Isolation and Characterization of Exosomes with HSV Glycoprotein D for Targeted Delivery into White Blood Cells

Submitted by:

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MS Healthcare Biotechnology

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2023

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2023

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DEDICATION

In heartfelt gratitude, To my beloved parents,

whose boundless love and steadfast support have illuminated my journey.

And to myself,

for persistently overcoming challenges and accomplishing what I set out to achieve.

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Shaheera Fatima

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List of Abbreviations

EVs	Extracellular Vesicles
BBB	Blood Brain Barrier
HVEM	Herpesvirus-entry mediator
HSV gD	herpes simplex virus Glycoprotein D
WBCs	White Blood Cells
NHL	Non-Hodgkin lymphoma
AML	Acute myeloid leukemia
CML	Chronic Myeloid Leukemia
GBD	Global burden of disease
PEG	Polyethylene Glycol
RVG	Rabies Viral Glycopeptide
GS linker	Glycine-Serine Linker
LB	Luria Broth
HEK293	Human Embryonic Kidney 293
DMEM	Dulbecco's Modified Eagle's media
FBS	Fetal Bovine Serum
qRT-PCR	Quantitative Real-time PCR
PBS	Phosphate Buffer Saline
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
PVDF	Polyvinylidene difluoride
TBST	Tris-Buffered Saline with Tween 20
LDS	Lithium Dodecyl Sulfate
BSA	Bovine Serum Albumin

TAE	Tris Acetic acid EDTA
UV	Ultraviolet
NF	Nuclease Free
O.D.	Optical Density

Abstract

Exosomes are naturally occurring extracellular vesicles with diameters varying from 30nm to 150nm that are enclosed by a lipid bilayer membrane like that of a human cell membrane. They are secreted by different types of cell lines and cells such as neural, hematopoietic, fibroblast, epithelial, muscle, stem cell, and tumor cells, and have been found in surrounding biological fluids. These nanoscale exosomes efficiently transport a variety of cargo, including lipids, metabolites, functional proteins, and nucleic acids, to the target cell, where they play a crucial role in intra- and intercellular communication. Their natural delivery capability makes them an ideal candidate for therapeutics delivery systems compared to synthetic counterparts, which often exhibit limitations such as immunogenicity, cytotoxicity, biocompatibility, and adverse side effects. Both natural exosomes and those that have been modified with other substances to increase their target delivery ability have been used.

This study has focused on natural delivery vehicles such as exosome-based targeted drug delivery vehicles that provide unique opportunities for the delivery of therapeutics across specific physical barriers and address challenges posed by synthetic drug delivery systems. In this study, exosomes were modified for targeted therapeutics delivery by surface engineering with the peptide of Herpes simplex virus Glycoprotein D that binds to the herpes virus entry mediator (HVEM) receptor. The cell-targeting function of these engineered exosomes was introduced by expressing HSV glycoprotein D in HEK293T cell and followed by isolation of exosome from supernatant of engineered cells. Characterization using

scanning electron microscopy confirmed the cup-shaped morphology and size of the exosomes, and the presence of the target gene was validated through western blotting. These engineered exosomes exhibit selective binding to white blood cells via the HVEM receptor that is present in blood cell populations such as white blood cells (Natural killer cells, monocytes, T cells, B cells), epithelial cells, and endothelial cells, offering potential application in treating leukemia (, *B-cell leukemia*, T-cell leukemia, Myeloid leukemia), autoimmune diseases, immunodeficiency diseases, and tumor, etc. by loading therapeutic agents into them.

CHAPTER 1

Introduction

Exosomes are naturally occurring nanocarriers that contain a variety of chemicals inside a phospholipid-bilayer membrane and function as intracellular messengers to mediate both normal and pathological processes (Amiri et al., 2022). The estimated diameter of exosomes is 30-150nm secreted by living cells through the exocytosis process and is present in several biological fluids, including serum, saliva, and urine (Fang et al., 2022). Additionally, when examined under transmission electron microscopy, exosomes exhibit a distinctive cup-like appearance (Di Bella, 2022). More importantly, natural exosomes deliver messages to target cells by a variety of mechanisms, including membrane fusion, surface receptor contact, receptor-mediated endocytosis, phagocytosis, and/or micropinocytosis. Exosomes can deliver therapeutic and bioactive components to targeted tissues and cells by utilizing these mechanisms. Exosomes have been regarded as attractive drug carriers for targeted therapy because of their characteristics of natural generation, nano-scaled size, minimal immunogenicity, and the ability to overcome various biological barriers (Chen et al., 2021).

