Development of Hydrogel Encapsulated with plant- derived exosome- like- nanoparticles for Wound Healing for Type-2 Diabetes

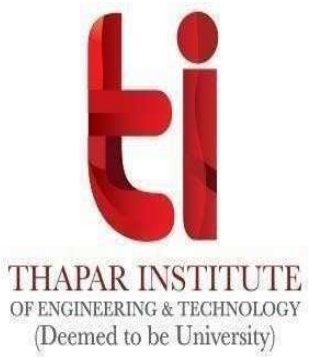
A Thesis

Submitted in partial fulfilment of the requirements for the award of the degree of

Master of Science

in

Biotechnology



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This is to certify that Ms. Arohi has undergone six months research training at NABI from 15.01.2025 to 14.06.2025 (without stipend) under the supervision of Dr Nitin K. Singhal, Scientist –E, NABI.

Her work has been found satisfactory and we wish her best of luck for her future endeavours.

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This is to certify that the thesis entitled **Development of Hydrogel Encapsulated with Hybrid Exosomes for Wound Healing for Type 2 Diabetes** being submitted by **Arohi** (Reg. No. 302301030), in partial fulfilment of the requirements for the award of the degree of **Master of Science** in **Biotechnology**, T**hapar Institute of Engineering and Technology, Patiala, Punjab** is a bonafide work carried out under the guidance and conception of **Dr. Nitin K. Singhal (NABI)** and based on the declaration by the candidate no part of this thesis has been submitted for the award of any other degree. The thesis is being submitted under the supervision of Dr. Ovais S. Qadri

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Candidate's Declaration

I hereby certify that the work, which is being presented in the thesis, entitled, **Development of hydrogel Encapsulated with Hybrid Exosomes for Wound Healing for Type 2 Diabetes** in partial fulfillment of the requirements for the award of the degree of **Master of Science** and submitted to the institution is an authentic record of my own work carried out during the period **January 2025** to **June 2025** under the giodance **of Dr. Nitin K. Singhal, Scientist E, National Agri-Food Biotechnology Institute**. I have also cited the references of the text(s)/figure(s)/table(s) from where they have been taken.

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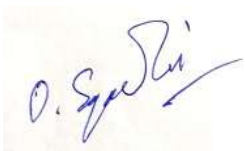

Dr Ovais Shafiq Qadri

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I regard this opportunity as a significant turning point in my professional progress. I will work diligently to use the knowledge and abilities I have acquired to achieve my career objective.

Arohi

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Abbreviations

EV-Extracellular vesicles
MVB-Multivesicular body
ADEV-Animal-derived Extracellular vesicles
PDEV-Plant-derived Extracellular vesicles
PMSF-Phenylmethylsulfonyl fluoride
PBS-Phosphate-buffered saline
TEM-Transmission electron microscopy
SEM -Scanning electron microscopy
BCA-Bicinchoninic acid assay
DLS-Dynamic light scattering
SDS PAGE - Sodium dodecyl sulfate-polyacrylamide Gel electrophoresis
ATM-Atomic force microscopy
T2D-Type 2 diabetes
IPA -Isopropanol
qRT-PCR -Real-Time Quantitative Reverse Transcription PCR
PCR -Polymerase chain reaction
MQ- milli-q
NaCl – Sodium Chloride
KCl – Potassium chloride
QVID- Quartz curvette
Na_2HPO_4 - Sodium Phosphate Dibasic

Abstract

The prevalence of type 2 diabetes mellitus (T2DM) is a growing global health concern, often accompanied by complications such as impaired wound healing due to vascular damage and chronic inflammation. This thesis presents the development and evaluation of innovative hydrogel patches encapsulated with hybrid exosomes derived from ginger and garlic, aimed at accelerating wound healing in T2DM patients. Ginger and garlic are renowned for their potent anti-inflammatory and antioxidant properties, which are harnessed in this study to address the delayed wound healing associated with diabetes.

The hydrogel patches were synthesized using a biocompatible polymer matrix, ensuring optimal delivery and sustained release of the encapsulated exosomes at the wound site. The hybrid exosomes were characterized for their size, morphology, and bioactive compound content, confirming their potential to modulate inflammatory responses and oxidative stress. In vitro assays demonstrated the ability of the exosome-loaded hydrogels to enhance cellular proliferation and migration, critical processes in wound repair. Furthermore, in vivo studies using a diabetic wound model revealed significant improvements in wound closure rates and histological markers of healing, compared to conventional treatments.

This research underscores the therapeutic potential of ginger and garlic hybrid exosomes in promoting efficient wound healing in diabetic patients, offering a promising avenue for the development of advanced wound care solutions. The findings contribute to the growing body of knowledge on the application of natural bioactive compounds in regenerative medicine, with implications for improving the quality of life for individuals suffering from diabetes-related complications. Future work will focus on optimizing the formulation and exploring the mechanistic pathways involved in the observed healing effects, paving the way for clinical translation of this novel therapeutic approach.

Chapter 1

Introduction

Normal wounds follow four distinct phases of healing: coagulation, inflammation, proliferation, and maturation. Chronic wounds tend to be stuck in the inflammatory phase such that they never move towards healing. Chronic wounds comprise different types of ulcers, i.e., diabetic foot ulcers, venous leg ulcer wounds, and pressure ulcers. The financial burden of all chronic non- healing wounds across the US alone has been estimated to exceed $50 billion annually. Diabetic foot ulcers confer a 5-year mortality (30.5%) comparable to cancer (31%). With the growing populations, particularly in developed nations, and increasing age the incidence and burden of chronic wounds is likely to continue rising. Traditional wound dressings are not made to facilitate the closure of hard-to-heal chronic wounds. One of the most common features of chronic wounds is long-term inflammation. Several studies show that non-healing wounds are stuck in a chronic inflammatory state that prevents normal healing progression. Particularly, recent research into chronic wound fluid and tissue shows competition between pro-inflammatory and anti- inflammatory signals that creates a redox imbalance and inhibition of the proper occurrence of wound healing. This traps the wound in an ongoing state of chronic inflammation that inhibits advancement to wound closure. As a result of the chronic inflammation, resident infiltrating neutrophils and macrophages reside in the area and they produce reactive oxygen species (ROS) to fight colonization with microorganisms .The higher ROS levels in chronic wounds have adverse consequences on wound healing since they can induce cellular damage ,tissue damage, and damage to the extracellular matrix (ECM), and trigger latent extracellular proteases (such as matrix metalloproteinases (MMPs)) and inflammatory cytokines .The continuous state of raised inflammation and levels of ROS render the wound incapable of moving away from the inflammatory phase. In extreme cases, cells in and near the bed of the wound die through programmed cell death (i.e. apoptosis, pyroptosis, ferroptosis) as a result of excessive oxidative stress, which causes a chain of reactions in surrounding cells that lead to similar cell death. This is probably the cause many chronic wounds become necrotic, which requires radical interventions like tissue debridement or worse, amputation, to save the life of the patient. Diabetes mellitus may result in vascular complications such as peripheral artery disease, which compromises blood supply to the lower limbs. Reduced circulation leads to impaired healing of wounds and predisposes them to ulcers and infection. Chronic wounds can have a great adverse effect on the quality of life of a diabetic individual, inducing pain, discomfort, and immobility. Successful healing of the wound

can provide relief from these symptoms and improve general

well-being. Diabetic patients require special wound care that considers the specifics of their condition. This involves frequent monitoring, the achievement of optimal blood glucose levels, and the utilization of sophisticated wound care technologies and modalities to facilitate healing and avoid complications.

Hydrogels have also been found to be a potential biomaterial in the realm of biomedicine, especially in wound healing, because of their distinctive characteristics that include high water content, biocompatibility, and extracellular matrix-mimicking capabilities. The next-generation chronic wound dressings must be conceptualized for holistic treatments with antioxidative and antibacterial/antibiofilm capabilities. While past research has looked into potential solutions, each has been limited. So far, no dual-functional treatments of wounds are independent or non-tainting since they are based on the natural immune response, photothermal irradiation, release of antibiotics or metal oxides. Our dressing takes advantage of the porosity of the hydrogel, recent developments have investigated the use of plant-derived natural exosomes like ginger and garlic in hydrogel matrices to increase their therapeutic capabilities. This new strategy takes advantage of the bioactive compounds contained in these exosomes, providing a new method for stimulating wound healing. Exosomes are small extracellular vesicles, usually between 30 and 150 nanometers in size, that cells secrete and utilize for intercellular communication. In plants such as ginger and garlic, exosomes are generated through a complex process involving the endosomal pathway. They are first formed inside multivesicular bodies (MVBs) within the cell. When MVBs fuse with the plasma membrane, the vesicles get secreted into the extracellular environment as exosomes.

Ginger exosomes are full of bioactive molecules like gingerols, shogaols, and paradols, which exhibit anti-inflammatory and antioxidant activity. Garlic exosomes also have allicin and other sulfur-containing molecules, which are characterized by their antimicrobial and immune-modulating activity. The bioactive molecules are trapped within the lipid bilayer of the exosomes, which ensures stability and targeted delivery to desired cells or tissues. After preparation of the hydrogel matrix, ginger and garlic exosomes are added to the hydrogel. This is accomplished either by mixing the exosomes with the polymer solution prior to gelation or by incorporating them into the hydrogel after formation. The process of incorporating ensures that exosomes are uniformly distributed in the hydrogel matrix. The bioactive molecules in ginger and garlic exosomes assist in diminishing inflammation at the site of the wound, healing faster, and relieving swelling and pain. Garlic exosomes are especially known to offer antimicrobial action that prevents infection, which is one of the most common wound

healing complications.

Exosomes enable cell-to-cell communication, which assists in the proliferation and migration of skin cells required for closing the wound and regenerating the tissue. The antioxidant effect of ginger exosomes aids in the neutralization of free radicals, lessening oxidative stress and also aiding healing.

In summary, the creation of hydrogels containing ginger and garlic exosomes is a novel method for wound healing that brings together the efficacy of natural bioactive molecules with innovative biomaterial technology. This new strategy is highly beneficial for enhancing patient outcomes and for the further development of regenerative medicine, providing a generic and valuable treatment for many different wound care issues.

Chapter 2

Objective

The objective of the work includes:

- Development of Hybrid Exosomes and doing Stability assessment.
- Hydrogel fabrication with exosomes encapsulation and Stability analysis of Hydrogel.
- Evaluation of wound healing properties of hydrogel encapsulated with exosomes .

Chapter 3

Review of Literature

3.1 Exosomes

Exosomes are tiny, membrane-bound vesicles produced by most eukaryotic cells, playing a key role in cell-to-cell communication. These extracellular vesicles (EVs) are part of a broader category that includes various sizes and types, ranging from 50 to 5000 nanometers, and are secreted by cells across different organisms, including bacteria, plants, and mammals [4]. Initially, EVs were thought to be a way for cells to dispose of waste, packaging and releasing it into the surrounding space [5]. However, we now know that they carry a mix of proteins, nucleic acids, and other cellular materials [6], wrapped in a phospholipid bilayer that allows them to move between cells [4].

EVs are categorized into three main types based on their size and origin: exosomes, microvesicles, and apoptotic bodies [7]. Exosomes are the smallest, ranging from 30 to 150 nanometers, and originate from the endosomal compartment within cells. They were first identified in 1983 as intraluminal vesicles formed by multivesicular bodies, a type of endosome [8]. Microvesicles, also known as ectosomes, are larger, between 50 and 1000 nanometers, and are created by the outward budding of cell membranes [9]. Apoptotic bodies are even larger, ranging from 1 to 5 micrometers, and are fragments released during programmed cell death [10].

The process of forming exosomes involves a unique inward budding of the membrane within multivesicular bodies, which is different from typical budding events inside cells [11]. This process seems to be linked to changes in the lipid composition of the membrane. While membrane exchanges between cells have been observed, such as between T cells and antigen-presenting cells or dendritic cells, it's still unclear if exosomes are involved in these exchanges [5][11]. Despite the complexity, exosomes are increasingly recognized for their potential in facilitating communication and material transfer between cells.

3.1.1 Animal-derived EVs (ADEVs) and Plant-derived EVs (PDEVs)

Extracellular vesicles (EVs) are tiny, nanoscale structures that play a crucial role in transferring genetic material between cells. They are made up of various biological molecules, including lipids, proteins, carbohydrates, nucleic acids, and small metabolites. EVs can be secreted by a wide range of sources, such as normal and infected cells, yeast, blood, fungi, plant tissue [12] [13][14], plasma [15][16][17], and milk [18].

Plant-derived EVs (PDEVs) are similar to those from animals (ADEVs) in that they have a phospholipid bilayer and carry proteins and nucleic acids [19]. PDEVs range in size from 100 to 1000 nanometers and have a negative zeta potential, which varies depending on the plant species [20]. Compared to ADEVs, PDEVs tend to have less protein content but are richer in lipids, including phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidylcholine (PC), digalactosyl diacylglycerol (DGDG), and monogalactosyl diacylglycerol (MGDG) [21]. These lipids are thought to contribute to the biological activity of PDEVs [22].

For instance, lipids from ginger-derived EVs have been shown to inhibit the activation of the NLRP3 inflammasome in bone marrow-derived macrophages [23]. Additionally, the PA in ginger-derived EVs has been identified as the active component that inhibits the growth of the gingival pathogen Porphyromonas gingivalis by interacting with bacterial cell membrane proteins [24]. PDEVs rich in PA tend to accumulate and stay longer in the intestine, while those high in PC migrate from the intestine to the liver, affecting their uptake in both eukaryotic and prokaryotic cells [20].

EVs are packed with nucleic acids, proteins, lipids, and other bioactive molecules, and they can enter recipient cells through fusion, endocytosis, or binding to cell membrane receptors. This ability makes them key players in cell-to-cell communication, allowing the exchange of genetic information [25][26]. Among these nucleic acids, microRNAs (miRNAs/miRs) are particularly important. These small, single-stranded RNAs, about 22 nucleotides long, can bind to mRNAs, leading to gene silencing and regulation. Plant miRNAs have a unique feature called 2′-O-methylation, which enhances their stability and protects them from enzymatic digestion [27] [28].

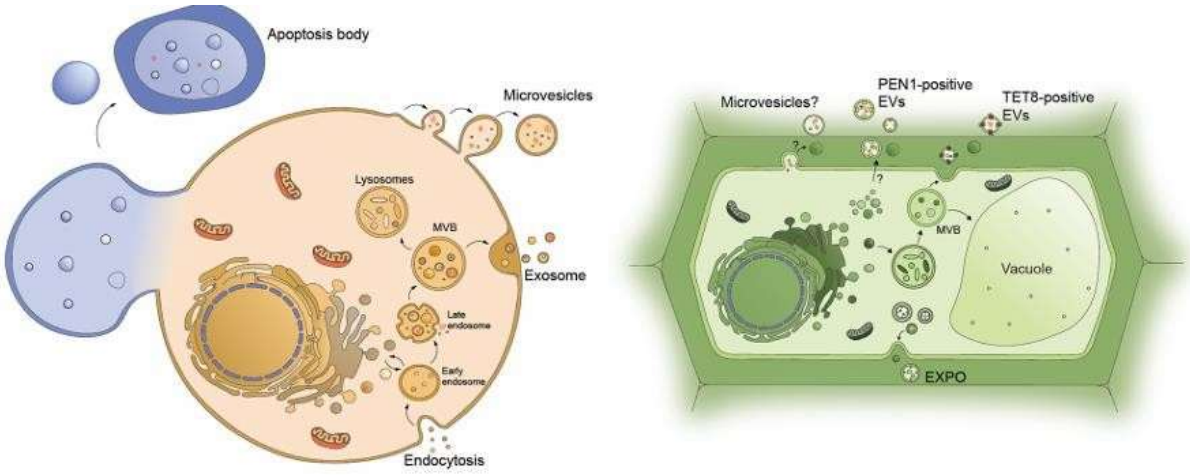


Figure 1 - Biogenesis pathway of EVs in mammalian cell and PDEVs.

3.1.1.a The Role of Proteins in PDEVs.

Proteins are an essential component of plant-derived extracellular vesicles (PDEVs), playing a significant role in various biological processes. Recent studies have explored the proteomic makeup of PDEVs from different plants, such as Arabidopsis thaliana [29], Allium sativum (garlic) [30], Citrus limon (lemon) [31], and Zingiber officinale (ginger) [32]. While their role in the plant immune system has been suggested, detailed reports are limited. For example, research by Rutter and Innes found that proteins abundant in EVs from A. thaliana are involved in responding to environmental stresses [29].

In garlic-derived EVs, the digestion of membrane surface proteins with trypsin resulted in reduced uptake compared to undigested EVs. This suggests that the interaction between garlic EV membrane proteins, such as mannose-specific binding protein, and the CD98 receptor on HepG2 cells is crucial for their internalization [33]. Unlike animal-derived EVs (ADEVs), PDEVs lack distinct protein markers for categorization [34]. However, proteomic analysis has identified several protein families in PDEVs across various plants, which might serve as potential markers. Common proteins found in PDEVs include heat shock protein 70 (HSP70) and aquaporins, which are prevalent in most plant-derived EVs [35].

EVs perform various functions in biological processes. Microvesicles are involved in cell signaling, apoptotic bodies transfer contents from dying cells to healthier ones, and exosomes facilitate intercellular communication in both normal and pathological conditions. These functions highlight the diverse roles of exosomes in their natural state [36].

Ginger exosomes have anti-inflammatory activity by regulating the polarization of macrophages and inhibiting pro-inflammatory cytokines. They also have antimicrobial activity, which prevents infection in chronic wounds. Ginger-derived exosome-like nanoparticles (ENs-6-shogaol) alleviated colitis and induced intestinal healing in mice, indicating systemic anti-inflammatory potential . Ginger exosomes in hydrogels or dressings can be used to enhance localized delivery for stimulating fibroblast proliferation and angiogenesis. Ginger exosomes also possess miRNAs and lipids that modulate oxidative stress (e.g., through Nrf2 pathways) and collagen production

. Garlic exosomes are able to inhibit inflammation by targeting signaling pathways. For instance, they engage with microglial cells to inhibit the c-Myc/STING/IFNIDO1 inflammatory cascade to lower neuroinflammation and safeguard nerve cells. In acute liver failure models, garlic exosome-like nanovesicles (GaELNVs) lowered inflammatory cytokines (IL-6, IL-1β, TNF-α), suppressed NF-κB activation, and lowered recruitment of inflammatory monocytes into the liver via downregulation of CCR2/CCR5 signalling .Ginger-Derived Exosomes Ginger exosomes have anti-inflammatory activity by regulating the polarization of macrophages and inhibiting pro-inflammatory cytokines. They also have antimicrobial activity, which prevents infection in chronic wounds . Ginger-derived exosome-like nanoparticles (ENs-6-shogaol) alleviated colitis and induced intestinal healing in mice, indicating systemic anti-inflammatory potential. They reprogram macrophages to the M2 phenotype, establishing a pro-healing immune microenvironment . Garlic exosomes are able to inhibit inflammation by targeting signaling pathways. For instance, they engage with microglial cells to inhibit the c-Myc/STING/IFNIDO1 inflammatory cascade to lower neuroinflammation and safeguard nerve cells. In acute liver failure models, garlic exosome-like nanovesicles (GaELNVs) lowered inflammatory cytokines (IL-6, IL-1β, TNF-α), suppressed NF-κB activation, and lowered recruitment of inflammatory monocytes into the liver via downregulation of CCR2/CCR5 signalling of Ginger and Garlic- derived exosomes . Ginger exosomes have anti-inflammatory activity by regulating the polarization of macrophages and inhibiting pro-inflammatory cytokines. They also have antimicrobial activity, which prevents infection in chronic wounds .[32] Ginger-derived exosome-like nanoparticles (ENs-6-shogaol) alleviated colitis and induced intestinal healing in mice, indicating systemic anti-inflammatory potential.Garlic exosomes are able to inhibit inflammation by targeting signaling pathways. For instance, they engage with microglial cells to inhibit the c-Myc/STING/IFNIDO1 inflammatory cascade to lower neuroinflammation and safeguard nerve cells. In acute liver failure models, garlic exosome-like nanovesicles (GaELNVs) lowered inflammatory cytokines (IL-6, IL-1β, TNF-α), suppressed NF-κB

activation, and lowered recruitment of inflammatory monocytes into the liver via downregulation of CCR2/CCR5 signalling .[33]

3.1.2 Composition of Exosomes

3.1.2.a Lipids

Phospholipids (e.g., phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine)

Sphingolipids (e.g., ceramides, sphingomyelin)

Cholesterol, which contributes to membrane rigidity and stability

Glycosphingolipids, which play a role in cell signaling and recognition [42]

The lipid composition of EVs is less comprehensively described than their protein and nucleic acid composition. Several hundred lipid species in EV membranes have been revealed using mass spectrometry.[43] The most frequent lipids in platelet-derived exosomes are cholesterol (42.5% of lipids), phosphatidylcholine (15.9%), and sphingomyelin (12.5%), along with their derivatives. [44]Surprisingly, exosomes and their parent cells have lipid compositions that vary greatly from one another[45], but less so from one another if both are from the same cell line. [44] The more elevated level of sphingomyelin, disaturated lipids, and cholesterol in the plasma membrane of exosomes compared to their parent cells can be attributed to the rigidity of exosomes and to their lack of degradability, which allow them to serve as good carriers of proteins and nucleic acids. The lipid distribution in the inner and outer leaflets of the exosome membrane is not equal, adding to the stability of exosomes.[46] In contrast to exosomes, microvesicles also possess a lipid composition close to their parent cells, and apoptotic bodies contain more phosphatidylserine than their parent cells. [47]

Lipids also play a regulatory role in EVs.[48] One of the best-documented examples is that phosphatidylserine on the outside of apoptotic body membranes serves as an "eat me" signal for phagocytes.[49] Further, lipids may influence inflammation: ceramide phosphates in bronchoalveolar lavage fluid membrane exosomes modulate an anti-inflammatory effect upon second-hand smoke exposure. Lipids are crucial to EV function, and a detailed examination of lipids in EVs could potentially reveal other physicochemical properties with which to separate exosomes from other subtypes of EVs.[48]

3.1.2.b Proteins

Tetraspanins (CD9, CD63, CD81, CD82) – Involved in exosome biogenesis and cell targeting

Heat Shock Proteins (HSP70, HSP90) – Assist in stress response and protein folding

Alix and TSG101 – Associated with exosome biogenesis via the ESCRT pathway

Integrins and Adhesion Molecules – Aid in cell targeting and uptake

Enzymes (e.g., peroxidases, superoxide dismutase) – Provide antioxidant activity [50]

Single EV would hold fewer than 100 total proteins. [51] Approximately 1 μg of total protein may be isolated from ≈109–1010 EVs. [51] Protein cargos are also heterogeneous between EVs of the same type, but certain proteins are always present in a particular EV type because of the functions of these proteins in EV biogenesis and protein sorting.[52] The three types of EVs can be separated according to their protein composition, and proteins are employed as markers in clinical diagnosis and EV characterization.[53]}. In exosomes, signature proteins are CD9, CD63, and CD81, tetraspanin proteins that are typical of exosome membranes, and are involved in membrane fusion, signaling, and protein targeting. [54] ALIX, flotillin, and TSG101 are engaged in the biogenesis of exosomes [59]. ESCRT and the related proteins hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), flotillin, TSG101, and ALIX are involved in MVB formation and ILV engulfment and are shared exosome cargo. [55] Rab27a and annexin are transport and fusion vesicle proteins responsible for docking MVBs to the plasma membrane and regulating exosome release. [56] Cytoskeletal proteins actin and myosin and heat shock proteins Hsp70 and Hsp90 are also present in exosomes. [57,60] These proteins combined are markers that differentiate exosomes from the rest of the EVs and may be used in exosome detection and isolation.[58]

Other EVs have their own characteristic protein cargo which is utilized to further differentiate exosomes. Microvesicles have proteins that are usually more highly post-translationally modified.[59] Microvesicle membrane proteins are CD40, integrins, glycoprotein Ib, and P-

selectin.[60] Tumor microvesicle membranes are present with the small GTP-binding protein ARF6, which is involved in protein selection in tumor cells. [61] Apoptotic bodies both have cytoplasmic and nuclear proteins and therefore have more intricate protein cargo compositions than other EVs.[62] Membranes of apoptotic bodies include calreticulin (CRP55) and calnexin (CNX), which facilitate efferocytosis and phagolysosome formation, as well as thrombospondin receptors for (TSP) and the complement protein C3b receptors that bind TSP and C3b and facilitate recognition of apoptotic bodies by phagocytes. These proteins are markers for further differentiation of exosomes from microvesicles and apoptotic bodies.[63]

Exosomes have crucial functions in the activation and inhibition of the immune system, and may regulate immune responses through antigen presentation on their surfaces.[64] For instance, exosome membranes merge with MHC–antigen complexes and cause antigen-specific T cell responses in initiating and developing inflammation. [65] The surface receptors CD86 and lymphocyte function-associated antigen 1 (LFA-1) on exosomes trigger inflammatory pathways that activate immune cells.[66] Surface proteins on exosomes also mediate immune suppression: exosomes from cancer cells expressing programmed death-ligand 1 (PD-L1), an inhibitory checkpoint molecule, can inhibit cytotoxic T cell function and enhance the immune escape of cancer cells.[67] In addition to surface proteins, exosomes were also found to deliver protein, DNA and RNA cargos that can trigger immune responses as well as other physiological activities.[68].

