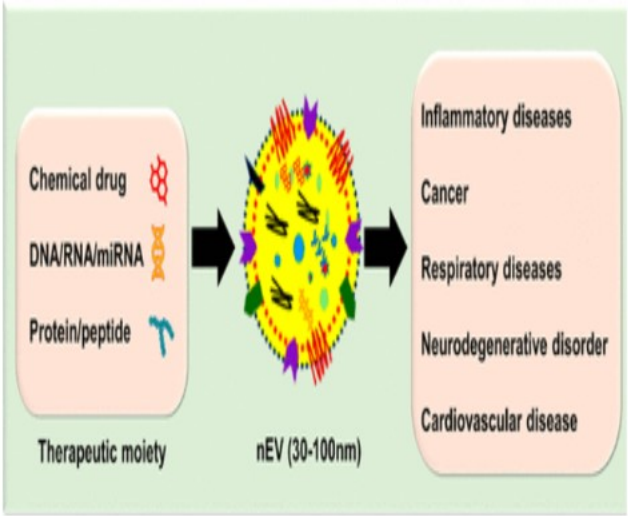

Figure 4: Nanosized extracellular vesicles are being evaluated as carriers of chemical drugs and therapeutic RNA, miRNA, proteins, and peptides for the treatment of various ailments, including inflammatory diseases, neurodegenerative disorders, and cancer

References

[1] Patel, G.K.; Khan, M.A.; Zubair, H.; Srivastava, S.K.; Khushman, M.; Singh, S.; Singh, A.P. Comparative Analysis of Exosome Isolation Methods Using Culture Supernatant for Optimum Yield, Purity and Downstream Applications. *Sci Rep***2019**, *9*, 1–10, doi:10.1038/s41598-019-41800-2.

[2] Zaid Alkilani, A.; Musleh, B.; Hamed, R.; Swellmeen, L.; Basheer, H.A. Preparation and Characterization of Patch Loaded with Clarithromycin Nanovesicles for Transdermal Drug Delivery. *J FunctBiomater***2023**, *14*, 57, doi:10.3390/jfb14020057.

[3] Patel, G.K.; Patton, M.C. Pancreatic Cancer Exosomes: Shedding Off for a Meaningful Journey. *PancreatDisordTher***2016**, *06*, e148, doi:10.4172/2165-7092.1000e148.

[4] Patel, G.K.; Khan, M.A.; Bhardwaj, A.; Srivastava, S.K.; Zubair, H.; Patton, M.C.; Singh, S.; Khushman, M.; Singh, A.P. Exosomes Confer Chemoresistance to Pancreatic Cancer Cells by Promoting ROS Detoxification and MiR-155-Mediated Suppression of Key Gemcitabine-Metabolising Enzyme, DCK. *Br J Cancer***2017**, *116*, 609–619, doi:10.1038/bjc.2017.18.

[5] Patton, M.C.; Zubair, H.; Khan, M.A.; Singh, S.; Singh, A.P. Hypoxia Alters the Release and Size Distribution of Extracellular Vesicles in Pancreatic Cancer Cells to Support Their Adaptive Survival. *J Cell Biochem***2020**, *121*, 828–839, doi:10.1002/jcb.29328.

[6] Kanchanapally, R.; Deshmukh, S.K.; Chavva, S.R.; Tyagi, N.; Srivastava, S.K.; Patel, G.K.; Singh, A.P.; Singh, S. Drug-Loaded Exosomal Preparations from Different Cell Types Exhibit Distinctive Loading Capability, Yield, and Antitumor Efficacies: A Comparative Analysis. *Int J Nanomedicine***2019**, *14*, 531–541, doi:10.2147/IJN.S191313.

[7] Kanchanapally, R.; Khan, M.A.; Deshmukh, S.K.; Srivastava, S.K.; Khushman, M.; Singh, S.; Singh, A.P. Exosomal Formulation Escalates Cellular Uptake of Honokiol Leading to the Enhancement of Its Antitumor Efficacy. *ACS Omega***2020**, *5*, 23299–23307, doi:10.1021/acsomega.0c03136.

[8] Munagala, R.; Aqil, F.; Jeyabalan, J.; Gupta, R.C. Bovine Milk-Derived Exosomes for Drug Delivery. *Cancer Lett***2016**, *371*, 48–61, doi:10.1016/j.canlet.2015.10.020.

[9] Tang, K.; Zhang, Y.; Zhang, H.; Xu, P.; Liu, J.; Ma, J.; Lv, M.; Li, D.; Katirai, F.; Shen, G.X.; et al. Delivery of Chemotherapeutic Drugs in Tumour Cell-Derived Microparticles. *Nat Commun***2012**, *3*, doi:10.1038/ncomms2282.

[10] Stowell, S.R.; Smith, N.H.; Zimring, J.C.; Fu, X.; Palmer, A.F.; Fontes, J.; Banerjee, U.; Yazer, M.H. Addition of Ascorbic Acid Solution to Stored Murine Red Blood Cells Increases Posttransfusion Recovery and Decreases Microparticles and Alloimmunization. *Transfusion (Paris)***2013**, *53*, 2248–2257, doi:10.1111/trf.12106.

[11] Tkach, M.; Kowal, J.; Zucchetti, A.E.; Enserink, L.; Jouve, M.; Lankar, D.; Saitakis, M.; Martin-Jaular, L.; Théry, C. Qualitative Differences in T-Cell Activation by Dendritic Cell-Derived Extracellular Vesicle Subtypes. *EMBO J***2017**, *36*, 3012–3028, doi:10.15252/EMBJ.201696003.