The most popular drug delivery systems currently include liposomes and polymeric nanoparticles. These nanoparticle drug delivery systems can effectively deliver drugs while also modifying their characteristics for improved drug release patterns and targeted distribution (Zhou et al., 2018). Liposomes are man-made vesicles made up of a phospholipid membrane that forms naturally in water-based conditions, resulting in a variety of sizes and shapes (Nsairat et al., 2022). Liposomes used as drug delivery vehicles offer various

advantages, including extended circulation, various methods for drug loading at high concentrations, and the ability to be created artificially. The immunological compatibility of liposomes is a topic of discussion, and little evidence supports the use of liposomes for targeting living organisms (Sercombe et al., 2015). Polymeric nanoparticles are used as drug delivery carriers by entrapping, encapsulating, or incorporating drug molecules into them (Begines et al., 2020). The application of polymeric nanoparticles as nanocarriers has various benefits, including the ability to release drugs in a consistent and controlled manner throughout transit, as well as increased drug administration efficacy (Zielińska et al., 2020). However, due to the smaller dimensions of these nanoparticles and their increased surface area, regulating particle accumulation becomes difficult, both in liquid and dry forms. Two main challenges occur when drug-loaded synthetic nanoparticles enter the circulatory system: toxicity and fast clearance by mononuclear phagocyte systems (Gutierrez-Millan et al., 2021). Numerous efforts have been made to address particle clearance and improve particle distribution in vivo. The most popular technique is the use of polyethylene glycol (PEG) to modify the surface of nanoparticles which can enhance their circulation in the body and reduce MPS drug absorption (Mitchell et al., 2021). In NP formulation, polyethylene glycol is a frequently used polymer and its addition improves therapeutic efficacy (Mohamed et al., 2019). Nonetheless, the NP modification may result in certain safety issues and PEGylation may also affect the therapeutic interaction with the target region (Luan et al., 2017).

Among other therapeutic approaches, nanoparticle delivery technologies are gaining importance, but the clinical applications of these systems also face challenges (Ren et al., 2016). These challenges include concerns about biocompatibility, poor biodistribution, cell targeting, short half-life in body fluid, poor efficacy while crossing the BBB, inherent immunogenicity, cytotoxicity of carriers and their breakdown products, fast renal clearance by the reticuloendothelial system, and the buildup of nanomaterials like polymers after

multiple administration (Gutierrez-Millan et al., 2021). These challenges can be overcome by shifting to a naturally developed therapeutic delivery system (Gaurav et al., 2021). Exosomes are more stable in circulation compared to liposomes and polymeric nanoparticles due to their endogenous source and distinctive surface composition (Kimiz-Gebologlu & Oncel, 2022).

Exosomes have many advantages over synthetic therapeutic delivery methods as a naturally occurring carrier: 1) The small size of exosomes enables them to efficiently traverse biological obstacles including the blood-brain barrier (BBB) and placental barrier (Caponnetto et al., 2017). 2) The endogenous lipids and protein contents of the exosomal membrane allow exosomes to enter the target cells effectively and deliver therapeutic drugs when administered intravenously (Peng et al., 2020). 3) Being naturally generated vesicles, exosomes have strong biocompatibility, stability, minimal immunogenicity, and cytotoxicity (Song et al., 2021). 4) Exosomes could be more easily home to target cells via surface modification approaches for targeted drug delivery and hence are expected to boost therapeutic effectiveness while decreasing side effects (Luan et al., 2017). 5) Exosomes can deliver therapeutics to the targeted regions, resulting in reduced systemic toxic reactions and improved stability and safety (Antimisiaris et al., 2018). 6) Exosomes possess a tendency to accumulate in particular organs, exhibit a longer half-life in blood plasma, and have the ability to bypass the reticuloendothelial cell, which makes them advantageous for targeted therapeutic delivery applications (Sushrut Kamerkar et al., 2017).