3.1.2.c Nucleic Acids

microRNAs (miRNAs) – Regulate gene expression and metabolic pathways

Messenger RNAs (mRNAs) – Can be translated into functional proteins in target cells

Long non-coding RNAs (lncRNAs) – Involved in epigenetic regulation

Small interfering RNAs (siRNAs) – Play a role in gene silencing

Extracellular vesicles (EVs) play a crucial role in transporting nucleic acids into target cells, where these molecules can be translated and transcribed, ultimately influencing cell behavior [69]. For example, research has shown that mitochondrial DNA carried by exosomes to breast cancer cells may contribute to hormone therapy-resistant breast cancer. This occurs by restoring the metabolic function of breast cancer cells that have become dysfunctional following hormonal treatment [70].

EVs commonly deliver mRNA and microRNA (miRNA) as part of cell-to-cell communication in both healthy and diseased states. The DNA and RNA found in EVs reflect the conditions of the parent cell. For instance, exosomes from virus-infected cells contain viral RNA, while those from cancerous cells have miRNA profiles that differ from those in normal cells [71]. This ability to mirror the parent cell's condition makes EVs a valuable tool for understanding and potentially diagnosing various health conditions.

The nucleic acid content within different types of extracellular vesicles (EVs) is quite varied, making it difficult to establish a definitive genetic profile for each type [57]. Despite this complexity, there are some notable differences in nucleic acid content among EV types. For instance, mRNA and microRNA (miRNA) are typically concentrated in microvesicles and exosomes [58], whereas ribosomal RNA (rRNA) tends to accumulate in apoptotic bodies. Additionally, DNA found in microvesicles and exosomes often includes a complete genome, while DNA in apoptotic bodies is usually fragmented [59].

Exosomes, in particular, have diverse miRNA profiles, with each exosome potentially containing up to around 500 different miRNA molecules [70]. However, the nucleic acid content and genetic profiles of exosomes, and how they differ from other EVs, are not well understood. To better characterize these differences, large-scale comparisons of EVs are needed. Such studies could help optimize and standardize the isolation of exosomes from other types of EVs, enhancing our understanding of their unique roles and functions [60].

3.1.2.d Plant Metabolic Compounds in PDEVs.

Doxorubicin (Dox) ■co-incubation 29 ■SW480colorectal cancer cell

[37] has-miR-184 ■transfection ■SW480colorectal cancer cells [38]

FA-arrow tail siRNA complex ■transfection 42,43,128 ■KB cervical tumor [39]

siRNA-CD98complex ■co-sonication with PDEVs lipids extract ■colon tissues[40]

doxorubicin (Dox)/FA ■co -sonication lipids extract ■colon-26 adenocarcinomatumors [41]

3.1.2.e Bioactive Compounds (Specific to Plant-Derived Exosomes)

Polyphenols (e.g., flavonoids, gingerol, allicin) – Possess antioxidant and anti-inflammatory effects

Terpenoids and Alkaloids – Have antimicrobial and metabolic regulatory functions

Vitamins and Minerals – Essential for cellular metabolism and immune modulation [11]

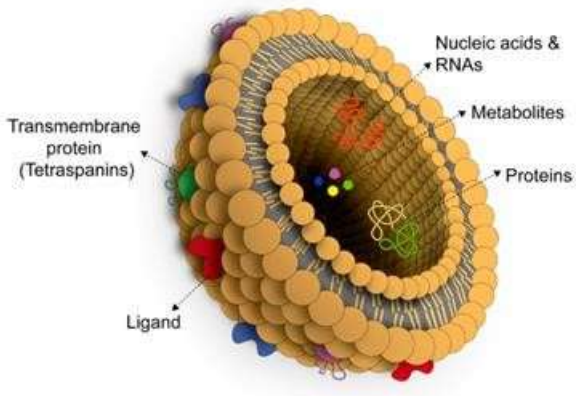


Figure 2 - An overview of the common structure and typical composition of extracellular vesicles (EVs).

3.1.3 Clinical Applications of Exosomes

Exosomes, tiny vesicles that transport various molecular cargo, play significant roles in the progression of several diseases. Understanding how exosomes influence disease pathways is crucial for developing therapies and diagnostic tools based on these vesicles. This section explores the involvement of exosomes in cancer, cardiovascular disease, neurodegenerative diseases, and HIV/AIDS, along with clinical trials that utilize exosomes for diagnostics and treatment [71].

In the context of cardiovascular disease, exosomes contribute to disease development in patients with diabetes and obesity through their antiangiogenic and proinflammatory properties, driven by the miRNA and proteins they carry. For example, diabetic rat heart cells release exosomes that are low in antiangiogenic miR-320 and high in proangiogenic miR-126, which suppresses blood vessel repair after damage from high glucose levels, increasing the risk of cardiovascular complications [65][67]. Similarly, exosomes derived from hypertensive rat macrophages are low in anti-inflammatory miR-17, and those from hypertensive human

macrophages may induce

inflammation in human coronary artery endothelial cells, contributing to cardiovascular disease [57].

In Parkinson's disease, exosomes facilitate the spread of infectious proteins between cells, contributing to disease progression. Parkinson's is characterized by the formation of Lewy bodies, aggregates of misfolded α-synuclein [67]. Exosomes can carry α-synuclein, promoting its aggregation and spreading between neurons and neuroglia. When neuroglia, such as astrocytes and microglial cells, endocytose α-synuclein-containing exosomes, they trigger a neuroinflammatory response central to Parkinson's pathogenesis [68]. This inflammatory cycle, driven by exosome delivery of α-synuclein, exacerbates neurodegeneration [69].

In Alzheimer's disease, exosomes have dual roles, with some advancing the disease and others preventing its progression [71]. Exosomes are abundant in Alzheimer's plaques, which are β-amyloid protein collections in the brain that block synaptic signals, as indicated by high levels of the exosomal protein marker ALIX in these plaques. Conversely, exosomes from human cerebrospinal fluid can prevent synaptic disruption caused by β-amyloid protein [72].

AIDS, caused by HIV infection, involves the immune system's invasion by the virus [73]. HIV-infected cells produce exosomes filled with viral products, affecting the exosomes' content [74]. These exosomes can influence disease progression by delivering Nef proteins to nearby CD4+ T cells, causing their apoptosis and depletion, a hallmark of AIDS pathogenesis [75]. Additionally, HIV miRNAs in exosomes suppress apoptosis, support chronic inflammation, and enhance viral transcription, all contributing to disease progression [76].

3.1.3.a Direct Approach: Exosomes as Therapeutic Agents

Exosomes are being explored as innovative drug-delivery vehicles due to their ability to transport proteins and nucleic acids between cells, offering promising therapeutic strategies for treating diseases and injuries [77]. In one clinical trial, patients with acute ischemic stroke were treated with exosomes derived from mesenchymal stem cells (MSCs) [78]. These exosomes were transfected with microRNA miR-124, known for its beneficial effects on brain injuries by promoting neurogenesis. The treatment enhanced neural functional recovery and stimulated neurogenesis and angiogenesis following a stroke [79]. Over a 12-month follow-up period, physiological adverse events such as brain , seizures, and stroke recurrence were monitored. Compared to conventional cell therapy, the exosomes proved to be more stable, immune-tolerant, and effective when delivered systemically [80].

Exosomes also have the potential to serve as drug carriers capable of penetrating the central nervous system by crossing the blood-brain barrier due to their small size [81]. For instance, studies have shown that exosomes derived from MSCs can promote neurogenesis and cognitive recovery in Alzheimer's disease (AD) mice. Exosomes from human umbilical cord MSCs have been approved for therapeutic use to alleviate neuroinflammation by activating microglia, leading to improved cognitive function and the removal of amyloid beta protein (Aβ) deposits in AD-affected mice [82]. Based on these promising results, biotechnology companies are developing MSC-derived exosomes for Alzheimer's treatment (Celltex Therapeutics), neural stem cell-derived exosomes for crossing the blood-brain barrier (Aruna Bio) [83], and an exosome-based vaccine platform for preventing infectious diseases (Codiak BioSciences) [84] [85].

3.1.3.b Indirect Approach: Exosomes as Biomarkers

In the field of biogenesis research, exosomes have been identified as valuable markers for various diseases and clinical conditions, as well as predictors of treatment response [86]. These tiny vesicles, found in circulation and all body fluids, are remarkably stable across a wide range of temperatures during isolation [87]. This stability reduces storage and transportation costs, enhancing their clinical value as both therapeutics and biomarkers. Researchers have harvested and analyzed exosomes from various biopsies to understand disease pathogenesis, evaluate the effectiveness of clinical therapies, and explore new therapeutic interventions [88].

For example, a clinical study involved isolating and characterizing urinary exosomes from 24 patients with hypertension to diagnose difficult-to-treat hypertension, a condition where blood pressure remains high despite the use of multiple antihypertensive drugs [89]. This condition is linked to abnormal renal sodium content, regulated by the renin–angiotensin–aldosterone system, which was assessed by detecting angiotensin (Ang) peptides like Ang II in patient plasma [90]. Urinary exosomes were a key outcome measure because they serve as markers of renal function, providing parameters for evaluating drug therapeutic action [91]. Exosomes released from tubular epithelial cells into urine contain sodium channels, such as the Na–Cl cotransporter and subunits of the epithelial sodium channel, which are targets of hypertension medications like furosemide and thiazide [92]. These properties allow for the assessment of renal performance and drug responsiveness [93]. This research highlights the potential of exosomes as biomarkers for diagnosing illnesses.

Moreover, studies have confirmed the significance of exosomes as therapeutic agents or biological markers post-treatment, particularly in cardiovascular diseases [94]. For instance, embryonic stem cell-derived exosomes have been shown to significantly improve doxorubicin-induced cardiac damage and cardiomyocyte pyroptosis by inhibiting caspase-1-mediated cell death and promoting M2 macrophages and anti-inflammatory cytokines. Additionally, the increased secretion of antihypoxic cardiac progenitor cell-derived exosomes may serve as a reliable biomarker and mechanism for treating ticagrelor, a selective oral non-thienopyridine P2Y12 inhibitor. These investigations demonstrate the dual potential of exosomes as both therapeutics and biomarkers [95].

3.1.3c Alternative Approaches: Eliminating Disease Promoting Exosomes

Another promising therapeutic approach involves removing disease-promoting exosomes to halt disease progression. This strategy aims to interrupt the flow of exosomes carrying harmful cargo, such as viral miRNA and proteins, immunosuppressive factors that aid tumor metastasis, or signals that promote tumor growth and resist treatment [96][97]. In a clinical trial, patients with head and neck squamous cell carcinoma (HNSCC) were treated with a blood-cleansing device called Hemopurifier on Days 1 and 21 to clear immunosuppressive exosomes from their circulation. This device had previously shown efficacy in removing viral particles from the plasma of hepatitis C and HIV patients and was used in a trial to eliminate the SARS-CoV-2 virus from COVID-19 patients. In the HNSCC trial, patients also received pembrolizumab, an FDA- approved monoclonal antibody used as a first-line therapy for HNSCC [98]. The therapy targeted circulating exosomes for clearance and used them as markers to assess treatment effectiveness [99]. The kinetics of exosome depletion and recovery were monitored before, during, and after treatment as secondary endpoints to evaluate the therapeutic effect [100].

Other research has focused on depleting harmful exosomes by inhibiting their biogenesis using specific drugs. For example, chemotherapeutics like tipifarnib and ketoconazole have been shown to reduce the biogenesis and secretion of exosomes in C4-2B and PC-3 cells in a dose-dependent manner [101][102][105]. These exosome-inhibiting drugs are currently being tested in clinical trials for treating HNSCC patients and in another trial for neoadjuvant treatment of recurrent glioma or metastatic breast cancer brain metastases [103][104]. These findings suggest that removing harmful exosomes, either through devices like Hemopurifier or by using biogenesis inhibitors, can be an effective treatment strategy [106].

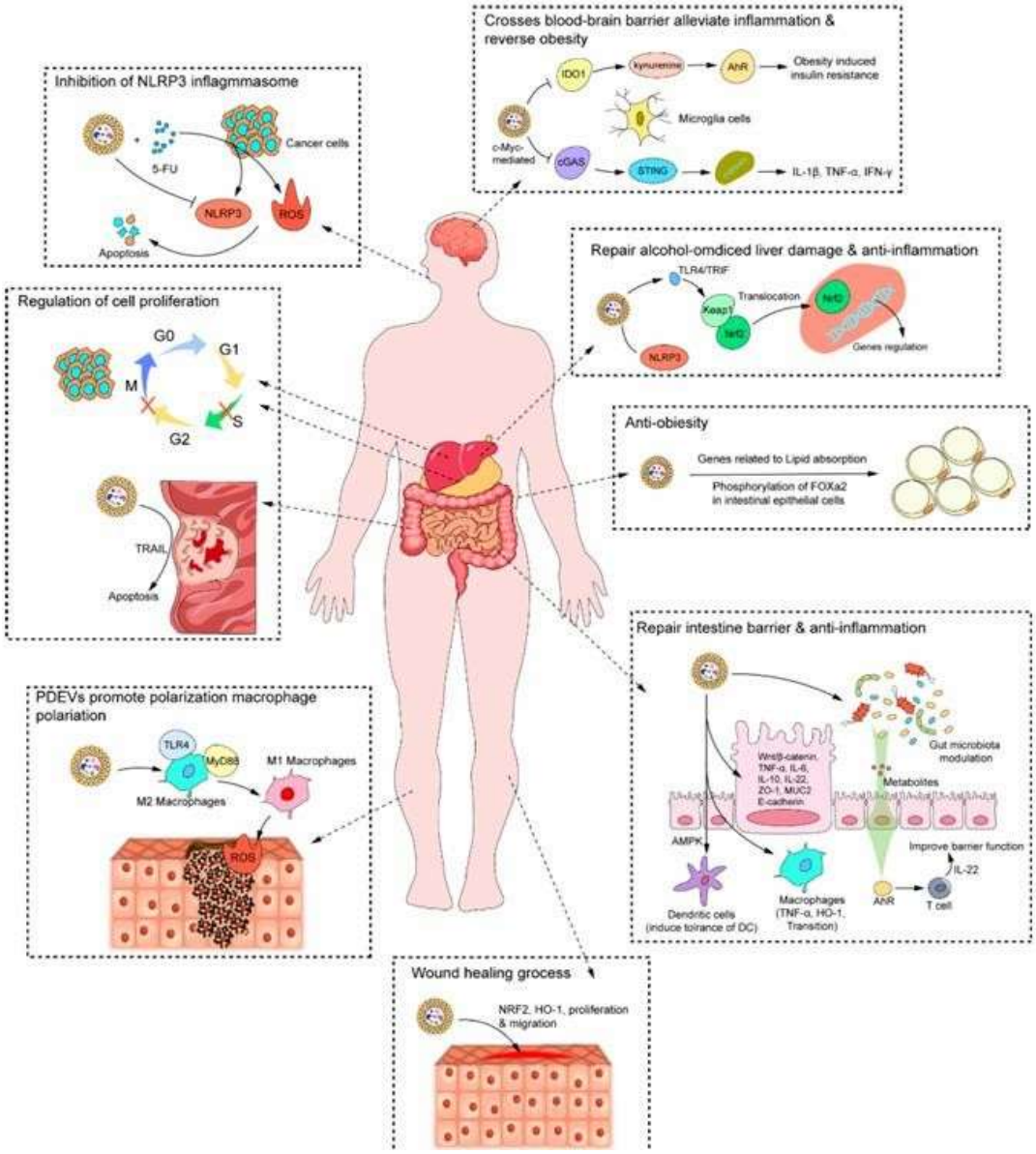


Figure 3 - The potential diseases therapy of PDEVs. The PDEVs exhibit various biological functions in the treatment of human diseases through diverse pathways.

3.1.3 d Exosomes in Clinical Trials

As our understanding of exosomes and their roles in disease mechanisms has grown, these tiny vesicles are increasingly being developed for therapeutic and diagnostic purposes. Although there is not yet an FDA-approved clinical exosome product, the number of active clinical trials exploring exosome-based therapies and diagnostics is on the rise. As of June 6, 2021, ClinicalTrials.gov listed 63 clinical trials involving exosomes, with a significant portion (26 out of 63) focused on cancer-related applications. Other trials are investigating the use of exosomes as diagnostic and therapeutic tools for a variety of conditions, including cognitive impairment,

Alzheimer's disease, heart failure, stroke, and periodontitis, showcasing the wide-ranging potential of exosome applications [107].

In response to the COVID-19 pandemic, eight clinical trials have been initiated to explore the use of exosomes, remaining in phases I and II just 16 months into the pandemic. This rapid development highlights the adaptability of exosome technology to meet emerging clinical needs. Current trials are examining exosomes as therapeutic agents, biomarkers, and targets for therapy. While exosome applications in cancer therapy and diagnosis have been extensively reviewed elsewhere, they have been used to deliver therapeutic agents targeting cancer stem cells, induce immune activation, promote antitumor immunity, and diagnose or monitor patient responses after treatment for gastrointestinal, lung, and breast cancers.

This section focuses on the use of exosomes in other diseases and the challenges associated with these applications, such as the complexity of body fluids, sample-to-sample heterogeneity, and limitations of existing exosome assays [108]. These challenges underscore the need for continued research and development to fully harness the potential of exosomes in diverse clinical settings.

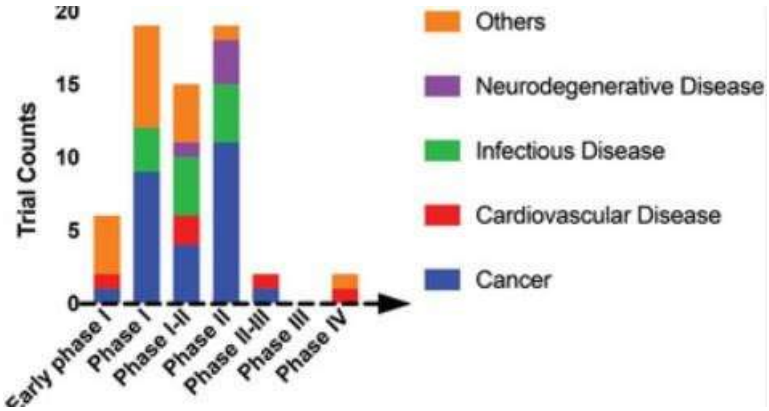


Figure 4 - Summary 63 clinical trials applying exosomes to diseases listed in ClinicalTrials.gov as of June 6, 2021.

3.1.4 Current knowledge on exosome biogenesis and release

Exosomes are nanoscale membrane vesicles that are secreted when a cell organelle called the multivesicular body (MVB) fuses with the plasma membrane [109]. These vesicles contain a complex mix of proteins, nucleic acids, lipids, and other metabolites, and are best visualized

using electron microscopy [110]. The release of exosomes involves several cellular steps: the

formation of intraluminal vesicles (ILVs) within MVBs, the transport of MVBs to the plasma membrane, and their fusion with the plasma membrane [111]. Although methodological challenges exist, studies using quantitative measurements and western blot analysis have shown that cells release only a small fraction of their content through exosomes [112].

Exosome biogenesis begins within the endosomal system, where early endosomes mature into late endosomes or MVBs. During this process, the endosomal membrane invaginates to form ILVs within the organelle lumen [113]. The endosomal sorting complexes required for transport (ESCRT) machinery play a crucial role in this process [114]. ESCRT consists of four distinct protein complexes: ESCRT-0, -I, -II, -III, and the related AAA ATPase Vps4 complex. Research has shown that knocking down ESCRT-III and related proteins like CHMP4C, VPS4B, VTA1, and ALIX can enhance exosome secretion. Conversely, depletion of proteins such as Hrs, TSG101, and STAM1 reduces exosome secretion, while VPS4B knockdown enhances it [115]. Silencing ALIX appears to alter the protein content of exosomes without affecting their secretion, suggesting a role in cargo loading or secretory MVB subtypes. However, ALIX depletion in dendritic cells has been shown to reduce exosome release in some donors [116].

Interestingly, MVB biogenesis can occur without ESCRTs, as ILVs can still form in MVBs even when all major ESCRT subunits are silenced, indicating the presence of ESCRT-independent processes [117]. Tetraspanins, transmembrane proteins enriched in exosomes, are implicated in ESCRT-independent exosome release. Another protein involved in exosome production is SIMPLE (or LITAF), a small integral membrane protein of the lysosome/late endosome. Transfection of COS cells with SIMPLE increases exosome secretion, while mutations in SIMPLE disrupt normal MVB formation [118].

Lipids also play a key role in vesicular transport, with membrane curvature influenced by the shape of membrane lipids, which depends on headgroup size and acyl chain length and saturation [119]. Phospholipase D2 (PLD2), an enzyme that produces phosphatidic acid (PA) from phospholipids, has been linked to exosome secretion in RBL-2H3 cells [120]. Exosome biogenesis has been characterized as both ESCRT-dependent and ESCRT-independent, but these routes may not be sharply distinguished. They could be synergistic, with different subpopulations of exosomes utilizing distinct mechanisms. Additionally, cell type and cellular homeostasis may significantly influence the regulation of exosome secretion [121][122].

3.1.5 Stability of exosomes

Exosomes are small, cup-shaped extracellular vesicles, ranging from 30 to 150 nanometers, with a lipid bilayer membrane structure [123]. They carry proteins, mRNAs, and microRNAs that facilitate intercellular communication [124]. Unlike other extracellular vesicles, exosomes are released into the extracellular space when multivesicular bodies (MVBs) fuse with the plasma membrane [125]. Exosomes can be secreted by nearly all cell types and are found in various biological fluids, including blood, urine, saliva, hydrothorax, and breast milk [126]. Numerous studies have demonstrated the roles of exosomes in disease progression and their clinical potential in diagnosis and therapy [127].

For consistent studies on exosomal content and function, it's crucial that storage conditions minimally affect exosomes. Research has partially verified the impact of different storage conditions on exosomes. Studies using exosomes derived from urine [128] and conditioned medium [129] have concluded that long-term storage at temperatures below −70 °C is optimal for exosome recovery, as determined by Western blotting. Conversely, Sokolova et al. [130] used nanoparticle tracking analysis (NTA) to examine size variations of exosomes at different temperatures, finding that storage at 37 °C resulted in greater size loss compared to storage at 4 °C, although no data on particle number changes were provided.

Other studies have explored the effects of pH, storage temperature, and freeze-thaw cycles on exosome isolation yield, but not on quantity changes during storage [131][132][133]. Thus, a definitive standard for exosome preservation conditions remains undefined. After isolation, exosome pellets were divided into aliquots and stored at various temperatures (−80 °C, −20 °C, 4 °C, 37 °C, and 60 °C), subjected to 1–5 freeze-thaw cycles at −80 °C, or stored at different pH levels (pH 4, pH 7, and pH 10). After 24 hours, NTA and Western blot analyses were conducted to assess the remaining exosome quantity. Exosomes stored at 4 °C were found to be the most concentrated.

To investigate the impact of storage conditions on exosome cellular uptake, PKH26-labeled exosomes were divided and stored under similar conditions. Current evidence suggests that −80°C is the most viable storage temperature for extracellular vesicles (EVs), though it may be limited by cost and transportation challenges. Therefore, alternatives like lyophilization and additives might be needed to enhance EV storage stability. Understanding optimal storage conditions for therapeutic EVs will help maximize their potential as a new class of therapeutics and drug carriers [134].

3.1.5 a Stability in Physiological Conditions

Ginger exosomes have been found to be highly stable under physiological conditions, making them a promising area of research [135]. While garlic exosomes are not as extensively studied, their chemical composition suggests they are rich in sulfur compounds and antioxidants, which may contribute to moderate stability. However, the presence of enzymes in garlic could lead to quicker degradation unless specially processed [136]. Both ginger and garlic-derived exosomes show significant potential as natural nanocarriers for therapeutic applications, with ginger exosomes being particularly stable and actively researched. Garlic exosomes likely have stability, but further research is needed to fully understand their behavior in vivo [137].