[12] Bruno, S.; Tapparo, M.; Collino, F.; Chiabotto, G.; Deregibus, M.C.; SoaresLindoso, R.; Neri, F.; Kholia, S.; Giunti, S.; Wen, S.; et al. Renal Regenerative Potential of Different Extracellular Vesicle Populations Derived from Bone Marrow Mesenchymal Stromal Cells. *Tissue Eng Part A***2017**, *23*, 1262–1273, doi:10.1089/TEN.TEA.2017.0069.

[13] Ju, S.; Mu, J.; Dokland, T.; Zhuang, X.; Wang, Q.; Jiang, H.; Xiang, X.; Deng, Z. Bin; Wang, B.; Zhang, L.; et al. Grape Exosome-like Nanoparticles Induce Intestinal Stem Cells and Protect Mice from DSS-Induced Colitis. *Mol Ther***2013**, *21*, 1345–1357, doi:10.1038/MT.2013.64.

[14] Fuhrmann, G.; Serio, A.; Mazo, M.; Nair, R.; Stevens, M.M. Active Loading into Extracellular Vesicles Significantly Improves the Cellular Uptake and Photodynamic Effect of Porphyrins. *J Control Release***2015**, *205*, 35–44, doi:10.1016/J.JCONREL.2014.11.029.

[15] Kim, M.S.; Haney, M.J.; Zhao, Y.; Mahajan, V.; Deygen, I.; Klyachko, N.L.; Inskoe, E.; Piroyan, A.; Sokolsky, M.; Okolie, O.; et al. Development of Exosome-Encapsulated Paclitaxel to Overcome MDR in Cancer Cells. *Nanomedicine***2016**, *12*, 655–664, doi:10.1016/J.NANO.2015.10.012.

[16] Bosch, S.; De Beaurepaire, L.; Allard, M.; Mosser, M.; Heichette, C.; Chrétien, D.; Jegou, D.; Bach, J.M. Trehalose Prevents Aggregation of Exosomes and Cryodamage. *Sci Rep***2016**, *6*, doi:10.1038/SREP36162.

[17] Johnsen, K.B.; Gudbergsson, J.M.; Skov, M.N.; Christiansen, G.; Gurevich, L.; Moos, T.; Duroux, M. Evaluation of Electroporation-Induced Adverse Effects on Adipose-Derived Stem Cell Exosomes. *Cytotechnology***2016**, *68*, 2125–2138, doi:10.1007/S10616-016-9952-7.

[18] Lewis, S.M.; Williams, A.; Eisenbarth, S.C. Structure-Function of the Immune System in the Spleen. *Sci Immunol***2019**, *4*, doi:10.1126/SCIIMMUNOL.AAU6085.

[19] Ohno, S.I.; Takanashi, M.; Sudo, K.; Ueda, S.; Ishikawa, A.; Matsuyama, N.; Fujita, K.; Mizutani, T.; Ohgi, T.; Ochiya, T.; et al. Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells. *Mol Ther***2013**, *21*, 185–191, doi:10.1038/MT.2012.180.

[20] Kooijmans, S.A.A.; Fliervoet, L.A.L.; Van Der Meel, R.; Fens, M.H.A.M.; Heijnen, H.F.G.; Van Bergen EnHenegouwen, P.M.P.; Vader, P.; Schiffelers, R.M. PEGylated and Targeted Extracellular Vesicles Display Enhanced Cell Specificity and Circulation Time. *J Control Release***2016**, *224*, 77–85, doi:10.1016/J.JCONREL.2016.01.009.

[21] Sato, Y.T.; Umezaki, K.; Sawada, S.; Mukai, S.A.; Sasaki, Y.; Harada, N.; Shiku, H.; Akiyoshi, K. Engineering Hybrid Exosomes by Membrane Fusion with Liposomes. *Scientific Reports 2016 6:1***2016**, *6*, 1–11, doi:10.1038/srep21933.

[22] Lee, H.; Park, H.; Noh, G.J.; Lee, E.S. PH-Responsive Hyaluronate-Anchored Extracellular Vesicles to Promote Tumor-Targeted Drug Delivery. *CarbohydrPolym***2018**, *202*, 323–333, doi:10.1016/J.CARBPOL.2018.08.141.

[23] Zhang, Q.; Xiao, Q.; Yin, H.; Xia, C.; Pu, Y.; He, Z.; Hu, Q.; Wang, J.; Wang, Y. Milk-Exosome Based PH/Light Sensitive Drug System to Enhance Anticancer Activity against Oral Squamous Cell Carcinoma. *RSC Adv***2020**, *10*, 28314–28323, doi:10.1039/D0RA05630H.

[24] Royo, F.; Cossío, U.; Ruiz De Angulo, A.; Llop, J.; Falcon-Perez, J.M. Modification of the Glycosylation of Extracellular Vesicles Alters Their Biodistribution in Mice. *Nanoscale***2019**, *11*, 1531–1537, doi:10.1039/C8NR03900C.

[25] Choi, E.S.; Song, J.; Kang, Y.Y.; Mok, H. Mannose-Modified Serum Exosomes for the Elevated Uptake to Murine Dendritic Cells and Lymphatic Accumulation. *MacromolBiosci***2019**, *19*, doi:10.1002/MABI.201900042.