Exosome-based drug delivery has been used to deliver several types of therapeutics to targeted tissues and cells. Most studies have focused on the transfer of nucleic acid such as small interfering RNA (siRNAs) and miRNA, with little emphasis on the ability to include

other types of therapeutics (Liang et al., 2021). Like liposomes, exosomes have an aqueous core and a bilayer lipid membrane, which makes it possible for both hydrophilic and lipophilic therapeutics to be incorporated into the exosome (Butreddy et al., 2021). However, conventional exosomes as nanocarriers should be altered for encapsulation of therapeutic substances via several in vitro techniques.

Indeed, Exosomes have been explored as a therapeutic delivery vehicle for many diseases therapy including bladder cancer, liver carcinoma, breast cancer, colorectal cancer, pancreatic cancer, hepatocellular carcinoma (Rufino-Ramos et al., 2017), as well as brain disorders such as neurological diseases, neuro infections, and brain tumors (Pistono et al., 2020). These engineered exosomes have been modified to carry specific cargo molecules, such as drugs, therapeutic agents, or genetic material, that are designed to combat the specific disease they are intended for, such as ischemia stroke, Parkinson's disease, and Alzheimer's disease, liver cancer, breast cancer, among others.

As mentioned earlier, exosome research is advancing rapidly, and we will now focus on developing exosomes with various functionalities that can target and treat multiple diseases. This innovative development holds considerable promise in the area of therapeutic therapies. Apart from their role in treating cancer and brain disorders, due to their potential to pass the blood-brain barrier and deliver therapeutic cargo to targeted cells. At present, we are utilizing this capability to create exosomes that exhibit significant potential as targeted therapeutic carriers for the treatment of hematological diseases. Their potential to treat hematological diseases will attract a lot of attention among many potential applications for exosome-based drug delivery. These disorders include a broad range of white cell disorders such as blood Cancer, lymphoma, leukemias, autoimmune diseases, and others. Moreover, these engineered exosomes can also be used as cancer immunotherapy and vaccine carriers.

White blood cells, commonly known as leukocytes, protect your body against disease. White blood cells play a crucial role in the immune system, which defends the body against pathogenic organisms and foreign substances. To effectively defend the body, an adequate number of white blood cells must receive a message that a pathogenic organism or foreign substance has infiltrated the body, go to the site of infection, kill and digest the dangerous organism or substance, and then return to the body (Glenn & Armstrong, 2019). Though they only make up about 1% of your blood, white blood cells are very important. The formation of white blood cells occurs in the soft tissue inside your bones (bone marrow). Two types of white blood cells (lymphocytes) are T cells (thymus gland) and B cells (lymph nodes and spleen). White blood cells can be divided into five main categories: Lymphocytes (T-cells, B-cells, natural killer cells), Neutrophils, Eosinophils, Basophils, and Monocytes, each with a distinct role in the immune response (U. o. R. M. Center, 2023).

White blood cell disorders, referred to as leukocyte disorders, are diseases that impair the production, function, or levels of white blood cells (leukocytes) in the body. White blood cell disorders include a variety of disorders that affect the immune system and hematological functions. It can vary from autoimmune diseases (in which the immune system targets the body's cells) to leukemia (a kind of blood cancer characterized by uncontrolled white blood cell proliferation) (Walkovich & Connelly, 2022). Treatment of these disorders can be challenging due to the intricate interactions of immune cells and the likelihood of diverse side effects with conventional drugs. To treat these diseases, therapeutic drugs must be delivered precisely to white blood cells, with minimal damage to healthy cells, limiting systemic adverse effects, and increasing treatment success.

The herpes simplex virus (HSV) glycoprotein D is a protein located on the virus's surface (Huang et al., 2022). It can interact with a receptor known as the herpesvirus entry mediator (HVEM), which is found on the surface of some white blood cells (Bivacqua et al., 2023). This contact is a natural process that the virus employs to gain access to cells. We will use this connection to directly target white blood cells with exosomes. To enable the tailored delivery of therapeutic medicines into blood cells, we will modify the outer surface of exosomes to achieve specific functions. In the present case, HSV glycoprotein D will be used to modify the exosome surface, resulting in exosomes that can recognize and bind to white blood cells expressing the HVEM receptor. This method basically gives exosomes a "homing mechanism" that directs them to their designated target cells.