Ginger and garlic-derived exosomes are stable within a pH range of 6–8, exhibiting minimal aggregation or degradation. They remain stable at 37°C for a few hours to days, with long-term storage being more effective at 4°C or -80°C. Ginger exosomes are resistant to serum-induced degradation, maintaining their integrity in blood plasma [138]. Garlic exosomes are less studied in serum, but their high antioxidant content may aid in stability. Factors that can lead to degradation include extreme pH levels (below 5 or above 9), high temperatures (above 40°C), and enzymatic digestion by proteases or lipases [139].

3.1.6 Exosome Processing and Isolation

Sample Types

The composition of the starting material plays a crucial role in determining the techniques used for exosome processing and their effectiveness [140]. Common starting materials for exosome isolation include cell culture media, plasma, serum, urine, saliva, cerebrospinal fluid (CSF), and milk [141]. Each of these materials requires different processing strategies due to their unique characteristics [142].

Plasma, for instance, is a complex biological fluid containing not only exosomes but also cell debris, apoptotic bodies, microvesicles, and plasma proteins. These components have similar sizes and biochemical features, making exosome isolation challenging [143]. Urine, on the other hand, has fewer interfering particles compared to blood or plasma, but it contains a lower concentration of exosomes, necessitating larger volumes to achieve the same yield [144].

Cell culture media is often used for exosome mass production because it is easy to handle, cost-effective, and does not require animal or human subjects [145]. It typically yields more exosomes than serum or plasma, while CSF provides the least amount of exosomes. Conducting a comprehensive comparison study of exosome isolation across various sample types would greatly benefit exosome research and technological advancements [146].

3.1.6 a Exosome Isolation Techniques

Ultracentrifugation is the go-to method for isolating exosomes, used in about 80% of processing cases [147]. It doesn't require complex sample preparation and is cost-effective, aside from the initial investment in equipment. However, it can be time-consuming and only moderately purifies exosomes [148]. This technique separates sample components based on density, but the similar density ranges of various extracellular vesicle (EV) types can make isolation challenging [149]. High centrifugal forces create layers in the sample, with denser particles settling at the bottom and lighter ones rising to the top. The spinning speeds for exosome separation range from 100,000 × g to 210,000 × g, but higher speeds can damage the exosomes. Differential centrifugation, a variant of ultracentrifugation, involves repeated centrifugation steps to separate exosomes from cell debris and other particles. This method requires constant user input to manage supernatants and pellets and prepare spin cycles [150]. Exosome loss is expected during repeated supernatant removal and transfers, so larger sample volumes are used initially to achieve optimal yield [151]. Despite these limitations, differential centrifugation is well-validated and consistently yields exosomes of moderate purity [152]. Combining it with density-gradient media can enhance purification [153]. Density-gradient ultracentrifugation, or isopycnic ultracentrifugation, uses layers of different densities to purify exosomes between sucrose or iodixanol layers [149]. Zonal ultracentrifugation, a form of density-gradient ultracentrifugation, employs a gradient of reduced densities to separate particles by size. Compared to differential centrifugation, zonal ultracentrifugation typically requires only a single prolonged high-spin rotation, lasting up to 18 hours. While density-gradient ultracentrifugation produces more purified exosome products, it has lower yield and throughput [154]. It is also time-consuming and costly due to the creation of polymer density gradients, making it generally unsuitable for large-scale exosome processing [155].

Ultrafiltration is characterized by the utilization of very small pores (≈100 nm diameter) and may be employed to fractionate exosomes by size [156]. Ultrafiltration techniques are fast—one filtration cycle takes from seconds to 30 min—enabling high throughput. Ultrafiltration separates vesicles by subjecting sample fluid to pressure to force it through membranes having

≈100 nm

pores [157]. Membranes with larger or smaller pore sizes can be employed in subsequent steps for removal of other unwanted particles [158]. The process is quicker than ultracentrifugation, yet pressure employed causes damage to exosomes through shear stress, and can lead to exosome loss through membrane adhesion and blockage of membranes from particle accumulation, decreasing exosome yield and increasing processing time [159]. Measures like membrane washing have been implemented to address such issues [160]. Exosome ultrafiltration techniques are sequential filtration, tandem filtration, centrifugal ultrafiltration, and tangential flow filtration. Sequential and tandem filtration are dead-end filtration methods carried out using a syringe. Sequential filtration is characterized by several cycles of filtration each having a different molecular weight cutoff; tandem filtration uses several filters in one syringe. The size- exclusion limits for exosomes are 20–200 nm; in tandem filtration, the middle membrane traps exosomes [159]. Centrifugal ultrafiltration is a combination of dead-end filtration and centrifugation to isolate exosomes through nanoscale pores. A nanoporous membrane glued within a tube is rotated, imparting a centrifugal force that forces sample content to flow through the membrane. Centrifugal ultrafiltration is usually preceded by initial centrifugation or dead- end filtration at 0.22 μm to eliminate large particles like cells, cell debris, and protein aggregates, and to avoid clogging [161]. Tangential flow filtration (TFF) was recently used for the isolation of exosomes. Contrary to the above methods, TFF does not involve applying pressure perpendicular to the membrane, but samples are passed tangentially over the membrane [160]. This method prevents clogging of the membrane by particle deposition onto the membrane. TFF is capable of treating greater volumes of fluid with increased reproducibility than ultracentrifugation methods and is less harsh to the sample. However, the processing time is longer for TFF than for other filtration methods [160].

Precipitation methods are widely used for characterizing extracellular vesicles (EVs). A global survey indicated that precipitation is the preferred method for EV RNA analysis [162]. These methods use volume-excluding polymers to bind water molecules and force less soluble components out of solution. Biological materials are excluded from the solvent spaces occupied by these polymers and are brought together until their solubility limit is reached, leading to precipitation. This technique is often combined with other isolation processes. Although it yields more exosomes, the purity of the product is lower [163]. Since the introduction of polyethylene glycol (PEG) in 1964, nonionic volume-excluding polymers like dextrans and other hydrophilic polymers have become the preferred precipitation agents because they are less prone to protein denaturation at high temperatures and concentrations compared to ethanol and other organic agents. These polymer-based precipitation reagents are also used in

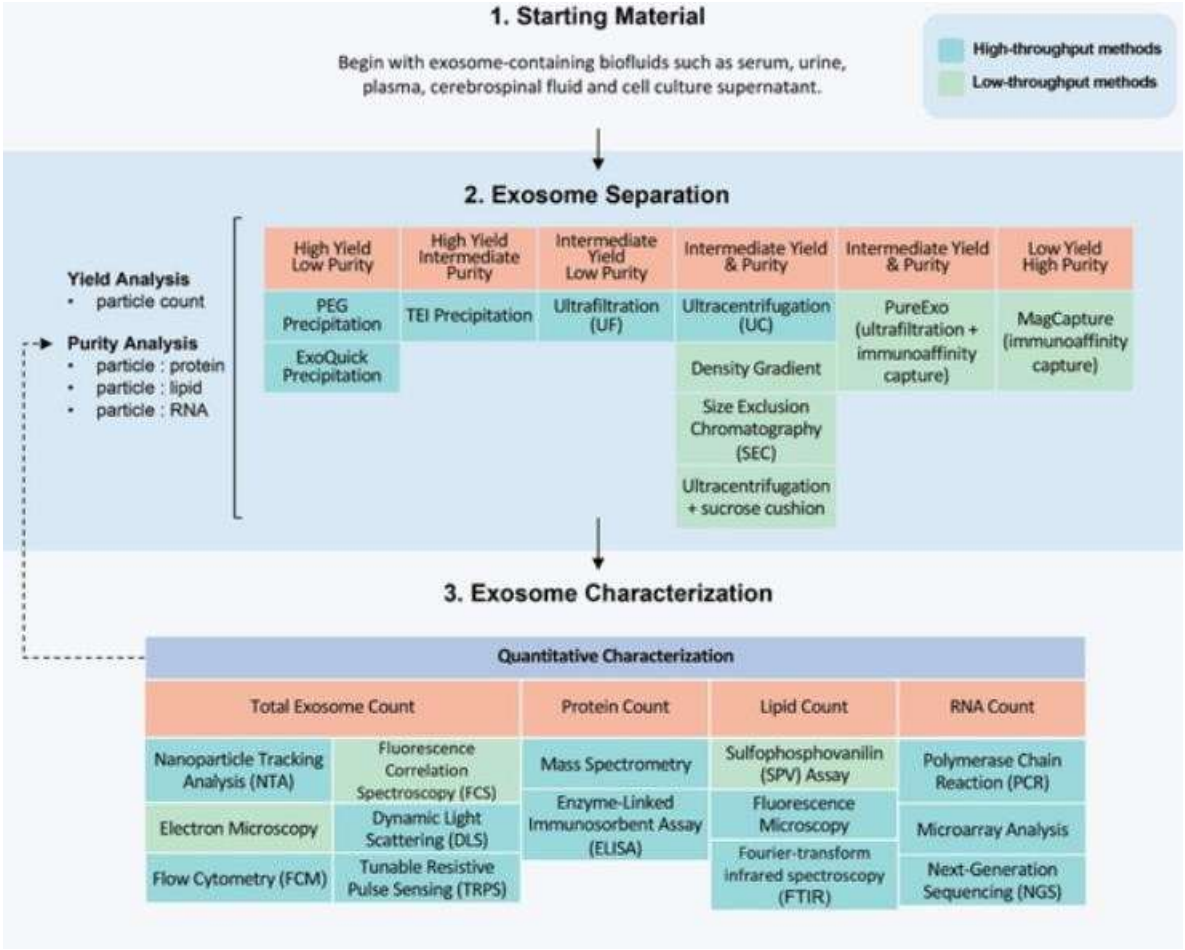
commercially available

exosome processing kits, such as ExoQuick (System Biosciences) and Total Exosome Isolation (Thermo Fisher) [164]. While these kits are not the most efficient for product purification, they offer advantages in terms of flexibility, affordability, and low equipment and training requirements. After treating samples with these kits, further centrifugation, filtration, or gel filtration may be used to enhance the purity of the exosome product [155].

Exosomes can be isolated based on their distinct surface markers using surface-immobilized antibodies on substrates like magnetic beads, chromatography resins, multiwell plates, and microfluidic devices [165]. This method, known as immunoaffinity capture, isolates exosomes with higher specificity than methods relying on physical properties. Immunoaffinity capture is sometimes combined with preprocessing strategies like size-exclusion chromatography (SEC) or centrifugation to remove protein aggregates and other large particulates [155]. Sample materials like plasma, whole blood, and cell culture media contain particles that can reduce the specificity of exosome capture by blocking antigen binding sites or encouraging nonspecific binding if not cleared in large amounts [166]. Immunoaffinity capture is a gentle method that preserves the biological function of exosomes post-isolation. A non-antibody-based method using TIM-4 and Ca+ dependent binding was developed to capture and elute exosomes without exposing the sample to non physiological conditions. Generally, this method is limited by the availability of antibodies and has a low yield due to the limited sample volume that can be used [167]. Additionally, extended incubation times are required. For example, the widely used magnetic Dynabeads (Thermo Fisher) require two 12-hour incubations, one for antibody conjugation and one for bead capture. These long incubation periods are necessary due to the large bead size (1.0– 4.5 μm), low mobility in solution, and low surface-area-to-volume ratio [168]. One solution to speed up this process is to use temperature- or pH-responsive magnetic nanoparticles, which reduce incubation and separation times to minutes due to their much higher surface-area-to- volume ratio (40 times that of 25 nm nanoparticles compared to 1 μm Dynabeads) and higher magnetophoretic mobility after aggregation from temperature or pH changes, facilitating swift magnetic separation [169]. Immunoaffinity strategies using Raman scattering also leverage magnetic properties to isolate and characterize exosomes. Raman scattering can detect molecules through their unique chemical fingerprints [170]. Magnetic surface-enhanced Raman scattering has been used to distinguish breast cancer in patient samples with high sensitivity and specificit y [171][172]. Incorporating characterization within sample processing is key to streamlining therapeutic and diagnostic applications of exosomes, setting this strategy apart from other immunoaffinity methods [173].

Size-exclusion chromatography (SEC) is the mildest chromatography method, generally used for purifying and isolating biopolymers like proteins and polysaccharides. SEC isolation of exosomes maintains vesicle integrity and biological activity while achieving high yield. SEC separates biomolecules based on hydrodynamic radius differences as they flow through a low-adsorption, unreactive resin of a porous matrix of beads packed within a column. Particles larger than the pores elute first, while smaller particles and molecules penetrate the pores to varying extents depending on their size, with increasing elution time as size decreases [174]. Optimal processing parameters like column size, bead packing, resin type, flow rate, and system volume are crucial for achieving high-resolution particle size. This technique can fractionate samples of various viscosities, from low-viscosity urine and cell culture medium to high-viscosity plasma. Samples must first be pretreated by ultracentrifugation or ultrafiltration to obtain EV preparations free from protein and lipoprotein contaminants [175]. To facilitate EV isolation via SEC, prepacked columns are commercially available, such as qEV (Izon Science) and HiLoad Superdex (GE Healthcare). SEC with prepacked columns produces a lower rate of exosome recovery and a more heterogeneous EV population than precipitation-based EV isolation, but is quick, easy, reproducible, versatile across many sample types, and doesn't require a chromatography system as it can function using a separate pump. However, SEC produces low- concentration samples, often necessitating an additional enrichment step [176]. Microfluidic devices can integrate multiple processes, including immunoaffinity capture, filtration, application of electrical or acoustical waves, and field flow fractionation, into a single one-step device with multiple channels that separate exosomes with high reproducibility and automation. Accurate control over fluid flow through these channels ensures laminar flow, which has more predictable fluid dynamics compared to turbulent flow. Microfluidic devices are small; for example, the immunoaffinity-based ExoChip device measures 75 mm × 25 mm and can be easily expanded by adding sampling wells. Due to their small size, these devices are portable and can be readily stored in well-packed lab settings. Microfluidics is particularly suited for diagnostic use because sample volumes range from nanoliters to microliters. Microfluidic devices can also incorporate modules for exosome detection, combining isolation and characterization [177]. Some devices feature arrays of silicon-nanowired micropillars that capture exosomes by size exclusion. When fluid flows through the device, exosomes are captured in the pores. This process is fast, but the captured exosomes must be eluted from the pores, which can take up to 24 hours, reducing the usefulness of this procedure in diagnostics. Size separation has also been achieved with viscoelastic-based isolation [174]. Sorting exosomes according to elastic lift forces with high sensitivity and specificity can be done using serum or cell culture media. Samples are mixed

with elastically responsive biocompatible polymers and deposited in viscoelastic media. As the liquid moves within the device, larger particles and extracellular molecules with greater elastic forces from the media are pushed away from the exosomes [178]. Acoustic microfluidic methods have been developed to differentiate exosomes by size. Acoustic waves are gentler than micropillar arrays and cause less contact. Waves are generated within a flowing sample using interdigital transducers. The wave frequency controls the particle size cut-off for separation into two channels, with waste like apoptotic bodies and larger microvesicles in one channel and exosomes in the other. Running a single sample takes just 25 minutes, and since it is contact-free, exosomes retain their biological activity. Besides acoustics, electrical waves have also been used in microfluidics to isolate exosomes label- and contact-free [179]. Ion-based separation exploits the more negative charge of exosomes compared to other particles. A microfluidic device designed by Mogi et al. uses two inlet channels and two outlet channels with high and low voltage to sort positively and negatively charged particles. One channel in each pair is high voltage and negatively charged, while the other is low voltage and positively charged. The perpendicular ion channel in the middle forms an ion depletion zone that drives charged particles into the channels, while uncharged particles remain near the middle. This instrument is calibrated for voltage and flow rate to maximize exosome retention and has shown far superior yield compared to ultracentrifugation [180].



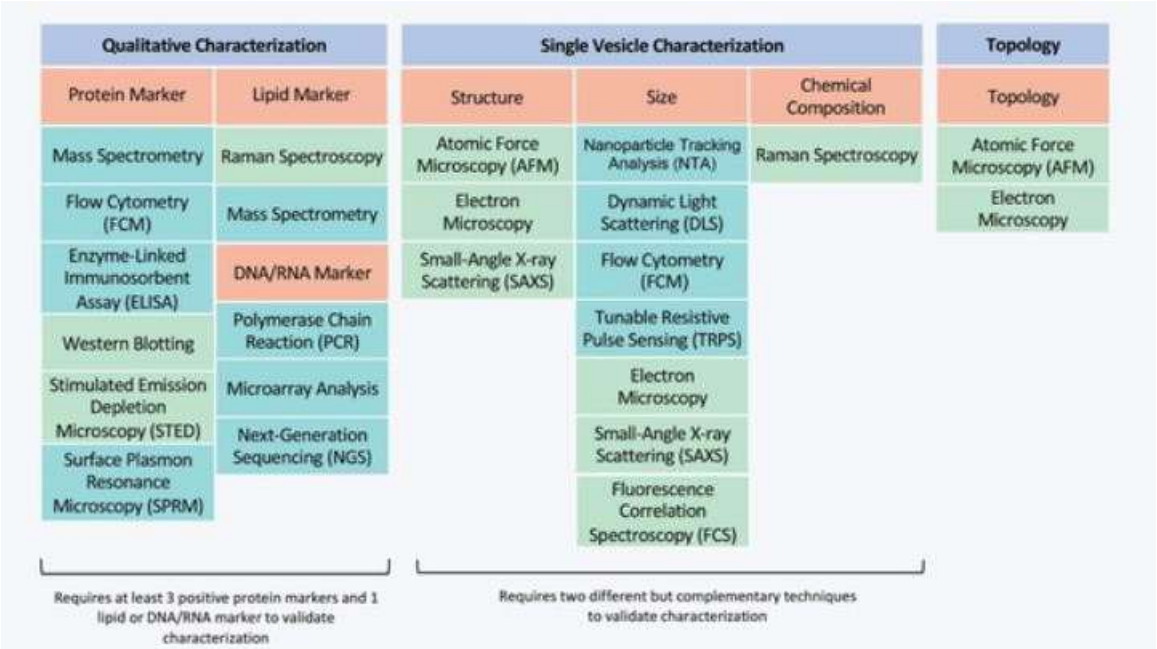


Figure 5 - Exosome Processing and Characterization Flowchart. This flowchart outlines the steps for processing and characterizing exosomes. It starts with selecting a processing method based on the starting material and desired yield and purity. The exosome separation section details common approaches and their expected outcomes. Once isolated, exosomes undergo

characterization to

assess the purity and yield of particles, proteins, lipids, and nucleic acids. If the sample doesn't meet the required standards, further processing is needed. The flowchart concludes with selecting methods for both bulk and single vesicle characterization, ensuring a thorough understanding of the exosome sample.

3.1.6b Exosome

Characterization Quantitative

Characterization

Quantitative characterization techniques are applied to determine the efficiency of exosome isolation and product quality, including yield and purity in terms of biomolecules like proteins, lipids, and nucleic acids.

Total Exosome Count

Exosome yield is measured using techniques that analyze particle count, such as nanoparticle tracking analysis (NTA), flow cytometry, fluorescence correlation spectroscopy (FCS), dynamic light scattering (DLS), resistive pulse sensing (RPS), and electron microscopy (EM) [181][182]. The most widely applied methods are NTA and FCS. NTA is a high-throughput imaging method that tracks the Brownian movement of particles suspended in a liquid. Particle concentration and size are determined from video of diffusing scattered light from randomly diffusing particles under illumination by a laser beam. FCS is an imaging statistical method that can be employed to describe molecule concentration, size, and diffusion rate. The method employs a laser to excite a very small volume (1 fL) of fluorescently labeled sample. Fluorescence intensity of the molecules varies as a result of Brownian motion, and particle concentration can be estimated on average by measurement of fluorescence intensity over time [183].

DLS and RPS tend to overestimate total particle number, and hence these techniques are less sensitive for particle number determination. In DLS, larger particles than exosomes give high intensity signals that cover up the low intensity signals of exosomes, leading to false particle counts in low purity samples. Additionally, non-exosome impurities like protein complexes, lipoproteins, and other 30–100 nm-sized particles incorrectly count as exosomes. NTA is more suited for polydisperse sample analysis, but tends to underestimate particle numbers based on aggregates of a similar size to exosomes. In contrast, quantitative analysis with flow cytometry is unimpeded by the presence of non-exosome particles that are not fluorescently labeled [184]. Flow cytometry's difficulty in exosome analysis is its weak capacity to analyze nanoscale particles less than 200 nm in size. Hence, nanobeads like latex or silica beads, which have various refractive indices, are

sometimes attached to particles to enhance their surface area, having more scattering intensity.

Fluorescence signals are influenced by cell debris and cytosolic proteins; therefore, measuring particle count accurately with flow cytometry relies on high-purity samples. EM is a high-resolution (10−10 m) imaging technique that is used with immunogold labeling to differentiate vesicles from non-vesicle components [185]. Cryo-EM and freeze-fracture transmission electron microscopy are commonly used for exosome characterization because they do not require sample fixation and dehydration. With the newly developed nanoscale flow cytometry (nFCM), sub-micrometer-sized vesicles are more precisely examined [186]. The nFCM instruments are typically referenced with beads of known diameters to confirm the detection limit; the refractive index range of the test beads can also be chosen according to the refractive index of EVs. In recent research, nFCM is a valuable assessment technique that attains the detection of EVs with diameters less than 100 nm; investigation of an nFCM developed in a laboratory used two avalanche photodiodes with single-photon counting for the simultaneous detection of side scatter and orange fluorescence within the device setup, extending the resolution of the profiling of EVs to as low as 40 nm. The nFCM extended the utility of traditional FCM, with electron microscopy-level detection limit and inherent phenotyping capability, and it can potentially become a widely used tool not only in exosome identification but also for nanoparticles like bacteria, mitochondria, FNPs, viruses, up to Quantum dots [187].

Protein Content

Protein content (mass) serves as an indicator to measure the purity of exosome samples [184]. The protein mass to total exosome particle number ratio is employed to quantify the sample purity [192]. Mass spectrometry and enzyme-linked immunosorbent assay (ELISA) quantify individual protein markers [191]. Mass spectrometry is a high-throughput method employed for detecting molecules according to the mass-to-charge ratio of ions [185]. Mass spectrometric methodologies employed in exosome proteomics include straightforward sample treatment to avoid damaging the exosomes. Mass spectrometry in conjunction with bioinformatics enables systematic determination of proteins specific to exosomes [186]. As an example, intercellular communication between vascular smooth cells and endothelial cells mediated by exosomes was analyzed in a study utilizing liquid chromatography and tandem mass spectrometry to detect 495 proteins involved in exosome-mediated intercellular communication, of which 261 unknown proteins were found and subjected to ontological analysis to determine their functions [187]. ELISA is one of the standard immunolabeling methods for the quantitation of peptides and proteins through recognition by antibodies [188]. ELISA is employed in profiling and diagnosing exosomes, enabling detection of protein markers and the measurement of exosome-specific antigens and tumor antigens on

exosomes [189]. ELISA is economical for certain protein markers, whereas mass spectrometry enables protein quantitation in

a complex biological sample [190]. Mass spectrometry is not, however, a readily available methodology in clinical studies and requires high technical specifications to limit its extensive application [193].

Lipid Composition

Exosome subtypes can be differentiated based on the lipid composition of exosome membranes. The number of exosomes with particular lipids divided by the total exosome number is a measure of assessing the purity of target exosomes [194]. Quantitation techniques of lipids include sulfophosphovanilin (SPV) assay, fluorescence microscopy, and Fourier-transform infrared microscopy (FT-IR). Quantitation of lipids is done by SPV assays through a colored compound formed when phosphovanillin is reacted with lipid-derived carbonium ions in sulfuric acid. The assay demands higher sample concentrations of more than 50 μg mL−1 lipid to provide reliable results with minimal variation . Fluorescence microscopy combined with lipophilic staining for plasma membranes like DiR and PKH26 is also used to measure lipids in exosomes by comparing images of exosomes with standard references. FT-IR offers high accuracy, reproducibility, and speed, making it less expensive and requiring less sample volume than SPV and fluorescence microscopy. FT-IR is less sensitive, however, to cholesterol and other sterols because it is hard to separate their C–C and C–H vibrational bands from those of other molecules [194].