CHAPTER 2

Literature Review

2.1. Hematological Disorders:

A subset of hematological disorders known as "white blood cell disorders" are characterized by abnormalities in leukocytes, or white blood cells, which are crucial immune system components. A wider range of conditions are referred to as hematological disorders, and they affect numerous components of the blood, including the lymph nodes, spleen, bone marrow, platelets, white blood cells, and red blood cells (Sinai, 2023). In the context of hematological illnesses, white blood cell disorders are medical problems characterized by abnormalities in the generation, function, or count of white blood cells (leukocytes) in the body. There are different possible causes of these disorders, including autoimmune diseases, hereditary conditions, infections, and in a small percentage of cases, cancer. There are several cases where the cause is unknown (healthdirect, 2021).

A common complication of all WBC disorders is the patient's higher likelihood of infection due to the dysfunction or absence of types of WBCs. It is crucial to understand that the immune system orchestrates all WBCs with the help of cytokines and other chemical mediators and any disorder or dysfunction that affects a particular type of WBC may severely compromise the immune system's ability to carry out its role.

A white blood cell disorder generally occurs when the number of white blood cell counts is abnormally low (leukopenia) or excessively high (leukocytosis), depending on the specific type of disorder. The terms leukocytosis and leukopenia refer to abnormal changes in the white blood cell (leukocyte) count in the blood. Leukocytosis is characterized by an excessive

white blood cell count in the blood. It can arise from bacterial or viral infections, inflammatory conditions (such as inflammatory bowel disease, rheumatoid arthritis, and vasculitis), cancers (such as leukemia, lymphoma, and myeloma), and allergic reactions (Clinic, 2023). Leukopenia is characterized by a reduction in white blood cell count, which can make the immune system less effective at battling infections. These disorders can be caused by a variety of reasons, including viral infections (such as HIV/AIDS and hepatitis), bone marrow disorders (aplastic anemia, myelodysplastic syndromes), autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis), certain medications (chemotherapy, immunosuppressive drugs), radiation therapy, and severe sepsis are all examples of leukopenia-related disorders (NHS, 2023)

Disorders associated with a high or low white blood cell count can indicate different underlying health problems. The white blood cell count is a vital component of a complete blood count (CBC) and gives important information about the body's immune system and general health (Health, 2023). White blood cell disorders are divided into two types: cancerous and non-cancerous. Leukemia, lymphoma, and myeloma are a few examples of cancerous white blood cell disorders, that are caused by uncontrolled proliferation and dissemination of abnormal white blood cells or bone marrow cells (Trust, 2020). Non-cancerous white blood cell diseases are a group of conditions in which white blood cells are overproduced, underproduced, or function poorly, resulting in immune system malfunction, autoimmune reactions, increased susceptibility to infections, and other symptoms (Britannica, 2023). The following are some of the most frequent WBC problems.

2.2. Leukemia

A form of cancer known as leukemia targets the body's blood-forming tissues, especially the lymphatic and bone marrow systems. It is caused by either an aberrant increase in white

blood cell production or a developmental problem that causes premature cells to be released from the bone marrow, resulting in abnormal responses when stimulated (Clinic, 2022). It can also suppress the normal blood cells, limit the capacity of the body to create healthy blood cells and impair the functioning of the immune system. Leukemia is defined as either acute (rapidly advancing) or chronic (slowly progressing) based on the kind of white blood cell affected. Acute lymphoblastic leukemia is a type of leukemia that primarily targets immature cells and progresses quickly. It is most observed in children and young people. Acute Myeloid Leukemia (AML) attacks myeloid cells, including granulocytes and monocytes, and tends to advance rapidly (Pelcovits & Niroula, 2020). Chronic lymphocytic leukemia (CLL) is a slow-growing cancer that primarily targets mature lymphocytes and is commonly diagnosed in elderly people. Chronic Myeloid Leukemia (CML) is caused by a genetic mutation in the bone marrow and affects myeloid cells, with a slower progression and the presence of the Philadelphia chromosome (Benchikh et al., 2022). The wide range of leukemia types emphasizes the importance of precise diagnosis and personalized treatment techniques, which may include chemotherapy, targeted treatments, stem cell transplantation, and other interventions depending on the exact subtype and the state of the patient's health.