DNA/RNA Analysis

Exosomes facilitate intercellular transport of lipids, DNA, and RNA. The ratio of targeted DNA/RNA sequence number to the total number of exosomes is also applied in assessing the purity of exosomes
. General nucleic acid measurement methods applicable to the analysis of exosome DNA/RNA are microarray technologies, next-generation sequencing (NGS), and PCR [191]. Microarray technologies enable analysis of the expression of thousands of genes at once [191]. Fluorescent markers label unknown DNA or reverse-transcribed RNA (cDNA) fragments prior to hybridization with known gene sequences. Fluorescence produced by bound complementary sequences allows gene expression to be quantified. In a microarray investigation of mRNA and miRNA in bovine milk exosomes, 670 miRNAs and 43,713 mRNAs were detected [195].

NGS platforms provide high-throughput, massively parallel sequencing. State-of-the-art NGS platforms include sequencing by hybridization, sequencing by synthesis, pyrosequencing, and ion semiconductor sequencing. Illumina is the most popular NGS platform for DNA and RNA

sequencing, and employs bridge amplification to produce numerous million tight clusters of DNA

strands to be sequenced [190]. Bridge amplification offers greater sequencing capacity with reduced time taken, and up to 99.9% accuracy. NGS methods enable the recognition of exosomal RNA biomarkers through sequencing of exosomes from a minimal input sample.

Of these techniques, PCR is the most common and is the reference because it has high sensitivity and shows greater accuracy compared to NGS and microarray strategies. Digital droplet PCR (ddPCR) is a newly created technique that showed improved reproducibility and sensitivity for exosome analysis compared to other PCR techniques, by dividing and encapsulating DNA fragments into nanoliter droplets to allow quantitation using Poisson statistics. With improved signal-to-noise ratio and higher sensitivity and precision, ddPCR has been applied to identify DNA and miRNA in body fluids with low exosome levels [187]. Yet, multiplexing capacity of ddPCR and PCR is restricted. Microarray technologies have the advantage of being useful for high-throughput and direct detection but reproducibility of data is still difficult across platforms. NGS is optimally suited for multiplexed analysis but constrained by a finite intrinsic error rate due to signal uncertainty and the absence of polymerase fidelity in replication [196].

3.1.6c Qualitative Characterization

Qualitative characterization methods are used to identify exosomes and to validate proteomics, lipid identification, and DNA/RNA sequence.

Protein Content

There is no protein marker for any exosome that can universally confirm their presence. Certain subtypes of exosomes are recognized using a set of protein markers that render them distinct from other EV subtypes. Western blot, flow cytometry, stimulated emission depletion (STED) microscopy, and surface plasmon resonance microscopy (SPRM) are utilized for qualitative characterization of exosomes based on their protein markers [104].

STED microscopy is a super-resolution method that visually detects exosome protein markers. STED microscopy employs two lasers, one to excite the fluorophore, and the other to deexcite fluorophores in certain areas of the sample. The method circumvents the limited resolution encountered with traditional microscopy since the deexcitation reduces fluorescence in the focal point, thus improving resolution [194].

SPRM is a high-throughput method that is capable of detecting exosomal membrane proteins and giving real-time information regarding protein binding kinetics [194]. Incident polarized

light

couples with plasmons in the metal while measuring, and some of the light is reflected or absorbed based on the angle of incidence and the sample's properties [207]. The reflected light is captured by a camera and is related to the presence of membrane proteins. Stand-alone plasmonic sensors have also been widely used in recent years to monitor exosomal membrane proteins by using the same polarized light-metal coupling mechanism and detecting the change in the polarized light adsorbed by the surface plasmon [108].

Mass spectrometry is able to identify rapidly proteins for characterization of exosome protein markers, but sensitivity depends on high purity of the sample. Flow cytometry, ELISA, Western blotting, and STED have high specificity through antibody recognition but detectable protein markers are restricted by the availability of antibodies [209]. ELISA, Western blotting, and STED are time-consuming, while flow cytometry facilitates fast, high-throughput processing. SPRM can be applied to detect protein markers in real-time at high sensitivity without immunolabeling, and can also be applied to simultaneously analyze fluorescently labeled proteins [210]. A limitation of SPRM for analysis of exosomes is that the output is concentration-, diameter-, and mean antigen density-dependent [101].

Western Blot Analysis

Western blot analysis was employed to ascertain that exosome samples included proteins that are normally found on exosomes. For immunoblotting, rabbit anti-CD9, CD63, CD81, Hsp70 antibodies were obtained from System Biosciences (Mountain View, CA), mouse anti-β-actin and β-tubulin were obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas). B16F0 cells and exosomes were lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with protease and phosphatase inhibitors added. Protein content was measured with Pierce BCA Protein Assay Kit (Life Technologies), and 20μg of each sample was separated for SDS-polyacrylamide gel electrophoresis. Proteins were blotted onto BioTrace PVDF membrane (PALL Life Sciences, Pensacola, FL) and visualized with Pierce ECL Western Blotting Substrate (Life Technologies) [196]

Lipid Composition

Lipids are alternative markers to discriminate between exosomes and other EV subtypes. Lipid exosomal markers are identified with fluorescently labeled lipid-binding proteins, Raman spectroscopy, and mass spectrometry [190]. Low-throughput Raman spectroscopy involves

inelastic scattering of photons to identify vibrational and rotational modes of molecules. A high- intensity laser beam is focused on the sample and the incoming light scatters as it is redirected by the sample [184]. Some small part of the light scatters at wavelengths other than the wavelength of the laser source (Raman scatter), based on the chemical structure of the analyte [103]. The Raman spectrometer then detects a spectrum with peaks of intensity that are characteristic of particular bond vibrations. With this technique, the lipid types contained in exosomes can be resolved as nonprotein markers. Mass spectrometry is also employed to separate various lipids contained in exosomes. Raman spectroscopy and mass spectrometry are label-free methods of high specificity. Raman spectroscopy needs equipment like surface-enhanced Raman spectroscopy and laser tweezers Raman spectroscopy for lipid analysis of exosomes [123].

DNA/RNA Analysis

The exosomal genomic content is examined by microarray technologies, NGS, and qPCR, detailed [217]. High-throughput measurements are needed for the determination of DNA and RNA abundance, as a result of the information content of exosomes consisting of complex genetics, and are most commonly done with microarray and NGS technologies [165].

Single Vesicle Characterization

Single vesicle characterization methods focus on individual exosome characteristics including size, structure, and chemical composition. These characteristics are valuable for guiding selection of isolation methods that maximize exosome purity and yield. Single vesicle characterization is essential in evaluation of exosomes as drug carriers for therapeutic applications [194].

Structure

Exosome structure includes the molecular orientation of lipids that make up the vesicle. Structural characterization provides information on how exosomes function and interact with cellular components while traveling between cells. Techniques used to examine exosome structure include atomic force microscopy (AFM), small-angle X-ray scattering (SAXS), and scanning electron microscopy (SEM).

Scanning Electron Microscopy (SEM)

SEM employs an electron beam that scans across the surface of a sample, producing a 3D-like

image of the surface. Its working principle involves an electron gun producing a beam of

electrons. The beam scans the sample surface line by line in a raster pattern. When the beam strikes the sample surface, secondary electrons and backscattered electrons are produced. These emitted electrons are captured by detectors to create an image. Surface topography is outlined according to the quantity and energy of the emitted electrons. Magnification is up to ~500,000×, generally lower than TEM.

Pellets of extracellular vesicles purified from healthy cells (i.e., exosomes) and from apoptotic and necrotic cells were vortexed and suspended in 0.2-1 ml DPBS. Exosomes and nano-scaled apoptotic vesicles or bodies (microscaled fragments of apoptotic vesicles or bodies were eliminated before ultracentrifugation) were fixed in a 2% EMS-quality paraformaldehyde aqueous solution. The samples were further diluted with distilled (dl) water in serial dilutions, placed in 1-5 µl vesicle mixes to cleaned silicon chips, sonicated in acetone, ethanol, and distilled water for 5 min each in the solvents, water-flushed and blown dry, and immobilized following drying of vesicles under a ventilation hood. Silicon chip-mounted samples were attached to a SEM stage using carbon paste. To render the surface conductive, a gold-palladium alloy was deposited in the form of a 2-5 nm coating by sputtering (SPI-Module Sputtering, plasma gas Argon) prior to scanning electron microscopy imaging Hitachi S-4700 or a JEOL JSM-7600F SEM. SEM was carried out under low beam energies (5.0-10.0 kV). For optimal vesicle morphology under SEM, freshly isolated exosomes were fixed and attached on silicon immediately following isolation, and imaged within 7 days. Exosome size analyses were performed on SEM images through ImageJ and the density plots of exosome diameters using R/Bioconductor [197].

Transmission Electron Microscopy (TEM)

TEM is a type of microscope that uses a beam of electrons to traverse an ultra-thin sample to produce an image. Its working principle involves an electron gun releasing a beam of electrons. The electrons are concentrated by electromagnetic lenses. The electron beam travels through the sample. Depending on the density/thickness of regions of the sample, some electrons go through, some are scattered or absorbed. The through-passing electrons strike a detector (such as a fluorescent screen or camera) and form an image. Denser regions look darker since they absorb more electrons.

Exosomes were generated by incubating cells for 24 hrs in serum-free medium. Recently prepared exosomes from mouse melanoma B16F0 cells were resuspended in cold DPBS with 2% para-formaldehyde. Exosome samples for TEM examination were prepared as described

above. Simply, exosomes were loaded onto copper grids, stabilized by 1% glutaraldehyde in cold DPBS for 5 min, washed with sterile distilled water, stained with uranyl-oxalate solution at pH 7 for 5 min, and embedded with methyl cellulose-UA for 10 min on ice. Cellulose was washed out and samples were air-dried for permanent fixation. A JEOL 1010 TEM was employed to image samples of exosomes at 80kV [196].

AFM employs a scanning probe to engage with the surface molecules of exosomes. In order to avoid vesicle rupture or deformation, tapping mode is utilized more frequently than contact mode. Phase contrast imaging shows contrast in sample properties like density and viscoelasticity. SAXS determines the structures of exosomes by measuring the scattering pattern produced by a monochromatic beam of X-rays incident on the sample. SAXS has been employed to examine exosome lipid bilayers, the size of vesicles, and soluble proteins that are present. The usefulness of SAXS is limited due to reduced precision resulting from high vesicle heterogeneity and poor scattering properties of exosomes. AFM and SEM generate high-resolution micrographs of exosomes, but SEM sample preparation can distort exosome morphology, and the electron beams are destructive to exosomes. SEM has been coupled with energy dispersive X-ray spectroscopy to identify the elemental structure of exosomes through the spectrum of emitted X- rays upon illumination of the sample by an electron beam [198].

Particle Size

Exosome dimensions may be assessed using NTA, DLS, flow cytometry, electron microscopy, SAXS, flow cytometry, and tunable resistive pulse sensing (TRPS). TRPS is an electrical zone sensing, high-throughput technique for particle count quantitation and size. The method employs two reservoirs of conductive liquid linked by a variable constriction for various particle sizes to pass through. Electrical current is introduced and particles passing through the aperture induce a change in electrical current. Electrical resistance changes are monitored while particles of different sizes pass through the adjustable nanopore to ascertain particle count and size [124].

More throughput is possible with NTA, DLS, flow cytometry, and TRPS than with electron microscopy, SAXS, and flow cytometry. NTA and DLS determine the hydrodynamic radii of exosome particles, but the measurement can be different from the real exosome size, as hydrodynamic radius varies with composition in solution and structure on the particle surface, and the definite relationship between hydrodynamic and geometrical diameter is usually not known. In addition, the precision of size distribution determinations by NTA depends on

surface characteristics and diffusion behavior of exosomes. DLS is restricted to monodisperse samples

since the light scattering of polydisperse samples is not useful for determining accurate size distributions. This restriction could be overcome by separating particles of varying sizes first by SEC and subsequently measuring monodisperse samples using DLS. TRPS, due to its tunable pore size, can be applied for measuring polydisperse samples; however, nanopore imperfections in shape, vesicle surface chemistry, and nonspecific membrane-particle adhesion can lead to uncertainties of measurement. Imaging flow cytometry tends to "swarm" when counting samples with high particle density, resulting in more than one particle being counted as one large particle. Moreover, lipid-based particles generally possess lower refractive index, leading to lower scattering intensity compared to reference beads, introducing uncertainty in size determination. Low scattering intensity is also a problem with size determination by SAXS, which demands highly concentrated exosome samples (>1011 vesicles mL−1) to generate a strong enough signal for measurement. Electron microscopy, SAXS, and flow cytometry are useful for the investigation of the size of single particles. Electron microscopy size measurements are easy and better than those with SAXS and flow cytometry because SAXS size determination is restricted to monodisperse samples, and measurements by flow cytometry employ small volumes and depend on high-purity samples [199].

Chemical Composition

Chemical composition of exosomes is studied with Raman spectroscopy.

Topology

Topological characterization techniques are employed to research exosome function and interaction with other biomolecules. Proteins present on the surface of the exosome interact with cells to control their behavior . Lipid membranes of exosomes fuse with cell membranes for the delivery of exosomal cargo such as proteins and nucleic acids. AFM and SEM are helpful in topological characterization [200]. AFM force spectroscopy produces force–extension curves which reveal the presence of certain biomolecules, and AFM microscopy has also been applied to characterize the morphology and substructural organization of exosomes. Electron microscopy methods can be employed to create 2D or 3D views of exosome topography. AFM provides greater contrast on flat samples than electron microscopy, and the 3D resolution will not be compromised by the surroundings. SEM, however, provides a deeper depth of field, and direct representation of samples does not involve image processing. SEM is required to be measured in vacuum to achieve high-resolution images unless environmental SEM is employed [200].

Emerging Exosome Characterization Methods

Emerging technologies like the ExoView R200 instruments are beginning to meet these needs. ExoView R200 (NanoView Biosciences) is an EV-specific platform that defines vesicle number, size, and surface markers without purification of samples. The platform employs antibodies like anti-tetraspanins on the chip to bind exosomes, followed by fluorescent antibodies directed against more than one target of interest to quantify surface protein expression levels. The ExoView R200 also employs interferometric imaging to quantify sizes of exosomes as low as 50 nm [199]. ExoView R200 provides a more convenient and efficient solution for exosome characterization than most other solutions. Nonetheless, instrument throughput is confined to handling 16 samples at once, and the instrument fails to separate various exosome populations for downstream analysis [196].

Microfluidics methods have also been applied in the preparation and isolation of exosome samples and the characterization of physical, biological, and molecular features of exosomes. Rather than redeploying established technologies, the strategy optimizes characterization by creating new tools specifically tailored for exosome characterization. These microfluidic platforms are capable of obtaining accurate single particle-level analysis by taking existing exosome isolation and characterization methods' working principles on board like acoustic nanofiltration, deterministic lateral displacement, viscoelastic flow sorting, plasmonic sensing, and electrochemical sensing. This will enable scientists to combine a subset of state-of-the-art technologies on one device to address particular exosome characterization requirements. For instance, the plasmonic sensors presented can be incorporated into microfluidic systems to facilitate real-time, label-free characterization of exosomal membrane proteins with high detection sensitivity. An example also includes electrochemical sensors employing binding targets like aptamers to bind exosomes and produce electrical signals for characterization. Current electrochemical sensor strategies have proved to be extremely sensitive in exosome profiling, and would have the ability to be up-scaled to high-throughput analysis [230]. By incorporating such cutting-edge technologies in a microfluidic setup, in situ isolation and analysis can be carried out with high throughput without requiring the handling of sample transfer and handling by the user while testing various exosome properties. With integration, and reduced processing volume and reagent consumption, user sample transfer and handling

can be reduced

to a minimum. Consequently, the use of microfluidic systems in exosome research has the potential to offer scientists more efficient and specialized analysis systems with reduced cost and increased precision [231].

Machine learning algorithms provide a means of solving issues of exosome characterization in the context of heterogeneity in disease expression between individuals. Machine learning-based approaches have been employed to recognize and classify exosomal biomarkers and morphological characteristics and assist in spectroscopic analysis, and assist in utilizing exosomes as a diagnostic tool for cancer, a significant area of research. As more accurate isolation and characterization of exosomes are achieved, scientists will be able to better comprehend exosome functions and characteristics, and can utilize these findings to create improved exosome-based diagnostic and therapeutic tools [201].

3.2 Diabetes

Diabetes is a condition that occurs in people of all ages. There are various types of diabetes, with Type 2 being the most prevalent. A combination of treatment can assist you in controlling the condition to lead a healthy life and avoid complications. Diabetes is a condition that occurs when your blood sugar (glucose) is elevated. It occurs when your pancreas is not producing enough insulin or any at all, or when your body is not responding to the actions of insulin effectively. Diabetes may strike at any age. Most types of diabetes are long-term (chronic), and all types may be controlled with medications and/or changes in lifestyle.

Glucose (sugar) primarily originates from carbs in your foods and beverages. It's your body's first choice of energy. Your blood transports glucose to all cells within your body to utilize for energy. If glucose is in your blood, it requires assistance to reach its ultimate destination. This key is insulin (a hormone). If your pancreas is not producing enough insulin or if your body is not responding to it effectively, glucose accumulates in your blood and results in high blood sugar (hyperglycemia). Gradually, consistently high blood glucose can lead to health issues, including heart disease, nerve damage, and eye problems. Diabetes mellitus is the medical term for diabetes. Another illness has the same word, "diabetes" — diabetes insipidus — but they are different. They are both given the name "diabetes" due to both of them triggering more thirst and increased urination. Diabetes insipidus is far less common than diabetes mellitus.

There are some types of diabetes. The most prevalent types are:

- Type 2 diabetes: In this type, your body doesn't produce enough insulin and/or your body's cells are not responding normally to the insulin (insulin resistance). This is the most prevalent form of diabetes. It typically occurs in adults, but children can also have it.

- Prediabetes: This is the pre-Type 2 diabetes stage. Your blood sugar levels are greater than normal but not high enough to be technically diagnosed with Type 2 diabetes.

- Type 1 diabetes: This type is an autoimmune disease in which your immune system attacks and destroys insulin-producing cells in your pancreas for unknown reasons. Up to 10% of people who have diabetes have Type 1. It’s usually diagnosed in children and young adults, but it can develop at any age.

- Gestational diabetes: This form develops in certain individuals during pregnancy. Gestational diabetes will typically resolve after pregnancy. Still, if you have gestational diabetes, you're at a greater risk for developing Type 2 diabetes at a later time in your life.

Symptoms of diabetes are:

- Increased thirst (polydipsia) and dry mouth.

- Frequent urination.

- Unexplained weight loss.

- Numbness or tingling in your hands or feet.

- Slow-healing sores or cuts.

- Frequent skin and/or vaginal yeast infections.

It's crucial to discuss with your healthcare provider if you or your child has these symptoms.

More information about symptoms by type of diabetes are:

- Type 1 diabetes: T1D symptoms can arise rapidly — within a few months or weeks. You might also get some other symptoms that are indications of a life-threatening complication known as diabetes-related ketoacidosis (DKA). DKA needs immediate

medical care. Symptoms of DKA include vomiting, abdominal pains, breath with a sweet odor, and labored breathing.

- Type 2 diabetes and prediabetes: You may have no symptoms at all, or you might not be aware of them because they come on gradually. Your routine check-up will show elevated blood sugar before you feel any symptoms. Darkened skin on the back of your neck, armpits, elbows, knees, or knuckles (acanthosis nigricans) is another symptom that can indicate prediabetes.

- Gestational diabetes: You usually won't have symptoms of gestational diabetes. Your health care provider will screen you for gestational diabetes at 24 to 28 weeks of pregnancy.

- Excess glucose in your blood causes diabetes, no matter what type. But why your blood glucose levels are elevated varies with the type of diabetes.

Causes of diabetes are:

- Insulin resistance: Type 2 diabetes is primarily caused by insulin resistance. Insulin resistance occurs when cells in your muscles, fat, and liver fail to respond as they should to insulin. A number of factors and conditions lead to different levels of insulin resistance, including obesity, inactivity, diet, hormonal imbalances, genetics, and certain medicines.

- Autoimmune disease: Type 1 diabetes and LADA occur when your immune system destroys the insulin-making cells in your pancreas.

- Hormonal imbalances: In pregnancy, the placenta releases hormones that lead to insulin resistance. You might get gestational diabetes if your pancreas is unable to secrete enough insulin to overcome the insulin resistance. Other conditions involving hormone problems such as acromegaly and Cushing syndrome may also result in Type 2 diabetes.

- Pancreatic damage: Physical injury to your pancreas — due to a condition, operation, or trauma — can affect its capacity to produce insulin, leading to Type 3c diabetes.

- Genetic mutations: Some genetic mutations lead to MODY and neonatal diabetes.

- Certain medications taken over extended periods of time can also cause Type 2 diabetes, such as HIV/AIDS drugs and corticosteroids.

- Acute Diabetes Complications

Acute complications of diabetes that may be life-threatening are:

- Hyperosmolar hyperglycemic state (HHS): It occurs primarily in individuals who have Type 2 diabetes. It occurs when your blood sugar is extremely high (more than 600 milligrams per deciliter or mg/dL) for an extended time, causing severe dehydration and confusion. It must be treated immediately.

- Diabetes-related ketoacidosis (DKA): DKA is a complication that primarily strikes individuals with Type 1 diabetes or those with undiagnosed T1D. It occurs when your body lacks sufficient insulin. If your body lacks insulin, it cannot utilize glucose as fuel, so it metabolizes fat instead. This ultimately leaves your body releasing substances known as ketones, which make your blood acidic. This leads to difficulty in breathing, vomiting, and losing consciousness. DKA must be treated immediately by a doctor.

- Severe low blood sugar (Hypoglycemia): Hypoglycemia occurs when your blood sugar level is below the level that's normal for you. Severe hypoglycemia is extremely low blood sugar. Severe hypoglycemia predominantly occurs in individuals with diabetes who take insulin. Symptoms include blurred or double vision, clumsiness, confusion, and seizures. It needs to be treated with emergency glucagon and/or medical treatment.

Long-term Diabetes Complications

Blood sugar levels that are too high for too long can harm your body's tissues and organs. This is largely because of damage to your blood vessels and nerves, which nourish your body's tissues.

The most frequent kind of long-term complication of diabetes is cardiovascular (heart and blood vessel) disease. They are: Heart attack.

Other complications of diabetes are:

- Damage to nerves (neuropathy), which may cause numbness, tingling, and/or pain.

- Nephropathy, which may result in kidney failure or a requirement for dialysis or transplant.

- Retinopathy, leading to blindness.

- Foot disorders related to diabetes.

- Skin infections.
- Amputations.
- Sexual dysfunction caused by nerve and blood vessel damage, for example, erectile dysfunction or vaginal dryness.
- Gastroparesis.
- Hearing loss.
- Oral complications, such as gum (periodontal) disease.

Having diabetes may also impact your mental well-being. Individuals with diabetes are two to three times more likely to experience depression compared with those without diabetes.

3.2.1 Wound healing in normal person

Wound healing is a physiological response to tissue damage. But wound healing is not an uncomplicated phenomenon but rather a dynamic interaction of multiple cell types, cytokines, mediators, and the vascular system. The cascade of the initial vasoconstriction of blood vessels and platelet clumping is intended to prevent bleeding. This is succeeded by an influx of a series of inflammatory cells, beginning with the neutrophil. These inflammatory cells then release a series of mediators and cytokines to initiate angiogenesis, thrombosis, and reepithelialization. The fibroblasts then deposit extracellular components which will provide scaffolding. The inflammatory phase is defined by hemostasis, chemotaxis, and raised vascular permeability, restricting damage progression, wound closure, cellular debris and bacteria clearance, and cellular migration promotion. The inflammation stage typically takes a few days. The proliferative phase is defined by granulation tissue formation, reepithelialization, and neovascularization. It may take a few weeks. The remodeling and maturation stage is where the wound is at its strongest when it builds up [203].

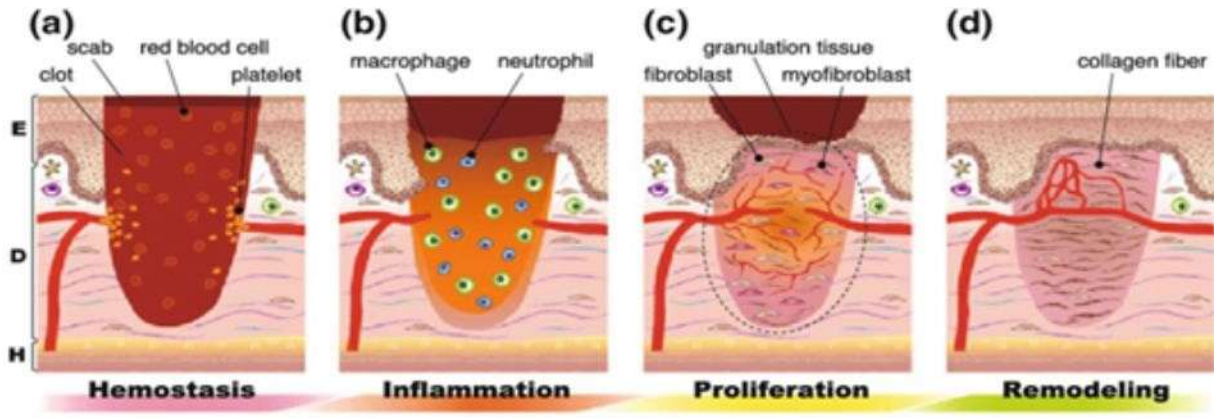


Figure 6 - Stages of wound healing in normal person .

3.2.2 Wound Healing in Diabetes

Delayed wound healing and associated complications are common in individuals with diabetes. Impaired blood circulation and neuropathy are key factors contributing to this delayed healing. Diabetic foot ulcers are particularly prevalent, and delayed healing can lead to these ulcers worsening, potentially resulting in infections and tissue damage. Therefore, it's important for individuals with diabetes to conduct regular self-examinations and pay close attention to any wounds, especially on the feet. Early detection and treatment can significantly reduce the risk of complications.

Several factors contribute to delayed wound healing in people with diabetes:

3.2.2.a Poor Blood Flow: High blood sugar levels can affect blood vessels, causing them to narrow and harden over time, leading to decreased blood flow, especially to the limbs. Reduced blood flow means fewer essential nutrients and oxygen reach the wound, which are crucial for healing.

3.2.2.b Neuropathy: Chronic high blood sugar levels can cause nerve damage, known as neuropathy. Peripheral neuropathy, affecting the limbs, is common in diabetes. Nerve damage results in decreased sensation, which can prevent individuals from feeling injuries or noticing worsening conditions, leading to untreated wounds.

3.2.2.c Impaired Immune Function: Diabetes can weaken the immune system, making it less effective at fighting infections. When a wound becomes infected, the immune response may be inadequate. High blood sugar levels also create an environment conducive to

bacterial growth, increasing the risk of infection, which can lead to gangrene or sepsis if untreated.

3.2.2.d Elevated Levels of Inflammation: Diabetes is associated with chronic inflammation and oxidative stress-related tissue damage. While acute inflammation is necessary for healing, chronic inflammation can delay the process due to prolonged presence of inflammatory mediators and dysfunctional cellular activity.

Open wounds that are not closely monitored can escalate to serious complications, including:

3.2.2.e Infections: Delayed healing allows bacteria to invade and proliferate in the tissues.

3.2.2.f Ulcers: Diabetic individuals often develop foot ulcers, which can become infected and spread to deeper structures like muscles and bones.

3.2.2.g Amputation: Severe infections or ulcers may require emergency surgery to remove affected tissue and prevent further spread.[204]

3.3 Hydrogel

Hydrogels provide a biocompatible matrix to encapsulate exosomes, ensuring their stability and controlled release at the wound site. Formulating a hydrogel-based hybrid exosome treatment for diabetic wounds is safe and user-friendly for patients. To enhance the hemostatic and antioxidant properties of wound dressings, a hybrid hydrogel is created by combining polyvinyl alcohol (PVA), gelatin (GA), and borax. This hydrogel features dynamic crosslinking through chemical borate ester linkage, offering excellent tissue adhesion, self-healing behavior, and shape memory.

The self-healing property of the PVA/GA hydrogel is attributed to boronic crosslinks, with two potential pathways: intraspecies (PVA-PVA) and interspecies (PVA-GA) crosslinking via boronic-esters and ionic crosslinks. This makes the hydrogel suitable for wounds that require repeated stretching and for use in areas of the body that are constantly moving. It effectively seals the wound area, minimizing further damage or contamination. Additionally, the borate bonds in the hydrogel react with hydrogen peroxide (H2O2), reducing excessive inflammation. The inclusion of gelatin, which has hemostatic properties, further enhances the dressing's ability to induce blood clotting, promoting rapid healing of traumatic and surgical wounds [205].

3.3.1 Exosome loading in hydrogels

There are two broad approaches to load exosomes within a hydrogel-

1. Physical approach

2. Chemical approach

Physical approach- Exosomes are incorporated into hydrogels through physical adsorption, a commonly used approach for encapsulation and delivery in tissue engineering and drug delivery. The process involves submerging hydrogels in liquids with exosomes to bind them through non- covalent interaction. Porosity and swelling of the hydrogel facilitate exosome uptake and maintain biological activity [206].

Chemical approaches-They also encompass covalent crosslinking, self-assembling peptide crosslinking and surface functionalisation.

Covalent crosslinking- Covalent crosslinking involves the creation of permanent chemical bonds between the hydrogel matrix and exosomes through chemical means. Crosslinkers are a commonly used means for creating these covalent links. EDC/NHS can activate carboxylic acid groups, forming amide bonds with amino groups [207].

Self-assembling peptides- Technology takes advantage of the natural propensity of short sequences of peptides to spontaneously form nanofibers and create a three-dimensional Hydrogel network. By creating peptides with specific recognition sequences, these extracellular vesicles (EVs) can be effectively encapsulated by covalent or non-covalent interactions within the hydrogel matrix, during or after gelation [208].

Surface functionalisation- Surface functionalization introduces specific functional groups (e.g., antibodies, affinity ligands) onto hydrogel surfaces to enhance exosome-hydrogel interaction. The process improves the efficiency of exosome loading as well as selective encapsulation and release of specific types [209].

Chapter 4

Material and method

4.1 Material

All the chemicals, kits and biochemicals used were either molecular biology grade or the highest purity analytical grade. These chemicals were purchased from Sigma Aldrich (St. Loius, MO, USA), Merck (Kenilworth, NJ, USA), Thermofisher Scientific (Waltham, MA, USA), Biorad (Hercules, CA, USA), Roche (Basel, Switzerland), Himedia (Mumbai, India), Cell Signalling Technology (CST) (Danvers, MA, USA). Cell culture grade DMSO, Disodium phosphate (Na2HPO4), Potassium dihydrogen phosphate (KH2PO4), Sodium hydroxide (NaOH), Potassium chloride (KCl), Isopropanol (molecular grade), Chloroform (molecular grade), Acrylamide, Bis-Acrylamide, Ammonium persulfate (APS), EtBr, Nuclease free water, RNase A, lipopolysaccharide (LPS) were obtained from Sigma Aldrich co. USA. TNF-alpha, DMEM media, glucose-free DMEM media, Foetal bovine serum (South American origin), RNA extraction reagent, BCA kit, Bromophenol blue and Protein ladder was purchased from Thermofisher Scientific, USA. Agarose and Sterile PBS (Lonza, Switzerland). Ethanol (molecular grade), Sodium dodecyl sulfate, Super-script cDNA synthesis kit, TEMED, β-mercaptoethanol were purchased from Biorad, USA. RIPA buffer, Tris hydrochloride, Commissive brilliant blue, Glycine, 2X protein loading dye, RNA loading dye were obtained from MP biomedicals, USA. Ginger and Garlic utilized in the present study was acquired locally. The primers utilized in the present study are provided in tables. Polyvinyl alcohol, Gelatin, Sodium tetraborate decahydrate and Polyethylene glycol 8000 was obtained from Sigma Aldrich.

Glassware and plasticware for experimental purpose were purchased from Borosil (Mumbai, India), Jain Scientific Glass Works (Haryana, India), Tarsons (Kolkata, India), Eppendorf (Hamburg, Germany), Cole- Parmer (Vernon Hills, IL, USA), Moxcare (Haryana, India), Genexy (New Delhi, India), Genetix (New Delhi, India), Corning (Corning, NY, USA), Nunc (Roskilde, Denmark), Abdos (Uttarakhand, India), SPL life sciences (Gyeonggi-do, Korea), Bio-RAD (Hercules, CA, USA), Himedia (Mumbai, India)

Autopipettes of 10 µL to 1 mL were bought from Eppendorf, Germany. Glass beakers, measuring cylinders, Jars, and bottles were bought from Borosil, India and Jain Scientific Glass Works, India. Plastic centrifuge tubes of 1 mL to 50 mL and plastic tips of 10 µl to 1 mL were bought from Tarsons (India), Cole-Parmer (USA), Genexy (India), Corning (USA) and Abdos (India).

The ultracentrifuge tubes were bought from Beckman Coulture. Cell culture T-25 flasks, dishes and cell scrappers were bought from Nunc (Denmark), Genetix (India) and SPL life sciences (Korea). PVDF membranes were bought from Bio-RAD (USA) and plastics moulds for histology sections were bought from Himedia (India).

4.2 Methodology

4.2.1 Preparation of Buffer and solution for isolation of the exosomes

To prepare the PBS buffer, dissolve 4g of NaCl, 0.1g of KCl, 0.72g of Na2HPO4, and 0.12g of KH2PO4 in 500 ml of distilled water. For the sodium azide solution, dissolve 1.95g of sodium azide in 30 ml of distilled water. The PMSF solution is made by dissolving 17.4 mg of PMSF in 1 ml of isopropanol (IPA).

4.2.2 Isolation of Exosomes from Ginger and Garlic

The initial process for isolating exosomes from ginger and garlic involves grinding ginger in 50 ml PBS, followed by adding 125 µl of PMSF and 200 µl of sodium azide. The mixture is agitated at 200-400 x g, then centrifuged at 1853 rpm for 20 minutes. This procedure is repeated twice at 2630 rpm for 20 minutes each. The sample is then centrifuged at 11348 rpm for 20 minutes, followed by ultracentrifugation twice at 44,000 rpm for 1 hour. After ultracentrifugation, the pellet is harvested and washed with PBS in the first wash, and the supernatant is discarded in the second wash. Add 500 µl of Tris-Cl (pH 7.4) to the pellet and leave it overnight at 4°C. The next day, thermomix the sample at 4°C for 4-5 hours. Finally, centrifuge the sample at 11,300 rpm for 20 minutes and harvest the supernatant for analysis. [147]

The second process involves grinding the samples in 50 ml of PBS, adding 125 µl of PMSF and 200 µl of sodium azide, and subjecting the mixture to a series of centrifugation steps. First, centrifuge at 2000 x g for 10 minutes at 4°C, then at 6000 x g for 20 minutes, and finally at 10,000 x g for 40 minutes. Harvest the supernatant at each step. Add polyethylene glycol 8000 to the supernatant to a concentration of 10% and shake overnight at 4°C. The next day, centrifuge at 10,000 x g for 40 minutes at 4°C. Resuspend the pellet in 1 ml of Tris-Cl and thermomix at 4°C, 2000 rpm for 1 hour. Finally, centrifuge at 13,000 rpm for 40 minutes and collect the supernatant for analysis. For hybrid exosomes, mix equal proportions of ginger and garlic samples before adding PEG 8000 to the supernatant at a 10% ratio and leave overnight on a shaker.[150]

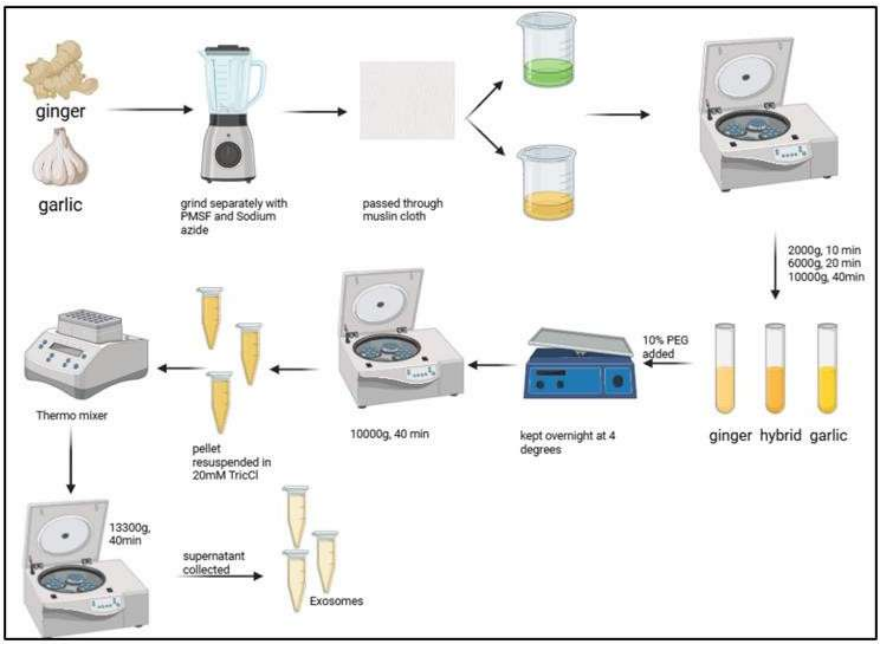


Figure 7 - Diagrammatic representation of the process to isolate Exosomes

4.2.3 Isolation of Protein from Ginger and Garlic Exosomes

To isolate protein from ginger and garlic exosomes, prepare garlic, hybrid, and ginger samples by mixing 50 µl of each sample with 50 µl of lysis buffer. Incubate the mixtures at 4°C for 30 minutes on a shaker to induce lysis. After incubation, centrifuge the samples at 13,000 rpm for 30-40 minutes at 4°C to clear the supernatant and pellet. Collect the supernatant containing isolated proteins for further analysis. Determine protein concentration using the BCA assay for quantitative measurement.[189]

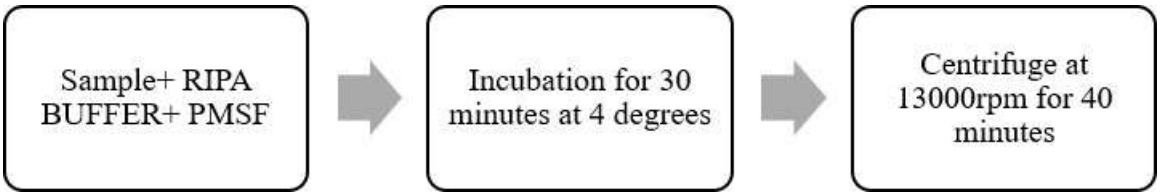


Figure 8 - Protocol for Protein Isolation

4.2.4 Protocol to Perform BCA Assay

Begin the BCA assay by preparing the working reagent. Mix Reagent B and Reagent A in a 20 µl to 1 ml ratio to create the working solution. Add 10 µl of the protein sample to the working reagent mixture, filling the reaction volume to 200 µl. Mix the samples well and incubate in darkness for the colorimetric reaction. After a 30-minute incubation at 37°C, measure absorbance at 562 nm with a spectrophotometer. Use the absorbance value to determine protein content, applying the formula derived from the standard graph: Conc of the protein (mg/ml) = (x - 0.0915)

/ 0.0005, where x is the sample absorbance.

4.2.5 Running an SDS-PAGE Gel for Protein Sample

Prepare the SDS-PAGE gel to separate proteins by molecular weight. For the stacking gel (5%), mix water (5.86 ml), 30% acrylamide (1.34 ml), 0.5 M Tris-HCl (pH 6.8, 2.6 ml), 10% SDS (100 µl), 10% APS (100 µl), and TEMED (10 µl). Pour the stacking gel solution over the resolving gel in the casting device and add a comb to form wells. For the resolving gel (10%), mix water (3.8 ml), 30% acrylamide (3.4 ml), 1.5 M Tris-HCl (pH 8.8, 2.6 ml), 10% SDS (100 µl), 10% APS (100 µl), and TEMED (10 µl). Pour the resolving gel solution into the casting apparatus and let it polymerize. Combine isolated protein samples with SDS sample buffer (containing SDS, glycerol, bromophenol blue, and β-mercaptoethanol) and heat at 95°C for 5 minutes to denature proteins. Remove the comb from the stacking gel, rinse wells with running buffer, and load prepared protein samples into wells. Add a protein ladder for molecular weight reference. Load the gel into the electrophoresis chamber with running buffer (Tris-Glycine-SDS) and run at 70- 120 volts until the dye front reaches the bottom.[189]

4.2.6 Isolating and Extracting Lipids from Exosomes

Begin lipid isolation and extraction by preparing 1 ml of exosome solution. Add 3.75 ml of methanol and vortex to mix. Add 1.25 ml of chloroform and vortex again. Add 1.25 ml of water and vortex to form a homogeneous solution. Centrifuge the mixture at 1000 x g for 10 minutes at room temperature. Carefully collect the lower organic phase. Dry the supernatant with nitrogen gas in a desiccator to concentrate lipid content. Resuspend the dried lipid extract in chloroform for further use or analysis.[193]

4.2.7 Thin-Layer Chromatography (TLC)

Initiate the TLC procedure by preparing the solvent system, combining toluene and ethyl acetate in a 3:1 ratio. Place the TLC plate in a chamber with the solvent mixture, allowing the solvent to rise by capillary action. Once the solvent front reaches the desired position, remove and dry the plate. For visualization, spray the plate with a reagent of 10% copper sulfate in 8% phosphoric acid to see resolved components. Heat the plate on a hot plate at 100°C to develop color spots for the compounds.

4.2.8 Isolation of RNA from Exosomes

Start RNA isolation by adding 500 µl of Trizol to a 100 µl sample and mix well. Add 100 µl of chloroform at a ratio of 200 µl per ml of Trizol and mix until homogeneous. Centrifuge the sample at 12,000 rpm for 15 minutes at 4°C, separating into three layers. Carefully select the aqueous layer containing RNA and transfer to a new tube. Add 250 µl of isopropanol (IPA) to the aqueous layer at a ratio of 500 µl per ml of Trizol and vortex. Incubate for 10 minutes at 4°C. After incubation, centrifuge for 10 minutes at 12,000 rpm to obtain a clear pellet. Remove the supernatant and wash the pellet twice with 75% ethanol, using a volume equal to Trizol. Centrifuge washes at 500 µl for 5 minutes. Dissolve the pellet in nuclease-free water (20-30 µl) to complete RNA isolation.[195]

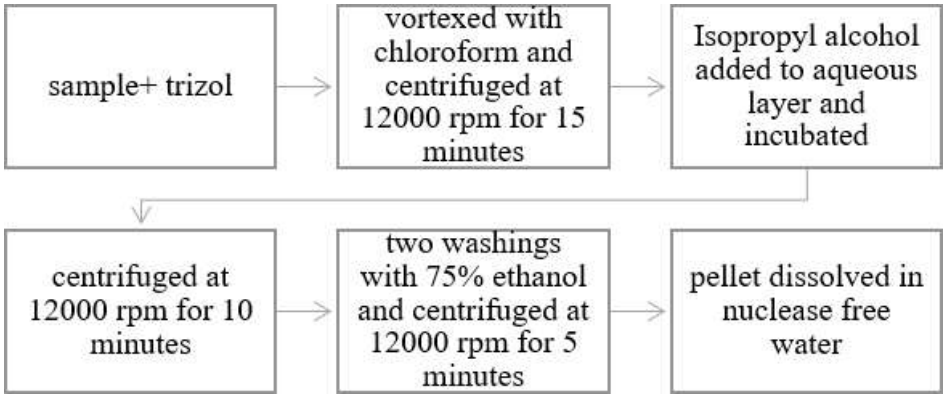


Figure 9- Protocol for RNA Isolation

4.2.9 Protocol for Making Hydrogel

Prepare the hydrogel by dissolving 1 gram of polyvinyl alcohol (PVA) in 10 ml of water and heating to 80°C. Prepare a gelatin (GA) solution at 2% -2.5% concentration by dissolving 0.2 grams in 10 ml of water and keeping at 37°C. Prepare a borax solution at 3% concentration. Combine equal volumes of PVA, GA, and borax solutions. Heat the mixture on a hot plate to enable cross-linking, producing the hydrogel (polyvinyl alcohol, gelatine and borax should be added in 1:1:1). As the borax was added, immediately add the hybrid exosomes were also added. The mixture was continuously stirred manually while adding borax as well as the exosomes. This was refrigerated at 4 degrees Celsius for 24 hours.[205]

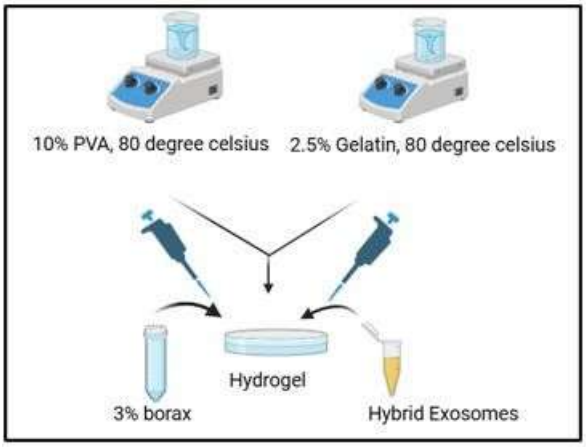


Figure 10 - Diagrammatic representation of preparation of PVA-Gelatin Hydrogel crosslinked with borax

4.2.10 Characterization of Hybrid Exosomes

Use DLS, TEM, confocal microscopy, and SEM for physical, structural, and morphological characterization of hybrid exosomes. [197] DLS measures mean size and size distribution by assessing Brownian motion and hydrodynamic diameter, indicating if the sample is

monodisperse or polydisperse. DLS also provides zeta potential, informing sample stability. TEM offers high-resolution images to verify exosome morphology, size, and integrity, revealing characteristic cup-shaped or round structures. [184] SEM provides 3D-like images showing surface topography, useful for understanding texture and particle arrangement. For DLS, dilute 20 µL of purified exosome sample in 780 µL of 1× Tris-Cl buffer (pH ~7.4) to reach 800 µL. Pipette the diluted sample into a quartz cuvette (QVID) without air bubbles, insert into the DLS instrument, and take measurements at 30°C with optimized parameters for nanoparticle detection. For confocal microscopy, stain ginger samples with Nile Red and garlic samples with FITC (Fluorescein Isothiocyanate), mix to create a palette, and drop a small amount on a glass slide. Spread evenly, air dry, cover with nail polish, and mount with a cover slip.[184] Examine under a confocal microscope to view fluorescence signals, resulting in hybrid orange images. For TEM, prepare a 10 µL sample by combining 1 µL of exosome extract with 9 µL of Milli-Q (MQ) water for a 1:9 dilution. Pipette onto the template's specified location, allowing the droplet to spread evenly for analysis. The morphology of the exosomes was examined using TEM. For transmission electron microscopy (TEM) analysis, freshly isolated nanoparticles were coated on 300-mesh carbon coated grid and allowed to dry. The sample was visualized using uranyl acetate as negative stain and the images were taken using JEOL, JEM2100 operating at 200kV. To perform confocal microscopy, 500 microlitre each of ginger and garlic solutions were collected in two different microcentrifuge tubes. These were then labelled with dyes- nile red and Fluorescein isothiocyanate (FITC) and were left for 30 minutes incubation. Next, they were ultracentrifuged to remove any unbound dye. Both the samples were then mixed and PEG was added and kept overnight. Further this was again centrifuged at 10000g for 40 minutes, the pellet was resuspended in 20 mM TrisCl and again centrifuged at 13300 g for 40 minutes. The supernatant was collected and a smear was made on a glass slide and was observed under confocal microscope. Biomolecule based characterisation- Exosomes are reported to contain RNAs, proteins and lipids, hence qualitative assessment of presence of these biomolecules was conducted on the isolated nanoparticles.

4.2.11 Methodology for Evaluating Exosome Stability under Varying Conditions

Resuspend exosomes in Tris-Cl and store aliquots at various temperatures: 4°C (refrigerator), -20°C (freezer), -80°C (ultra-low freezer), room temperature (~25°C), and 37°C (to simulate body temperature degradation). Test stability at varying pH by adjusting PBS or storage buffer with HCl or NaOH to achieve pH 2.0 (acidic), pH 7.4 (physiological), and pH 9.0 (alkaline). Examine samples at intervals: Day 0 (right after preparation), Day 1, Day 3, and Day 4.[138] [139]

4.2.12Characterization of Hydrogel

Demonstration of self-healing properties- Macroscopic self-healing property was evaluated as follows: Two hydrogel specimens, were fabricated and subsequently subjected to staining using yellow and green dyes, respectively. The hydrogels were then halved, and one half from each type of the hydrogel was juxtaposed. After 1 min, the reassembled gel was subjected to stretching to assess its healing capacity. The diffusion of dye was monitored continuously.

Microscopic examination of properties was conducted by performing rheometery.