2.3. Lymphoma

An immune system-attacking type of blood cancer is called lymphoma. Lymphocytes, which are white blood cells that play an important part in your immune system, are mostly affected. Lymphoma is also known as lymphatic cancer, and it has around 35 distinct subtypes. Lymphoma arises when abnormal lymphocytes, a kind of white blood cell, begin to proliferate and divide uncontrollably, resulting in tumors in lymph nodes or other lymphatic systems. (UK, 2023). This blocks the lymphatic system's natural function and can result in a variety of health problems. Lymphoma is classified into two types: Hodgkin lymphoma, also referred to as Hodgkin’s disease, and non-Hodgkin lymphoma, which proliferate and receives

treatment differently. Your lymphocytes are affected by each type. Hodgkin's lymphoma affects a subtype of B lymphocytes known as Reed-Sternberg cells, which are huge abnormal cells with distinct features when viewed through a microscope (López et al., 2022). Non-Hodgkin's lymphoma affects other B and T lymphocytes without the Reed-Sternberg cells observed in Hodgkin's lymphoma (Shaikh., 2023). While both types affect the lymphatic system, their basic biology, treatments, and cure rates differ. Hodgkin's lymphoma has five subtypes, while non-Hodgkin's lymphoma has thirty subtypes. A person's risk of developing lymphoma increases if they have Helicobacter pylori, Epstein-Barr virus (EBV), hepatitis B or C, or human T-lymphocytic virus type 1 (HTLV-1). The elderly have a significantly higher risk of lymphoma (Chabay et al., 2020).

2.4. Myeloma

A malignancy that affects plasma cells is called myeloma. One subset of white blood cells called plasma cells makes antibodies to help the body's immune system fight infection. A plasma cell multiplies quickly when it turns cancerous. We refer to this as multiple myeloma. Because the malignant plasma cells may crowd out the regular blood-forming cells already there, the bone marrow's capacity to make healthy blood cells may be diminished. In addition, the cancer cells start to make abnormal antibodies known as monoclonal proteins (m proteins) or paraproteins instead of antibodies that fight infections. They are known as Bence Jones proteins in the urine. These proteins do not combat infection (M. A. C. Center, 2023). This can result in a variety of complications, including anemia, bone degeneration, kidney failure, and infections. There are many different treatment options available, some of which include symptom management, immunotherapy, chemotherapy, targeted treatments, and stem cell transplantation. Though multiple myeloma is thought to be incurable, innovations in treatment have improved results, and many individuals may attain remission or manage the cancer as a chronic condition with appropriate medical treatment (Moreau et al., 2021).

2.4. Autoimmune Diseases:

When the body's immune system attacks its healthy tissues, it is referred to as an autoimmune disease. This can occur when the immune system mistakenly recognizes healthy cells as foreign intruders. This immune response targeting the body's cells and tissues can cause inflammation, tissue damage, and a variety of symptoms, depending on the autoimmune disease and the organs or systems affected. There are over 80 distinct types of autoimmune disorders, and they can affect any area of the body ((.gov), 2021). Rheumatoid Arthritis, Autoimmune Hemolytic Anemia, Celia disorders, Inflammatory Bowel Disease (IBD), Multiple Sclerosis, Hashimoto's Thyroiditis, Systemic Lupus Erythematosus (SLE), and others are some of the most prevalent autoimmune disorders. Although there is no known cure for autoimmune disorders, there are treatments that can help manage the symptoms, reduce immune system activity, block future tissue damage, and enhance quality of life (Miller, 2023).

Treatment for hematological disorders, including white blood cell abnormalities, differs based on the disease and its root cause. Treatments that are commonly utilized include chemotherapy, radiation therapy, bone marrow transplants, targeted therapies, and preventative measures (S. M. Center, 2023). These treatments have some limitations even though they have been successful in treating many hematological illnesses. Chemotherapy and radiation therapy can have serious adverse effects and destroy healthy tissues (Jameus et al., 2021). Although bone marrow transplantation can be curative for certain patients, it comes with challenges such as finding acceptable donors and the risk of complications (Mewawalla3., 2022). Targeted treatments and immunotherapy are promising, but they are not always effective, and they may result in drug resistance or unpredictable patient responses. A new area of research in the treatment of hematological disorders involves extracellular vesicles such as exosomes as drug delivery vehicles. Exosomes, which are tiny

vesicles generated by cells, could transport therapeutic materials, like RNA or proteins, directly to target cells within the hematopoietic system (Wang et al., 2023). They offer several benefits for targeted therapy. Exosome-targeted therapies have the benefit of precision targeting, as these tiny vesicles can be engineered to specifically transport therapeutic cargo to damaged cells or tissues. This precision provides the ability to prevent off-target effects and protect healthy cells while addressing the intricate details of some white blood cell diseases. Furthermore, exosome-based treatments might result in fewer systemic side effects, making them a good choice (Zhu et al., 2020).

2.5. Epidemiology:

Hematologic malignancies (Blood Cancer) are among the most frequent cancers, and understanding their prevalence and mortality is essential for properly targeting preventive, clinical practice improvement, and research resources. Blood cancer risk factors include radiation and chemical exposure, specific genetic problems, autoimmune diseases, and certain infections (Zhang et al., 2023). Leukemia, lymphoma, and myeloma represent the blood cancers that affect more males than women. Each year, an estimated 1.24 million cases of blood cancer are diagnosed worldwide, which accounts for approximately 6% of all cancer cases (Squibb, 2023). The most recent research report on the global burden of disease (GBD) of blood cancers was published in 2019. According to the report, there were an estimated 10.2 million new cases and 5.8 million deaths from blood malignancies in 2019. This is an increase from the predicted 9.7 million new cases and 5.5 million deaths in 2017 (Evaluation, 2019).

Blood cancers account for around 10% of all cancers diagnosed in the United States each year (Medicine, 2023). In the US, a new case of leukemia, lymphoma, or myeloma is diagnosed every three minutes. An estimated 184,720 Americans are expected to receive a

leukemia, lymphoma, or myeloma diagnosis in 2023. Leukemia, lymphoma, and myeloma are estimated to kill 57,380 people in the United States in 2023. It is anticipated that in 2023, leukemia, lymphoma, and myeloma cases will make up 9.4% of the 1,958,310 new cases of cancer that will be diagnosed in the US (Society, 2023). According to WHO, 173 937 new instances of cancer were reported in Pakistan in 2018; of these, 4.1% were leukemia, 3.4% were non-Hodgkin's lymphoma, 0.92 percent were Hodgkin's lymphoma, and 0.81% were multiple myeloma (Malik et al., 2021). According to published data, children's cancer represented 10% of all diagnosed cancers in 2017 (DAWN, 2020). According to Pakistan's Karachi Cancer Registry, the two most common children's cancers are leukemia (31%) and lymphomas (20%). Data from the Punjab Cancer Registry revealed that lymphomas (31%) are more common than leukemia (23%) overall (Shaukat Khanum Memorial Cancer Hospital and Research Center, 2017). The prevalence of autoimmune disorders varies between 3% to 8% worldwide, with women accounting for 78% to 85% of cases (Ershadinia et al.)

Leukemia was among the most common blood cancers, accounting for 37% of all diagnoses and 27% of all deaths. However, the present prognosis for leukemia is not encouraging, and it continues to pose a serious risk to human health. Leukemia was anticipated to be the 15th and 11th most prevalent cause of cancer incidence and mortality globally in 2020, accounting for 474,519 incident cases and 311,594 deaths, respectively. (Sung et al., 2021). Leukemia also accounts for the largest percentage of mortality among children under the age of five and is the most prevalent cancer in those children, putting a major burden on people, families, and governments (Lin et al., 2019). Non- Hodgkin Lymphoma is the fifth to ninth most prevalent disease in many countries, with an estimated 544,000 new cancer cases and 260,000 cancer deaths in 2020. NHL incidence rates in both men and women varied by around 5-fold across WHO regions (Ephrem Sedeta, 2022). In Pakistan, the prevalence of autoimmune disorders has been reported to be around 7%.