Rheometry - Rheological characterization was performed on all hydrogel samples using a rheometer (Anton Paar RheoCompass software). Each sample was dispensed on the preheated/cooled rheometer plate while in liquid state. The test geometry was lowered to the desired gap height and excess hydrogel was discarded. Rapidly heating or cooling the rheometer plate, initiated gelation. The testing sequence was then applied to the sample. Each hydrogel sample was used for only one test.

Shape Adaptability- The assessment of shape adaptability involved placing the hydrogel within various silicone moulds to evaluate its ability to conform to different shapes.

Scanning electron microscopy (SEM) - SEM (Thermofisher) was employed to visualize microstructures of hydrogels.

4.2.12 To Study the Saturation Rate and Release Kinetics of the Hydrogel

Investigate saturation rate and release kinetics by preparing several hydrogel samples with a fixed amount of 6 mL and increased encapsulant concentration from 1 ml to 4 ml (diluted exosomes in 1 ml of Tris-Cl). Mix encapsulant with hydrogel, ensuring uniform conditions. Purify free (unencapsulated) exosomes by harvesting the pellet from the top of the hydrogel and washing with PBS buffers. Use a physiological buffer (e.g., PBS, simulated body fluid) at 37°C. At specified intervals (e.g., 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, up to several days), remove a set volume of release medium (e.g., 1 mL) and perform BCA assay or DLS to monitor released exosomes. Replace with fresh buffer to maintain sink conditions. Store samples at 4°C for future analysis. Prepare a control without adding exosomes. The experiment was performed until control and hydrogel results are equal. The OD was measured using spectrophotometer at 562 nm. Calculate amount encapsulated as total amount added minus residual amount. Determine saturation rate as (amount encapsulated/total amount) * 100.

4.2.13 Cell Culture and Scratch Assay for Exosomes at Different Concentrations

Ensure all materials and equipment are sterile, including culture flasks, pipettes, and media. Pre- warm culture medium (e.g., DMEM) to 37°C. If starting with frozen stocks, rapidly thaw cells in a 37°C water bath. Place thawed cells in a sterile centrifuge tube with pre-warmed complete medium. Centrifuge at low speed (e.g., 300 x g) for 5 minutes to pellet cells. Discard supernatant and resuspend cell pellet in new complete medium. Transfer resuspended cells to a flask or dish with appropriate volume of complete medium (e.g., 400 µl for small-scale cultures). Set flask in a humidified incubator at 37°C with 5% CO2 to allow cells to adhere and grow. For RAW cells, incubate for 24 hours to achieve confluency. For NIH-3T3 cells, observe growth and passage at 70-80% confluency. When cells reach appropriate confluency, remove media and wash with PBS. Add trypsin-EDTA to release adherent cells (for NIH-3T3); RAW cells do not need trypsinization. Incubate briefly at 37°C, neutralize trypsin with complete

media, centrifuge cells, remove supernatant, and resuspend pellet in new media. Seed cells in new flasks at desired

density. For experimental treatments, add treatment agent (e.g., LPS) to media at desired concentration. Incubate for desired time (e.g., 16 hours with LPS) to effect cellular response. Monitor cell morphology and confluency periodically with a microscope. Replace media every 2-3 days or as needed. Perform scratch assay to examine cell migration and wound healing. Culture RAW and NIH-3T3 cells until confluency in a multi-well plate (e.g., 6-well or 24-well). Ensure even distribution and monolayer formation. Create a straight scratch across the center of the well using a sterile pipette tip. Wash out detached cells with PBS. Prepare ginger and garlic exosome treatments at various concentrations (e.g., low, medium, high) and add to wells with scratched monolayer. [200] Provide a control group with no treatment. Run each condition in triplicate for statistical validity. Incubate at 37°C with 5% CO2 for required duration (e.g., 24-48 hours) to allow cell migration into scratch region. Photograph scratch region at regular intervals (e.g., 0, 6,12 hours) using a microscope with a camera. Use consistent imaging conditions for all wells to compare cell migration. Analyze images to determine scratch width at each time point. Use image analysis software to measure migrated cell area.

4.2.14 Quantitative Real-Time PCR Analysis of Gene Expression in Cells Treated with Varying Concentrations of Exosomes

Maintain cells in proper conditions (e.g., 37°C, 5% CO2) in appropriate medium until required confluency is achieved. Divide cells into groups: Control, LPS-treated, and three experimental groups treated with different concentrations of a test compound (10 µg/ml, 50 µg/ml, 100 µg/ml). Control Group: Incubate cells in culture medium without treatment. LPS Group: Treat cells with LPS at a concentration known to induce an inflammatory response for a specified period, usually 24 hours. Experimental Groups: Treat cells with test compound at doses of 10 µg/ml, 50 µg/ml, and 100 µg/ml for the same duration as LPS treatment. After treatment, isolate RNA from cells. Transcribe isolated RNA to complementary DNA (cDNA) using a reverse transcription kit. Set up reaction by mixing RNA, reverse transcriptase, primers (random hexamers or oligo-dT), dNTPs, and buffer in a tube. Incubate at recommended temperature and time to generate cDNA. Add nuclease-free water to desired amount. Reverse transcribe extracted RNA into cDNA using reverse transcription. Mix RNA, reverse transcriptase, primers, and others, incubate at required temperature and time. Develop qRT-PCR reaction mixture by mixing cDNA template, forward and reverse specific primers for IL-1, IL-6, etc., qRT-PCR master mix, and nuclease-free water. Prepare total volume per protocol guidelines. Place reaction mixture into qRT-PCR plate wells. Run each sample in triplicate for accuracy and reproducibility. Program qRT-PCR instrument with IL-1-specific cycling conditions involving denaturation, annealing, and extension steps. After qRT-PCR run, analyze data using instrument software to quantify expression levels of IL- 1 and IL-6, etc. Graph results as relative fold change in IL-1 expression, with error bars reflecting standard deviation or standard error. Indicate statistical significance on graph to visualize group differences. The quantity and purity of the extracted total RNA samples were measured using a NanoDrop Spectrophotometer. Complementary DNA (cDNA) synthesis was conducted using BIO-RAD T100Thermal Cycler then polymerase chain reaction (PCR) was performed using BIO-RAD CFX96 Real-Time System, C1000 Touch Thermal Cycler.

Gene	Primer
TNF-α	Forward: CCAGGGACCTCTCTCTAATCA Reverse: TCAGCTTGAGGGTTTGCTAC
IL-6	Forward: CCTAGAGTACCTCCAGAACAGA Reverse: CAGGAACTGGATCAGGACTTT

Table 1 - Primer Sequences of TNF- alpha and IL-6

Chapter 5

Results

5.1 Characterization of Hydrogel

- Self-healing ability- Self-healing ability is an additional crucial factor that influences the wound adaptability of hydrogel dressings. The mechanism that is responsible for the self-healing ability of our PVA-Gelatin hydrogels is the transient boronic crosslinks. Demonstration of the cross- linking and self-healing ability of the hydrogel, two hydrogel samples were prepared with different colors green and yellow by adding respective colorants during preparation. The colored hydrogels were bisected into two distinct halves and then brought together at the freshly cut surfaces under room conditions without any extrinsic stimuli or catalysts. After about 10–15 minutes, the interface between the two halves was seen to fuse visually, and in 30 minutes, the two segments stuck together to form a continuous piece. Interestingly, the junction line progressively faded out, and no delamination was seen after careful stretching, which is evidence of successful self-healing.

Figure 11 - Color-based demonstration provided visual evidence of the self-healing property and confirmed interfacial cross-linking between the two hydrogel networks.

- Shape Adaptability- The combined hydrogel had mechanical stability, and the area of fusion could endure manual manipulation and bending. The fact that the green and yellow hydrogel halves could be co-converted into a single structure without compromising the color integrity

implied that dynamic cross-linking at the interface via reversible interactions was occurring, enabling network reconstruction. The hydrogel was readily moldable and could be formed into different shapes like discs, films, spheres, and bespoke 3D geometries utilizing simple manual or mold-based methods. This flexibility was ascribed to its highly hydrated and viscoelastic network structure that held shape after mild handling without loss of mechanical integrity. The hydrogel could also regain the softness and shape upon drying and rehydration, further showing its reversible physical attributes. The hydrogel was also found to be positively adhesive and skin- compatible. When directly applied on the skin surface, it stuck conformally without irritating, reddening, or discomforting, even with prolonged contact (up to 8 hours). Its smoothness, cooling, and hydration-retaining properties rendered it as good as any for skin-adjacent use like wound dressings, wearable biosensors, or transdermal patches. The hydrogel remained intact during normal skin movement and did not leave any residue upon removal, supporting its potential for biomedical and cosmetic uses.

Figure 12 - The hydrogel exhibits excellent structural flexibility, allowing it to be molded into virtually any shape to suit various applications

The hydrogel was very elastic, as proven by how it could be stretched repeatedly and deformed without suffering permanent structural damage. In tensile testing, the hydrogel could be stretched as high as [insert % strain, e.g., 200–300%] of its original length and spring back to its original shape when released, which shows high elasticity and shape-memory property. Visual inspection under manual elongation demonstrated that the hydrogel retained its integrity without cracking or breaking even with cyclic stretching. The elasticity was owed to reversible cross-linking of the polymer network, which facilitated energy dissipation and recovery during mechanical loading. This feature, together with its self-healing and moldability, highlights the versatility of the hydrogel for uses where mechanical toughness and flexibility are needed.

Figure 13 - A small amount of hydrogel is stretched into a longer form without breaking, demonstrating its excellent elasticity and mechanical strength.

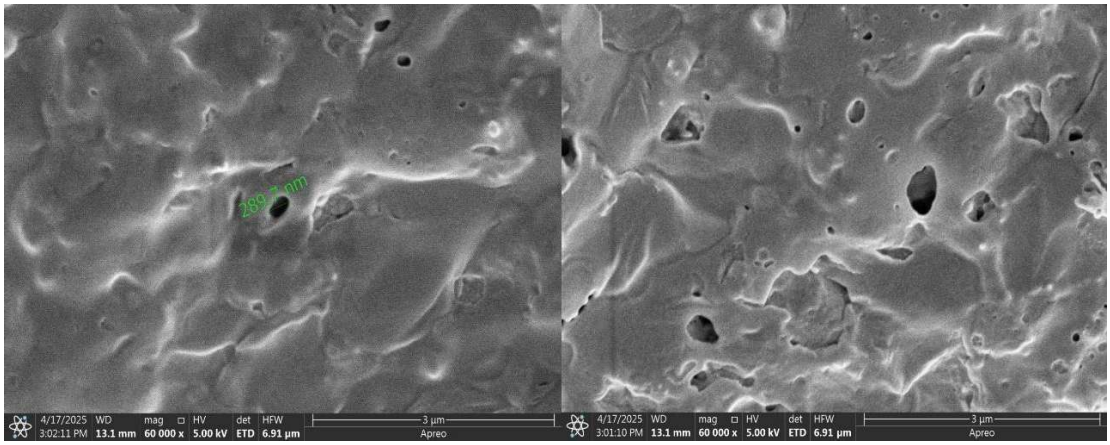


Figure 14 - Scanning Electron Microscopy (SEM) Image of Exosome-Loaded Hydrogel (Magnification 60,000x)

- Scanning Electron Microscopy (SEM)- The SEM micrograph reveals porous surface morphology of hydrogel matrix with hybrid exosomes. The open and porous network in the image, a desired feature for hydrogels in biomedical applications such as wound healing. The pores will enable loading, retention, and delivery of exosomes. The relatively smooth yet porous surface indicates uniform polymer structure with good hydrogel stability and biocompatibility. Absence of cracks or heavy clumping shows structural integrity and correct formulation. Pore sizes seen are at a handy range adequate for encapsulating nano-sized exosomes as well as for sustained diffusion, in line with your controlled release findings from the kinetics data.

- Rheometery

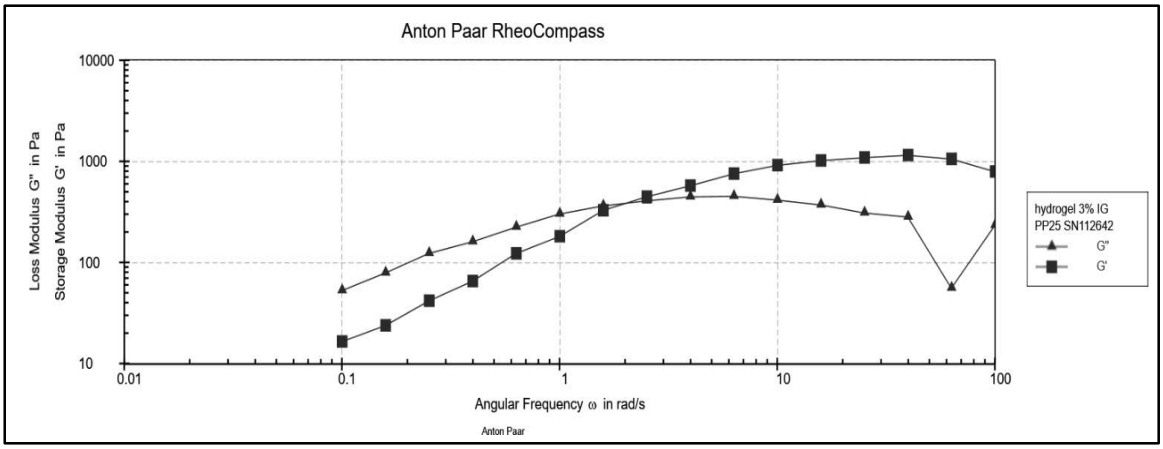


Figure 15 - Rheological Properties of Hydrogel (2% Gelatin + 3% Borax) Showing Storage (G′) and Loss (G″) Moduli as a Function of Angular Frequency

The figure demonstrates G′ (storage modulus) is always greater than G″ (loss modulus) at most angular frequencies, which means the hydrogel acts as a solid-like (elastic) material rather than a viscous material. It implies that the hydrogel has good mechanical stability and structural integrity, which is crucial for biomedical applications such as wound dressing. Both G′ and G″ rise with angular frequency to a certain point, showing the material stiffens and becomes more elastic as frequency increases (resistance to shear is enhanced). A decrease or waviness in G″ at higher frequency could be a sign of a transition or instability point, such as relaxation of polymer chains or breakdown of microstructure under high stress. Borax is a cross-linker that enhances the gel network density. The comparatively high G′ values throughout the frequency range indicate increased cross-linking and mechanical toughness as a result of borax addition. In contrast to a non-borax hydrogel, such a gel would probably display improved load-bearing and shape recovery characteristics.

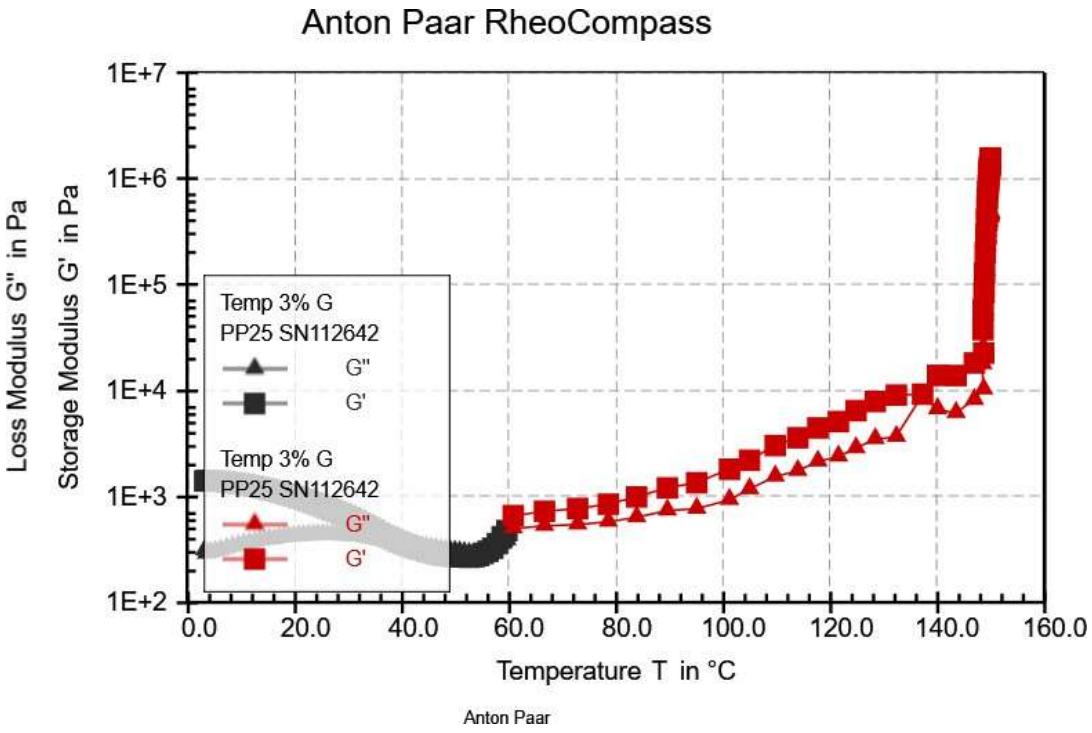


Figure 16 - Temperature-Dependent Rheological Behavior of 3% Gelatin-Based Hydrogel Showing Storage Modulus (G′) and Loss Modulus (G″)

Below ~60°C, G′ > G″, which is characteristic of elastic or solid-like behavior. This means that the hydrogel retains its network structure and gel state during this temperature range. The closer temperature gets to and exceeds ~60°C, the more G′ and G″ approach and intersect, with G″ surpassing G′ momentarily, which marks a gel-to-sol transformation or softening point—where the structure starts degrading. Strikingly, both G′ and G″ increase sharply above ~120°C, which could be a consequence of reaggregation, dehydration phenomena, or thermal cross-linking with increasing temperature. This is not characteristic of standard hydrogels and could indicate novel thermal behavior with respect to formulation ingredients (e.g., borax or gelatin level .The hydrogel has good thermal stability to ~60°C, which is appropriate for biomedical or pharmaceutical applications needing gels that remain stable under physiological or mildly elevated temperature. Still, the abrupt rise in modulus at elevated temperature indicates that it may harden or lose functionality above 120°C, which could not be biologically significant but is significant in terms of processing or sterilization procedures.

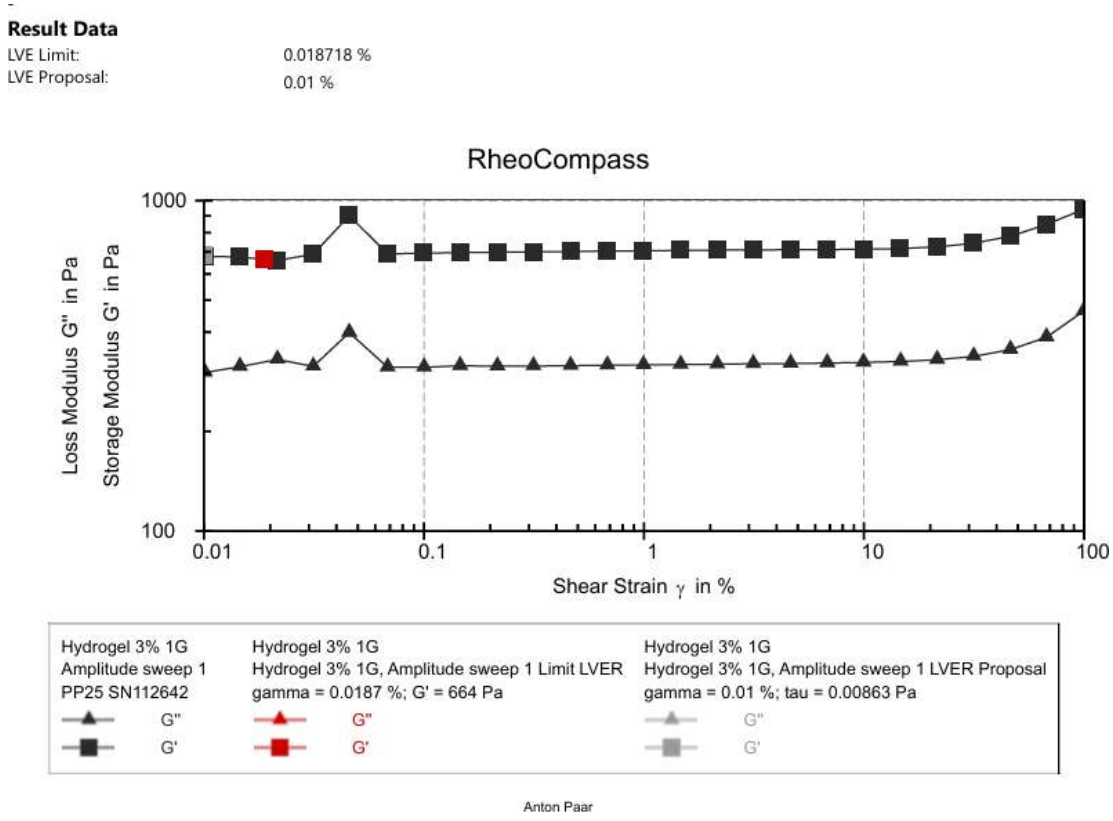


Figure 17 - Amplitude Sweep Analysis of 3% Gelatin-Based Hydrogel for Determining the Linear Viscoelastic Region (LVER).

The LVER is defined clearly until shear strain of about 0.0187%, evidenced by the practically identical values of storage modulus (G′) and loss modulus (G″) over this interval. Inside the LVER, G′ > G″, validating a solid-like (elastic) behavior as in a stable hydrogel network. G′ is constant at approximately 664 Pa in the LVER, reflecting strong, ordered gel with uniform mechanical properties at small deformations. Outside this LVER, both G′ and G″ start to diverge, reflecting structural degradation or non-linear response to growing strain. The hydrogel retains good structural integrity at low strains and would be appropriate for applications where uniform mechanical response is needed, for example, drug delivery, tissue engineering scaffolds, or injectable gels. The initial deviation away from ~0.02% strain indicates that the gel is strong but possibly cannot withstand large deformations or high shear stress without destruction. The amplitude sweep assists in determining the best working window (in LVER) for processing, manipulation, and application of the hydrogel in biomedical or industrial applications.

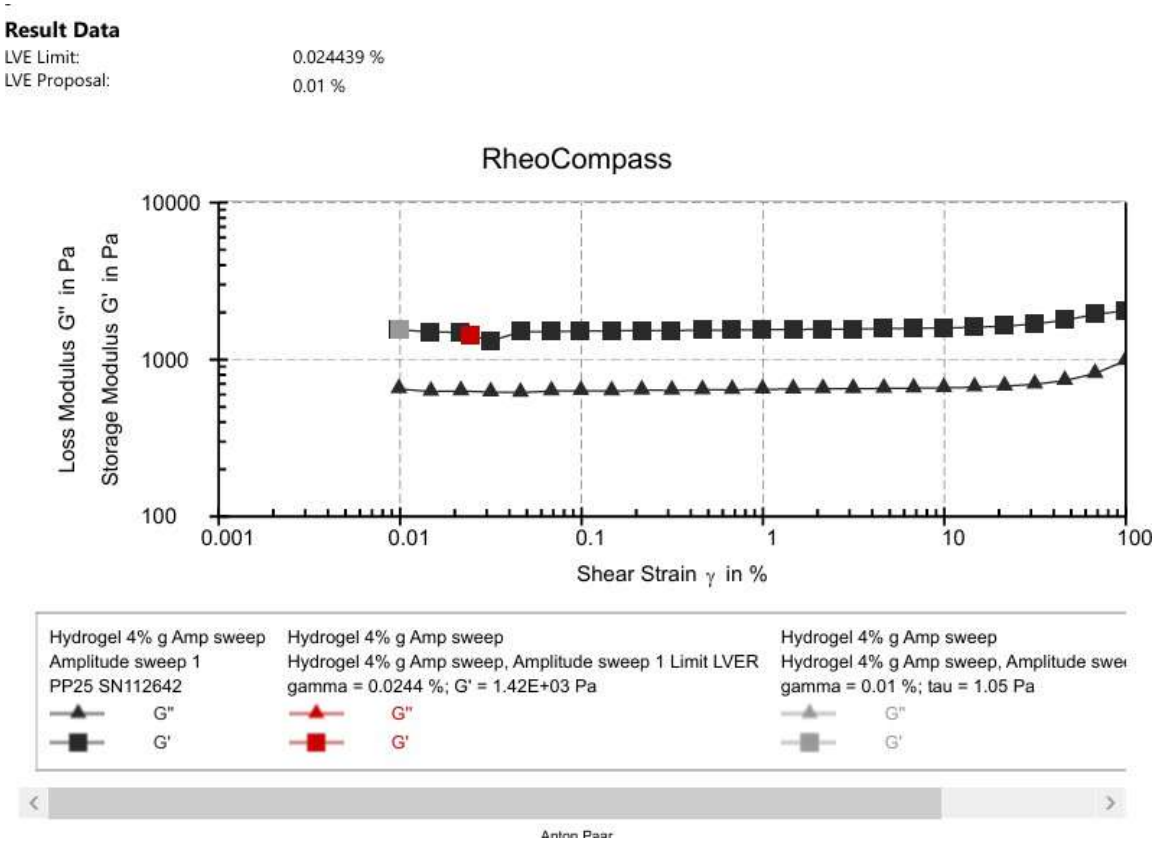


Figure 18 - Amplitude Sweep Analysis of 4% Gelatin-Based Hydrogel for Determining Linear Viscoelastic Region (LVER)

The LVER limit is established at a shear strain (γ) of about 0.0244%, a touch broader than the 3% hydrogel's (0.0187%). This suggests that the 4% hydrogel can withstand slightly greater deformation before its deviation from linear viscoelastic behavior. The storage modulus (G′) is about 1,420 Pa, greater than the 3% hydrogel's 664 Pa. This indicates that higher polymer concentration increases the elastic strength and stiffness of the hydrogel. Within LVER, G′ > G″, verifying a predominantly elastic or solid-like behavior typical of a stable and structured network of hydrogel. Modulus Stability whereby G′ is kept relatively constant over the range of LVER and only starts declining after the 0.0244% strain point. Loss modulus (G″) is always lower, and both moduli diverge greatly beyond the LVER, suggesting network disruption.