2.6. EVs and Types of Extracellular Vesicles (EVs):

EVs are a diverse group of intercellular messengers between cells that come from the plasma membrane or late endosomes (Abels & Breakefield, 2016). All cell types secrete EVs, which are small phospholipid bilayer-enclosed vesicles that are important for cell communication (Vader et al., 2016). Peter Wolf first described the EVs as "platelet dust" in 1967 and was thought of as cellular trash for over 20 years (Wolf, 1967). However, EVs are currently seen as growing tools for cellular communication and targeted therapeutic delivery. These EVs can be found in surrounding biological fluids such as synovial fluid, blood, breast milk, semen, saliva, bronchial fluid, amniotic fluid, tears, lymph, urine, bile, and gastric acid, cerebral spinal fluid (Caby et al., 2005). EVs can be classified into four categories depending on their size and biogenesis processes: microvesicles, apoptotic bodies, exosomes, and oncosomes. The final category is the most recently identified and is illustrated schematically in **Fig. 2.1** (Minciacchi et al., 2015).

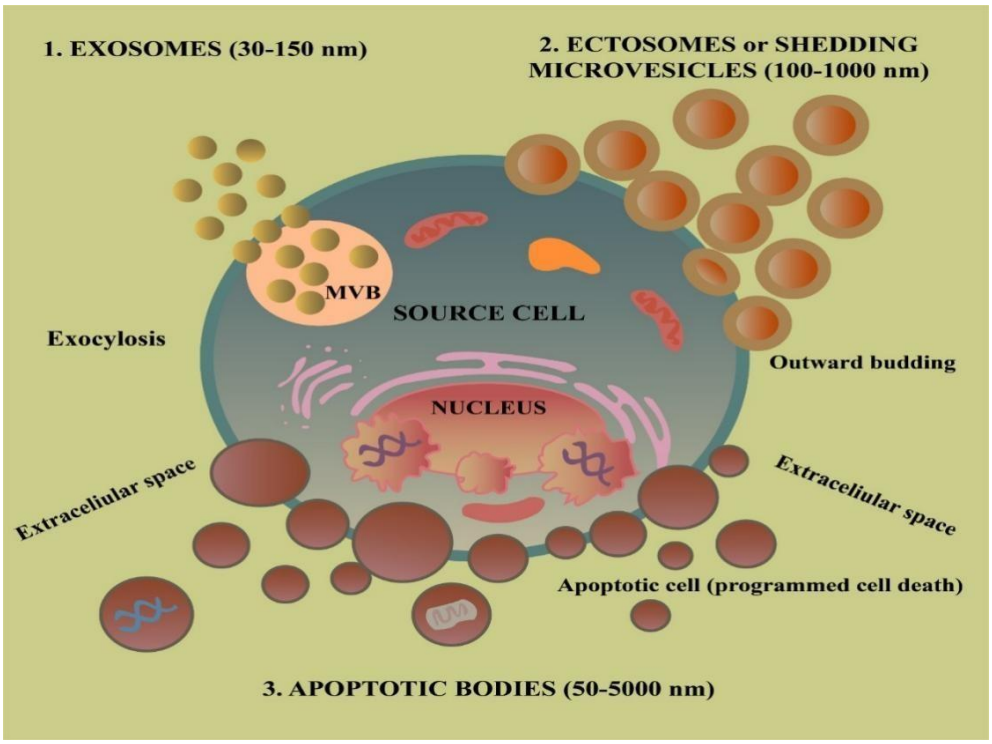

Figure 2.1. *Schematic illustration of three main types of EV namely exosomes, shedding microvesicles, and apoptotic bodies*

Purification techniques are inadequate because of the heterogeneity in the EV's size and other characteristics. This contributes to the incomplete characterization of these types (Hauser et al., 2017). Nonetheless, exosomes are considered the well-characterized type thus far and are the subject of this work (Hu et al., 2022). **Table. 2.1** provides a summary of the distinctive features of extracellular vesicles, including their shape, diameter, biogenesis, and contents, which might differ based on the specific type of vesicle.