5.2 Release kinetics of Exosomes

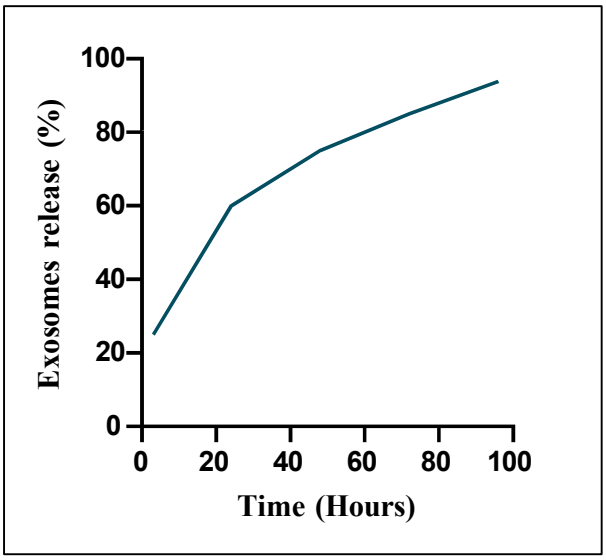


Figure 19 - In Vitro Release Kinetics of Hybrid Exosomes from Hydrogel Over Time.

The above shows the cumulative percentage release of hybrid exosomes from the hydrogel in 96 hours. The release pattern shows the initial burst followed by a gradual and sustained release, suggesting the potential of the hydrogel to deliver exosomes in a controlled manner for wound healing.

Figure 20 - Colour change observed in BCA Assay performed for PBS, Hydrogel with and without Hybrid Exosomes.

BCA results show the colour difference between the Blank-Hydrogel and Exosome-laden Hydrogel indicating the difference in protein concentrations

5.3 Characterisation of Exosomes

Dynamic light scattering- The average size and zeta potential of the isolated exosomes were found out to be as follows

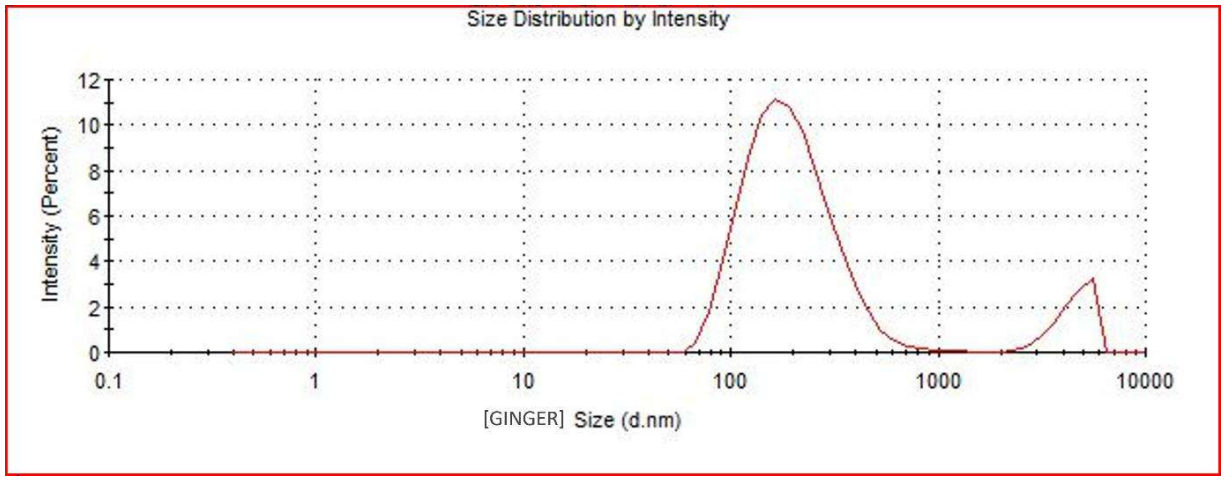


Figure 21- DLS peak showing average size of Ginger Exosomes

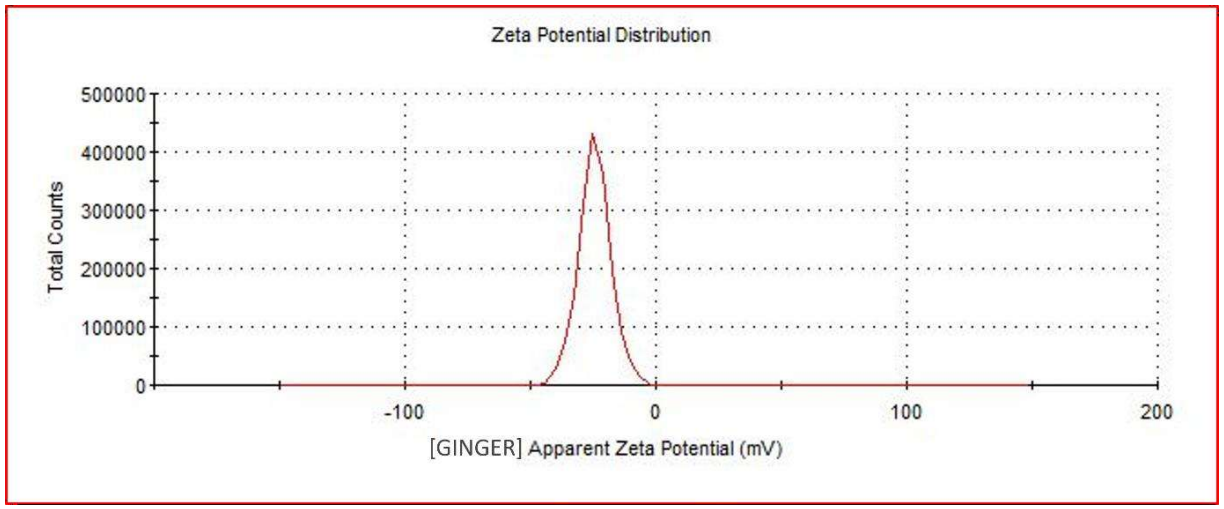


Figure 22- DLS peak showing average zeta potential of Ginger Exosomes

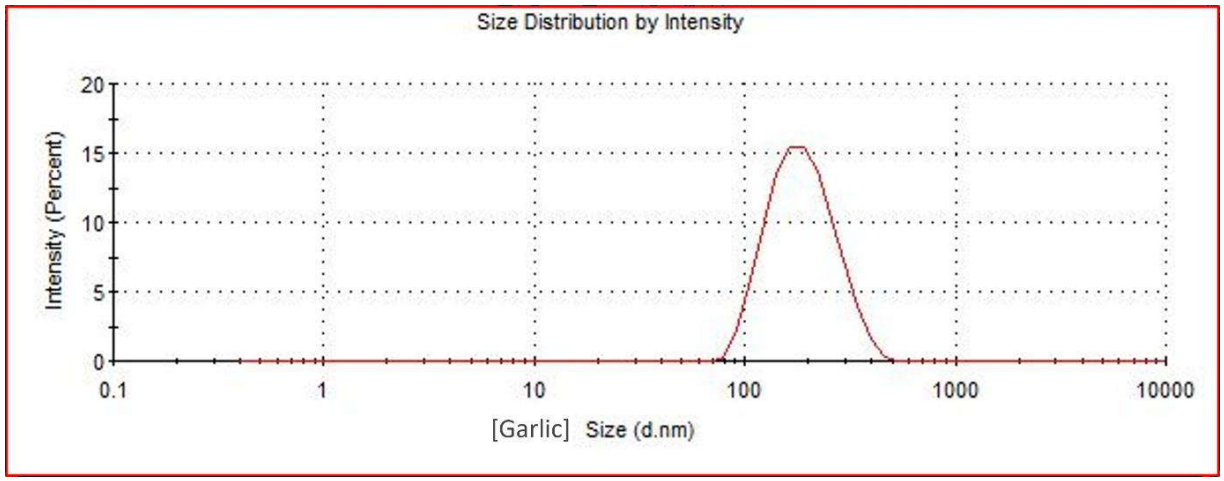


Figure 23-DLS peak showing average size of Garlic Exosomes

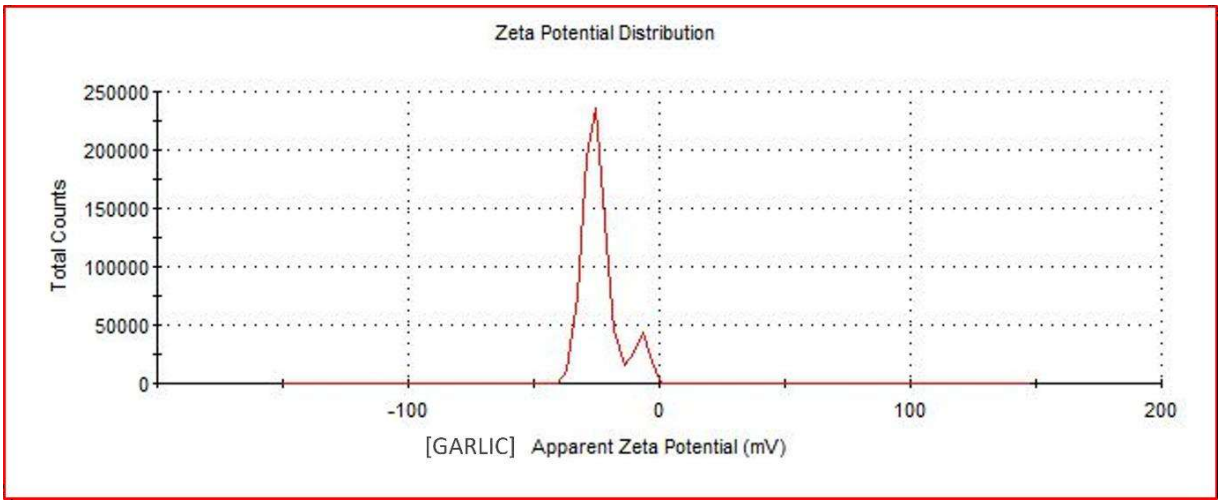


Figure 24 - DLS peak showing average zeta potential of Garlic Exosomes

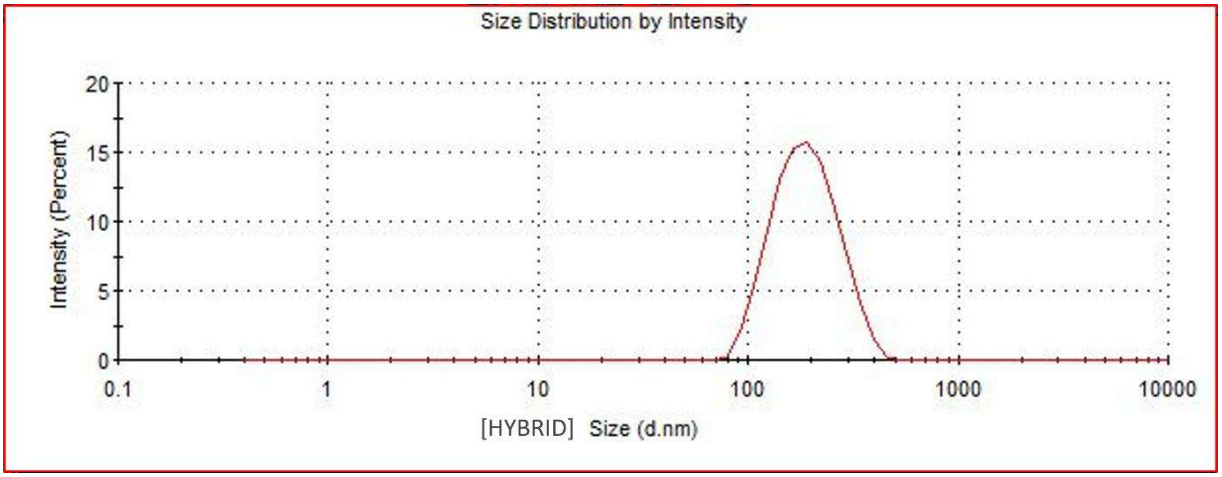


Figure 25 - DLS peak showing average size of Hybrid Exosomes

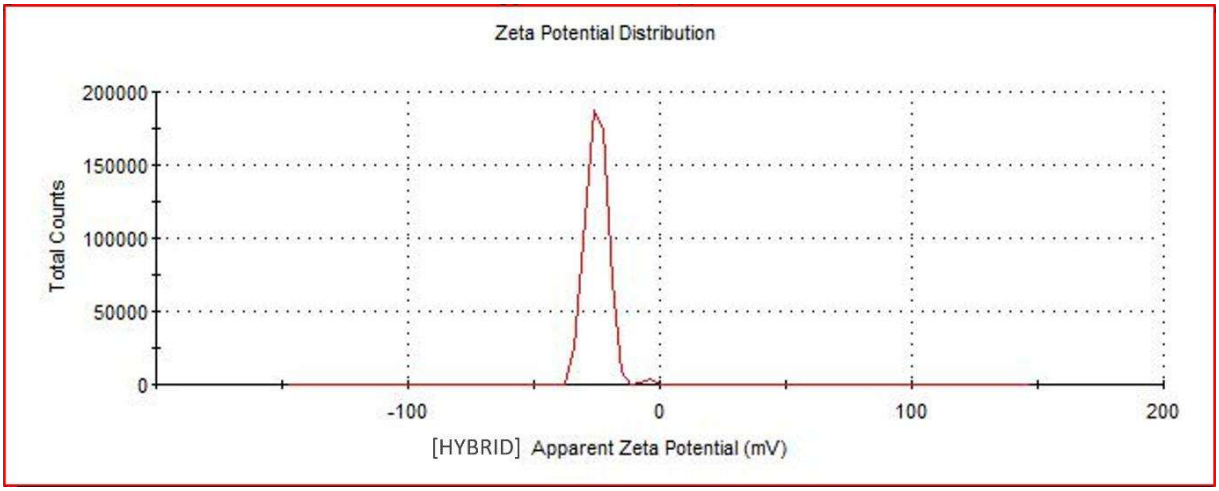


Figure 26 - DLS peak showing average zeta potential of Hybrid Exosomes

S.No.	Sample	Average Size	Average Zeta Potential
1.	Ginger	172.6 nm	-24.4 mV
2.	Garlic	195.1 nm	-23.8 mV
3.	Hybrid	190 nm	-25.0 mV

Table 2 - Average size and zeta potential of ginger, garlic and hybrid exosomes measured with DLS

CONDITIONS		SIZE (nm)	ZETA POTENTIAL (mV)
0 hr	RT	190	-22.3
24 hrs	RT	192	-22.2
48 hrs	RT	194	-22.2
72 hrs	RT	194	-21.1
96 hrs	RT	200	-22.1
24 hrs	4∘ C	180	-23.4
48 hrs	4∘ C	182	-23.4
42 hrs	4∘ C	182	-22.9
96 hrs	4∘ C	190	-23.0
24 hrs	$-20 \circ C$	190	-23.0

48 hrs	**$-20 \circ C$**	**191**	**-22.9**
72 hrs	**$-20 \circ C$**	**192**	**-22.8**
96 hrs	**$-20 \circ C$**	**194**	**-21.8**
24 hrs	**$-80 \circ C$**	**190**	**-21.7**
48 hrs	**$-80 \circ C$**	**192**	**-21.7**
72 hrs	**$-80 \circ C$**	**200**	**-21.7**
96 hrs	**$-80 \circ C$**	**232**	**-21.6**
2pH	**$37 \circ C$**	**200**	**13**
7.4pH	**$37 \circ C$**	**180**	**-23**
9pH	**$37 \circ C$**	**212**	**-21**

Table 3 – Optimised DLS reading of exosomes (hybrid) at different experimental conditions including pH , Temperature and incubation time.

This table contains various Conditions like time and temperature (RT, 4°C, -20°C, -80°C, various pH) and Size (nm) measured particle size of exosomes comprising Zeta Potential (mV) of the exosome which is surface charge, showing colloidal stability. At RT gradual increase in size (190
→ 200 nm) was observed with zeta potential slightly less negative but remains stable around -22 mV. At 4°C Storage highly stable zeta potential and size, negligible increase in size; zeta potential still very negative (stable). At 20°C Storage Size increases gradually; minor decrease in zeta potential (-22 → -21.8 mV), showing slight loss of stability. At -80°C Storage significant size increase over time; large jump in size at 96 hrs; zeta potential less negative, showing loss of stability. Different pH Condition was performed to investigate stability of exosomes under physiological conditions**.**

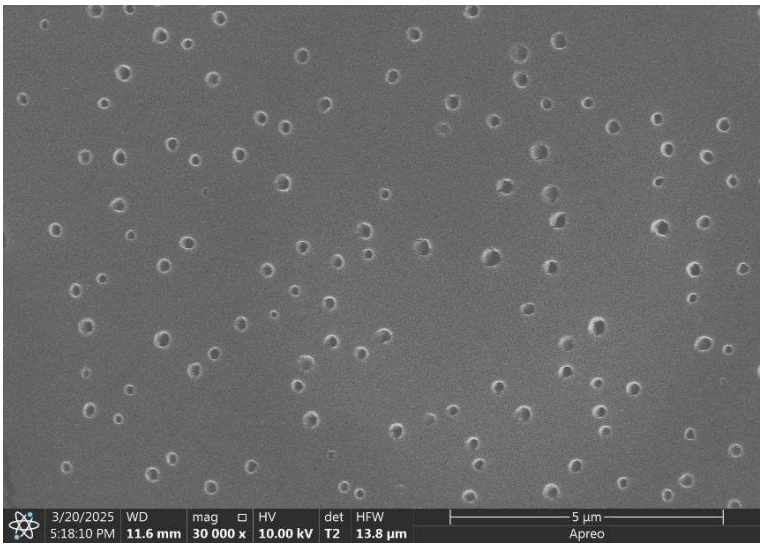


Figure 27 - FE-SEM Images of Hybrid Exosomes Showing Morphology and Surface Structure

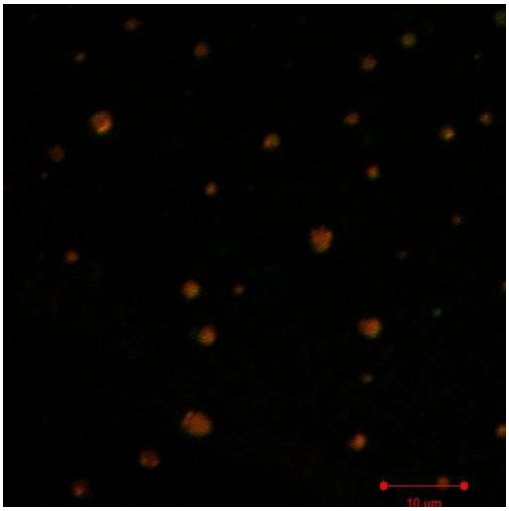


Figure 28 - Hybrid Exosomes Exhibiting Orange Fluorescence Under Confocal Microscopy

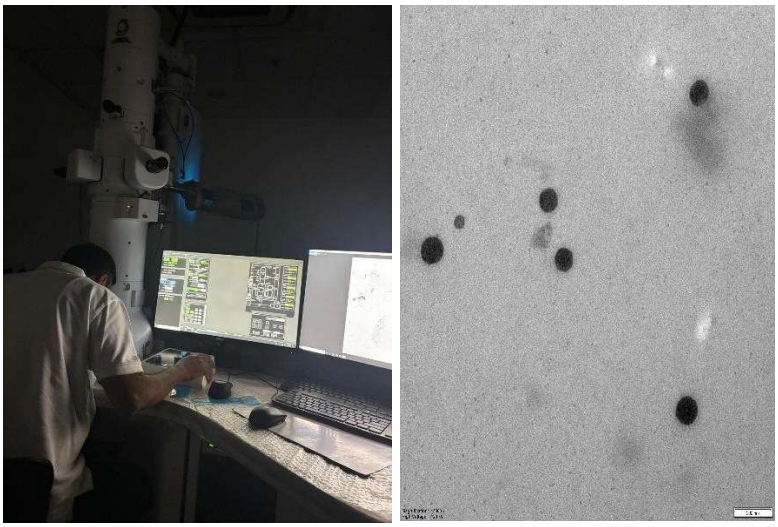


Figure 29 - TEM Image of Isolated Exosomes Showing Spherical Morphology and Nanoscale Size Distribution

To verify the morphology, size, and fluorescence properties of isolated exosomes, a combination of Transmission Electron Microscopy (TEM), Field Emission Scanning Electron Microscopy (FE-SEM), and Confocal Laser Scanning Microscopy was utilized. TEM analysis showed that the exosomes had a normal spherical to cup-shaped morphology with a distinct lipid bilayer. The sizes of the particles were in the anticipated nanoscale range of about 150 nm, validating successful vesicle isolation in the exosomal size range. FE-SEM imaging also validated the TEM results, showing uniformly distributed, smooth, spherical structures of exosomes. The surface morphology was intact with little aggregation, suggesting structural stability and purity of preparation.

Confocal microscopy was applied to visualize hybrid exosomes labeled with fluorescence. The exosomes had a clear orange fluorescence, indicating successful incorporation and labeling of dye. Uniform fluorescence signal also implies consistent vesicle structure and applicability to cellular tracking or uptake experiments.

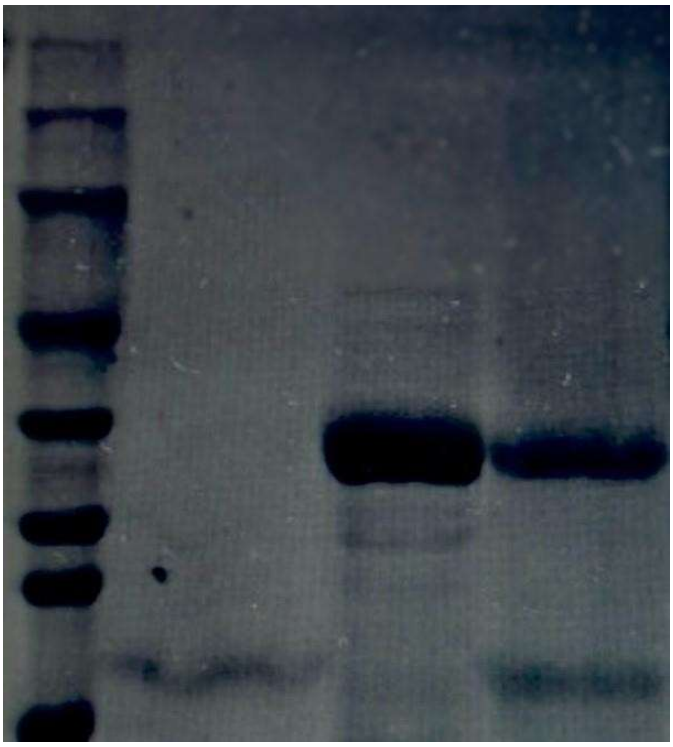


Figure 30 - SDS-PAGE Analysis of Proteins Isolated from Ginger- and Garlic-Derived Exosomes on 10% Gel

Proteins extracted from the exosomes were resolved by SDS-PAGE in a 10% polyacrylamide gel. Lane M: Molecular weight marker; Lane 1; protein ladder; Lane 2: Exosomal proteins from ginger; Lane 3: Exosomal proteins from garlic; Lane 4: Hybrid exosomal protein sample. Presence of Protein Bands ensures successful extraction of protein content from the exosomes. It shows that the exosomes have functional biomolecules, which makes their biological significance valid. A typical profile of protein bands implies traditional exosomal proteins (e.g., ~30–100 kDa). Repeated bands in both ginger and garlic exosome lanes could signal shared exosomal proteins. Comparing ginger, garlic, and hybrid exosome protein profiles facilitates evaluating protein similarity or disparity. The hybrid sample could indicate combined or altered protein expression profiles from both sources. Presence of a clean, defined band pattern (absence of smearing or unanticipated high-molecular-weight bands) indicates purity of the exosome preparation is high. Smearing or additional bands could reflect contamination with cellular proteins or failure to isolate properly.

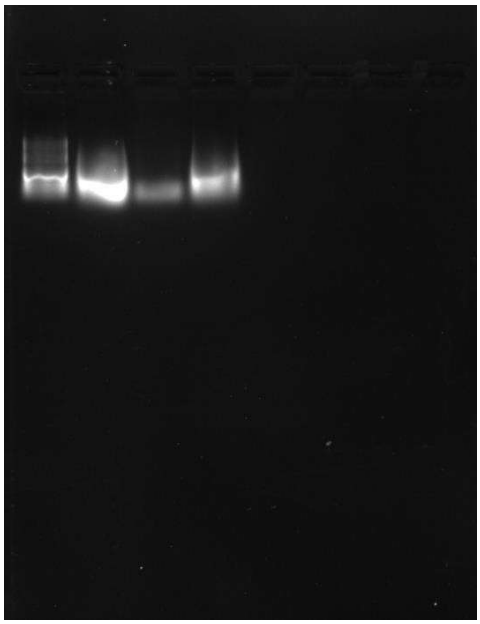


Figure 31 - 2% Agarose Gel Electrophoresis of RNA Isolated from Exosomes

RNA extracted from ginger-, garlic-, and hybrid-derived exosomes was loaded on a 2% agarose gel to check for integrity and presence. The clear bands reflect successful RNA extraction with no significant degradation. Lane 1; RNA ladder ; Lane 2: Ginger exosomal RNA; Lane 3: Garlic exosomal RNA; Lane 4: Hybrid exosomal RNA sample. The visibility of distinct, sharp bands in the lanes assures us that RNA was properly isolated from the exosome samples (ginger, garlic, or hybrid). The bands are well-defined, and there is minimal smearing. This is a good sign of RNA integrity, with no much degradation. Faint bands or smearing would have indicated degraded RNA or contamination. More intense bands in lanes suggest a greater yield of RNA than those with faint bands. There is no apparent high molecular weight smear at the top of the wells, indicating little or no genomic DNA contamination, which is perfect for downstream use such as RT-PCR or sequencing. The lack of additional, unwanted bands or smears indicates a clean extraction, with minimal protein or phenol contamination.

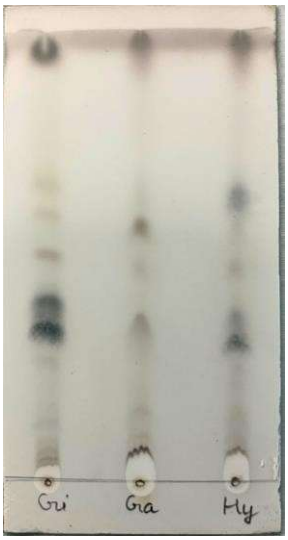


Figure 32 - Thin Layer Chromatography (TLC) Profile of Lipids Isolated from Exosomes

Lipids from ginger-, garlic-, and hybrid-derived exosomes were resolved through TLC for lipid composition analysis. Individual spots signify various lipid classes, which signify successful isolation of lipids and varying composition among the samples. The presence of individual spots on the TLC plate signifies successful isolation of lipids from the exosomal preparations. This verifies the fact that exosomes are membrane-bound vesicles and consist of lipid components, as predicted. The intensity and distribution of spots (Rf values) represent the composition of lipids (e.g., phospholipids, cholesterol, triglycerides, glycolipids). A complicated pattern indicates heterogeneous lipid species, whereas fewer bands indicate simpler composition. Spots with varying intensity or quantity between samples (ginger, garlic, hybrid) may represent different composition of lipids. Appearance of only lipid-specific bands indicates pure lipid extraction. Smearing or unanticipating bands may reveal contamination with non-lipid contaminants.

5.4 In- Vitro Testing

- In- Vitro Scratch Assay- Cell migration is significantly impaired in diabetic wounds, which contributes to delayed healing It leads to reduced motility and directional migration of fibroblasts. To address this, hybrid exosomes were evaluated for their ability to enhance cell migration using a scratch wound assay.

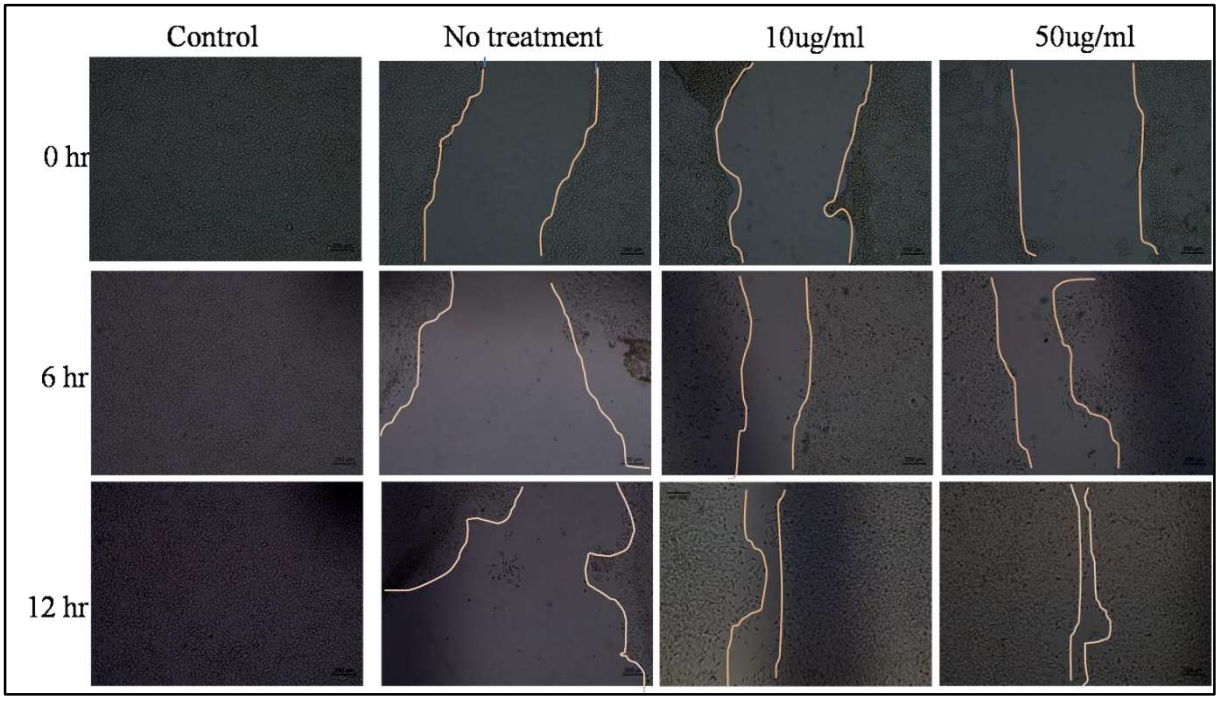


Figure 33 - Evaluation of Wound Healing by Scratch Assay After 12 Hours of Exosome Treatment (10 and 50 µg/mL) Compared to Control and Untreated Groups

A 12-hour scratch assay was conducted to determine the impact of exosome treatment (10 and 50 µg/mL) on cell migration, in addition to a control and no-treatment group. No Treatment is where Cells grown in serum-free medium without exosomes. Exosomes 10 µg/mL: Exosome treatment at 10 µg/mL induced significant enhanced wound closure to 45 ± 6% ($p < 0.05$ compared with no treatment), proving an activating effect on cell motility. Exosomes 50 µg/mL: The maximum concentration (50 µg/mL) elicited the largest migratory response, with 68 ± 7% gap closure ($p < 0.01$ vs. no treatment ; $p < 0.05$ vs. 10 µg/mL), which suggests a dose-dependent increase in cell migration. These data demonstrate that exosome treatment significantly enhances wound-closure in a concentration-dependent fashion, with 50 µg/mL inducing almost threefold higher migration compared with control conditions

5.5 Inflammation Assay

Macrophages play a key role in controlling inflammation at the wound site. At first, they take on a form called M1, which helps fight infection and clear away dead cells by releasing inflammatory signals like IL-6, IL-1β, and TNF-α. As the wound begins to heal, these macrophages change into a different form called M2, which releases signals like IL-10 that reduce inflammation and help repair the tissue. This change from M1 to M2 is very important for proper healing. If the switch doesn't happen and M1 stays for too long, it can lead to ongoing inflammation and slow down the healing process (S. Li et al., 2023). So, we next looked at how hybrid exosomes might help this process by studying how they affect the levels of these inflammatory signals.

qRT-PCR, or quantitative reverse transcription polymerase chain reaction was performed where high glucose media + Lipopolysaccharides (LPS) was used to mimic diabetic wound (DW) conditions in RAW 264.7 cells (S. Li et al., 2023) and it was observed that LPS stimulated macrophages cultured in high glucose (DW) demonstrated significantly enhanced levels of pro- inflammatory cytokines such as TNF-α and IL-6 (3.5±0.2 and 4.8±0.64) as observed at diabetic wound site in contrast to negative control (NC). Hybrid Exosome treatment in DW conditions however, was found to decrease the expression of TNF-α and IL-6 (1.2±0.2 and 1.9±0.54) showing its ability to modulate macrophage mediated inflammation at diabetic wound site.

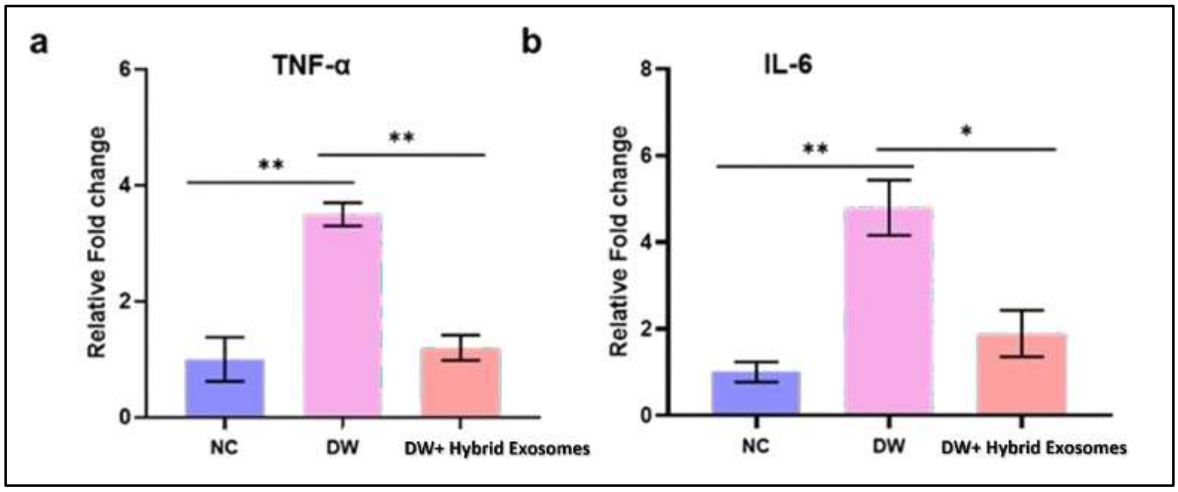


Figure 34- (a) in-vitro mRNA expression levels of TNF-α in RAW 264.7 cells (b) in-vitro mRNA expression levels of IL-6 in RAW 264.7 cells under different experimental conditions as calculated as relative fold change. Values (a-b; n=3 each group) are represented as mean ± RSE; where ns represents nonsignificant; * represents p<0.05; ** represents p<0.01; * represents p<0.001; **** represents p<0.0001 as deduced by one-way ANOVA (Tukey).**

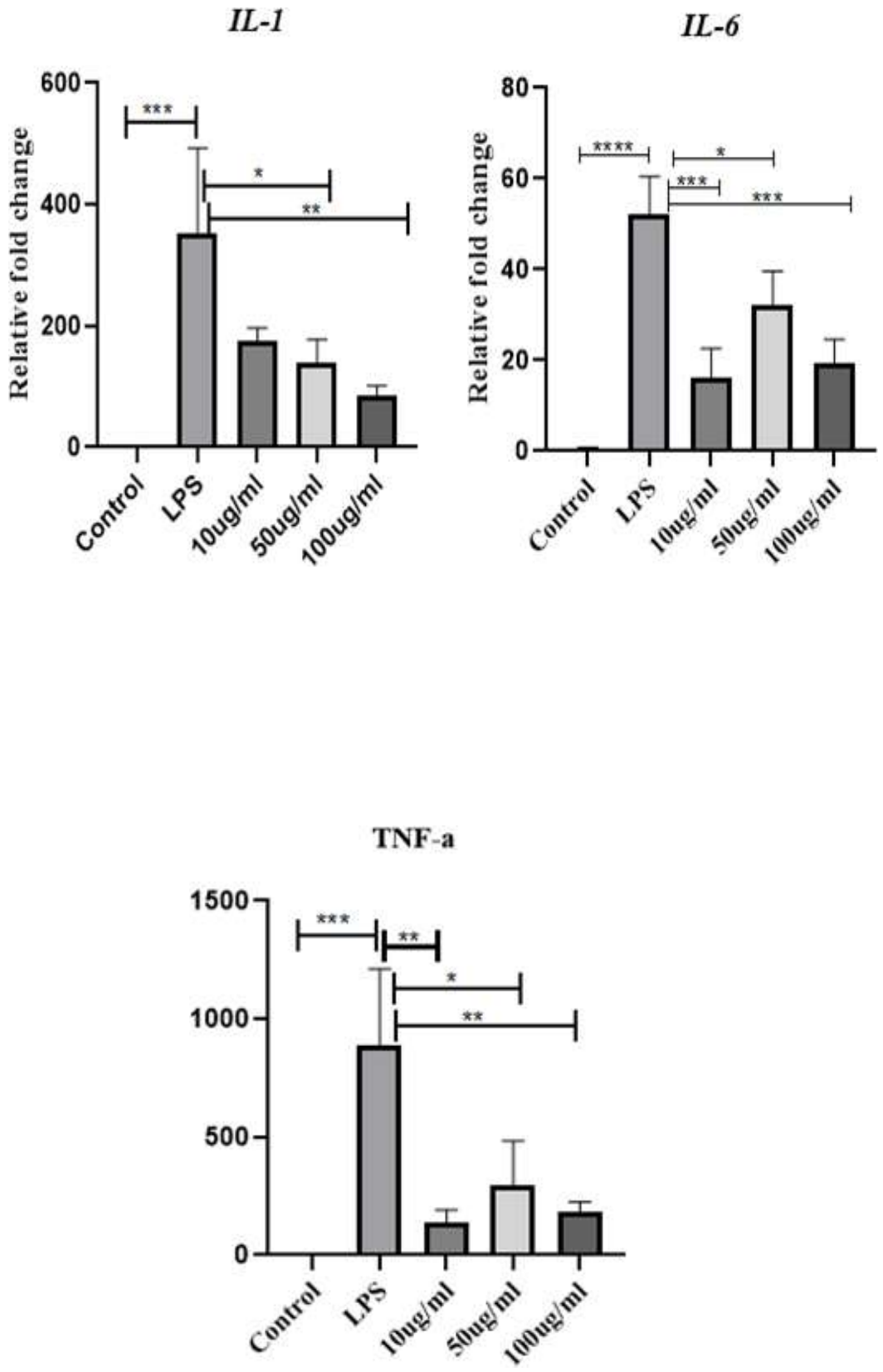


Figure 35 - qRT-PCR Analysis of Pro-Inflammatory Cytokines (IL-1, IL-6, and TNF-α) in Response to LPS and Exosome Treatments (10, 50, and 100 µg/mL)

These qRT-PCR results show that LPS highly upregulates pro-inflammatory cytokines (IL-1, IL- 6, and TNF-α), as would be expected by its established action as a strong inflammatory agonist. Conversely, exposure to exosomes at 10, 50, and 100 µg/mL reduced the expression of all three cytokines in a dose-response manner.

This implies that the exosomes are anti-inflammatory and can reverse LPS-induced inflammation. The higher suppression seen at 50 and 100 µg/mL suggests that increased exosome concentrations are more potent in inhibiting inflammatory cytokine expression.

Chapter 6

Discussion

Ginger is well known for its anti-inflammatory, antioxidant, and antimicrobial activity, primarily due to its bioactive compounds like gingerol, shogaol, and paradol. In wound healing, ginger plays a role mainly exhibiting Anti-inflammatory activity Through the inhibition of pro-inflammatory cytokines (e.g., TNF-α, IL-1β), ginger inhibits swelling and redness, hastening the resolution of the inflammatory phase. Ginger scavenges ROS, preventing tissue oxidative stress and promoting fibroblast function. It stimulates collagen deposition, supporting proliferation and remodeling stages of healing. Ginger has been found effective against ubiquitous wound infective pathogens (e.g., Staphylococcus aureus), preventing infection. [31] The potential of garlic's wound healing lies mainly in the sulfur-containing compounds present in it, notably allicin. Its most significant contributions are broad-spectrum antibacterial and antifungal effects, which play a critical role in preventing infection and infection control in wound locations.

Garlic induces vascular endothelial growth factor (VEGF), stimulating new blood vessel formation, an essential process for tissue repair. Garlic increases leukocyte function and cytokine production, aiding in immune defense at initial stages of wounds. This allows for extracellular matrix structure and regeneration of epithelium. When combined, ginger and garlic can also have synergistic effects, including increased wound healing activity above and beyond what each agent contributes separately. The hybrid drug formulation takes advantage of the bioactive phytochemicals both plants contain to regulate the inflammatory response while protecting tissue from oxidative insult so that the healing process occurs in a more balanced manner. The blend offers a wider range of microbial inhibition, decreasing the chance for infection more than single- agent use. The hybrid promotes the acceleration of collagen deposition, activation of fibroblasts, and angiogenesis, which are essential for wound closure and contraction. When formulated in hydrogels or topical creams, the hybrid provides a moist and stable environment for healing, optimal for tissue repair. Plant RNA molecules, such as small RNAs (sRNAs) and microRNA- like sequences, have the ability to modulate host gene expression via cross-kingdom interaction mechanisms. Ginger and garlic sRNAs could interact with NF-κB and MAPK pathways and inhibit transcription of TNF-α and IL-6. This inhibits excessive inflammatory signaling and facilitates the shift to the proliferative phase. Plant RNAs can suppress activation of NLRP3 inflammasome, which is a central regulator of IL-1β

secretion, and thereby decrease pyroptosis and inflammatory injury at the wound. miRNAs from ginger might be involved in targeting

STAT3 or other pro-inflammatory transcription factors, regulating the inflammatory process. These actions lead to a more regulated inflammatory process, mitigating tissue injury and orderly wound healing. The lipid components of ginger and garlic, such as essential fatty acids and sterol derivatives, have immediate actions on cytokine production. Bioactive lipids like linoleic acid and oleic acid present in garlic suppress TLR4/NF-κB signaling activation and subsequently lower IL-6 and TNF-α secretion from macrophages. Ginger lipids promote the generation of resolvins and lipoxins, which actively resolve inflammation by inducing macrophage polarization to an anti-inflammatory (M2) type. Lipids stabilize the cellular membranes of immune cells, preventing their hyperactivation and mitigating cytokine storms. By suppressing the expression of IL-1β, IL-6, and TNF-α, lipids of these plants truncate the inflammatory period, allowing quicker advancement towards tissue regeneration. Bioactive proteins and peptides of ginger and garlic have a very crucial role to play in immune regulation and cytokine suppression. Garlic proteins (e.g., alliinase) are known to suppress pro-inflammatory gene expression, notably NF-κB and AP-1, both of which regulate IL-1β, IL-6, and TNF-α. Anti-inflammatory peptides in ginger proteins can suppress COX-2 expression and consequently decrease IL-6 and TNF-α. Enzymic antioxidants (e.g., catalase, peroxidase) lower oxidative stress, a recognized inducer of over-expression of cytokines, notably TNF-α. Amino acids as immunomodulators: Sulfur amino acids of garlic (e.g., cysteine) assist in glutathione synthesis, an essential regulator that preserves redox homeostasis and inhibits pathological cytokine production. Such proteins promote immune homeostasis via restoration, constraining cytokine-induced tissue damage and facilitating fibroblast growth and matrix deposition. Exosome hybrids are 200 nm-sized nano-vesicles that are created by the integration of natural exosomes (such as from ginger and garlic) with other components to enhance delivery and therapeutic response. They are generally spherical or cup- shaped, and may be visualized with TEM with an evident bilayer structure. Their size and homogeneity are established with methods such as Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA). The majority of exosome hybrids lie in the 180 nm- 200nm size range based on their cargo and surface modifications. Zeta potential indicates how stable, viable, and bioavailable the exosome hybrid is. A zeta potential of −20 to −30 mV is ideal for therapeutic delivery systems such as ginger–garlic exosome hydrogels. Hydrogels are mostly water-swollen polymer networks that can also be used to mimic the natural extracellular matrix, and they find biomedical applications in wound healing, among others. The hydrogel utilized in this research is highly biocompatible, and it evokes no cytotoxic reaction when exposed to skin cells (such as keratinocytes or fibroblasts). Its non-immunogenic and non-toxic character allows for its safe use on damaged, as well as sensitive, skin. The therapeutic, soothing environment

provided by the hydrogel also helps in natural tissue regeneration and minimizes infection. The porosity of the hydrogel is essential in its function to Encapsulate exosomes effectivel , Protect exosomes from early degradation and Regulate the release of exosomal material over time. SEM images verify that the hydrogel exhibits a well-connected and porous structure, with enough room for loading exosomes. Facilitates a sustained and controlled release, improving the therapeutic window for wound healing. Porosity also enhances oxygen and nutrient diffusion, further enhancing tissue repair. Very good stretchability and flexibility, enabling it to conform to numerous body contours and movement without cracking or losing adhesion. This mechanical resistance avoids wound reopening and allows constant healing in mobile regions such as joints or fingers. The hydrogel lacks chemical irritants, heavy metals, or crosslinkers that could harm skin tissue. It preserves skin pH equilibrium and does not disrupt the skin's natural microbiome. Biodegradable ingredients guarantee that any residue will not be accumulated or result in prolonged toxicity.

Chapter 7

Conclusion

The current research emphasizes the fabrication and characterization of a biocompatible hydrogel system containing ginger–garlic-derived exosome hybrids for improved wound healing applications. The hydrogel exhibited good porosity, mechanical stretchability, and non-toxicity, rendering it suitable for direct skin application. [205] The porous nature of the hydrogel permitted effective encapsulation and shielding of exosomes, and also permitted a stable and controlled release over time. This extended release is essential in sustaining therapeutic concentrations of bioactive molecules at the wound bed and aiding continuous tissue regeneration. Exosome hybrids purified from ginger and garlic maintained major bioactive constituents such as proteins, lipids, and RNAs that played a crucial role in anti-inflammatory, antioxidant, and regenerative activities.[31] These molecules assisted in pro-inflammatory cytokine downregulation like IL-6, IL-1β, and TNF-α, which are important to prevent chronic inflammation and enhance the wound closure rate. Structural integrity, particle size 200nm, and stability of the exosome hybrids were verified by characterization methods like SEM, SDS-PAGE, RNA and lipid analysis, TLC, and rheology studies. Zeta potential measurements (usually −20 to −30 mV) also confirmed their colloidal stability and bioavailability.

In summary, this hydrogel-based exosome delivery system offers a safe, stable, and effective platform for promoting wound healing through the combined therapeutic potential of plant-derived exosomal biomolecules and a supportive hydrogel scaffold. It holds promise as a next-generation wound dressing with natural, non-toxic, and regenerative benefits.

Future Work

Based on the encouraging in vitro findings, subsequent studies will examine the in vivo efficacy of ginger–garlic exosome-loaded hydrogel in a diabetic mouse model of wound healing.

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