Vesicles	**Size (Ephrem Sedeta)**	**Shape**	**Biogenesis**	**Content**	**Reference**
Exosomes	30-150nm	Cup/ round shape	Exocytosis	Proteins, nucleic acid, Lipids, cytosol	(Raposo & Stoorvogel, 2013)
Microvesicle	100-1000nm	Heterogenous	Membrane Budding	Proteins, nucleic acid, Lipids, cytosol	(Raposo & Stoorvogel, 2013)
Apoptotic bodies	50-5000nm	Heterogenous	Fragmentation/ Apoptosis	Proteins, Lipids, DNA, rRNA, organelle, and cytosol	(Xu et al., 2019)
Oncosomes	1-10um	Heterogenous	Membrane	miRNA, oncogenic	(Minciacchi

	(100-400nm		Budding/Shedding	transcripts, oncoproteins	et al., 2015)

Table. 2.1. *Extracellular vesicle types and their characteristics.*

2.7. What are Exosomes?

Exosomes are naturally occurring, lipid membrane-enclosed microsized extracellular vesicles with diameters ranging from 30nm to 150nm, recently gaining much attention from scientists (Cocucci et al., 2009). Rose Johnstone coined the term "exosomes" in 1970 after discovering exosomes in sheep reticulocytes (Trajkovic et al., 2008). They are secreted by different types of cell lines and cells such as neural, hematopoietic, fibroblast, epithelial, muscle, stem cell, and tumor cells (Raposo & Stoorvogel, 2013) and have been found in surrounding biological (Caby et al., 2005).

When cells released exosomes, it was initially thought that they were either a byproduct of homeostasis or cellular waste that resulted from injury to the cells and that they had no effect on the cells surrounding (Johnstone et al., 1987). A later study, however, revealed their role as important cell-cell communication vehicles that function through the targeted delivery of protein, lipid, and nucleic acid to the target cells and have been related to a variety of physiological and pathological functions (Mathivanan et al., 2010).

2.8. Biogenesis and secretion

Exosomes are produced via the endocytic cellular pathway, which has three stages: early endosomes, late endosomes, and multivesicular bodies (MVBs). **Fig. 2.2** depicts the three stages of exosome biogenesis and secretion: plasma membrane invagination produces early endocytic vesicles (early sorting endosomes) with distinct biomarkers on their surfaces, like RabGTPases (Schmidt & Teis, 2012; Xie et al., 2022). Along with cell-surface protein, some

extracellular contents may also enter the ESEs during this process. After then, the ESEs mature into late-sorting endosomes. The late limiting membrane can invaginate inward to form Intraluminal vesicles (IVs), which assemble in the late endosome lumen (Gatta & Carlton, 2019). The accumulation of certain cargoes such as protein, lipids, or nucleic acid into ILs via ESCRT-dependent or ESCRT-independent processes takes place during the second step (Colombo et al., 2013). The late endosome is also called a multivesicular body because it accumulates a large number of IVs. The final stage of this process involves the fusion of these multivesicular bodies with either lysosomes/ autophagosomes for degradation or may fuse with the plasma membrane to secrete ILs into extracellular space and released ILs are exosomes (Bebelman et al., 2018). It has been proposed that the exosome biogenesis and secretion have been linked to ESCRT (endosomal sorting complexes) proteins, lipid compounds, the Rab-GTPase family, phospholipids, SNARE (soluble NSF attachment protein receptors), TSG101 (tumor susceptibility gene 101), syndecan-1, and tetraspanins (Rahbarghazi et al., 2019). The entry of secreted exosomes into a recipient call can occur through different channels including direct fusion with the plasma membrane of recipient cells, receptor-mediated endocytosis, and can also take up via phagocytosis, pinocytosis, and endocytosis, which are all mediated by lipid rafts, caveolin or clathrin (Mathieu et al., 2019).

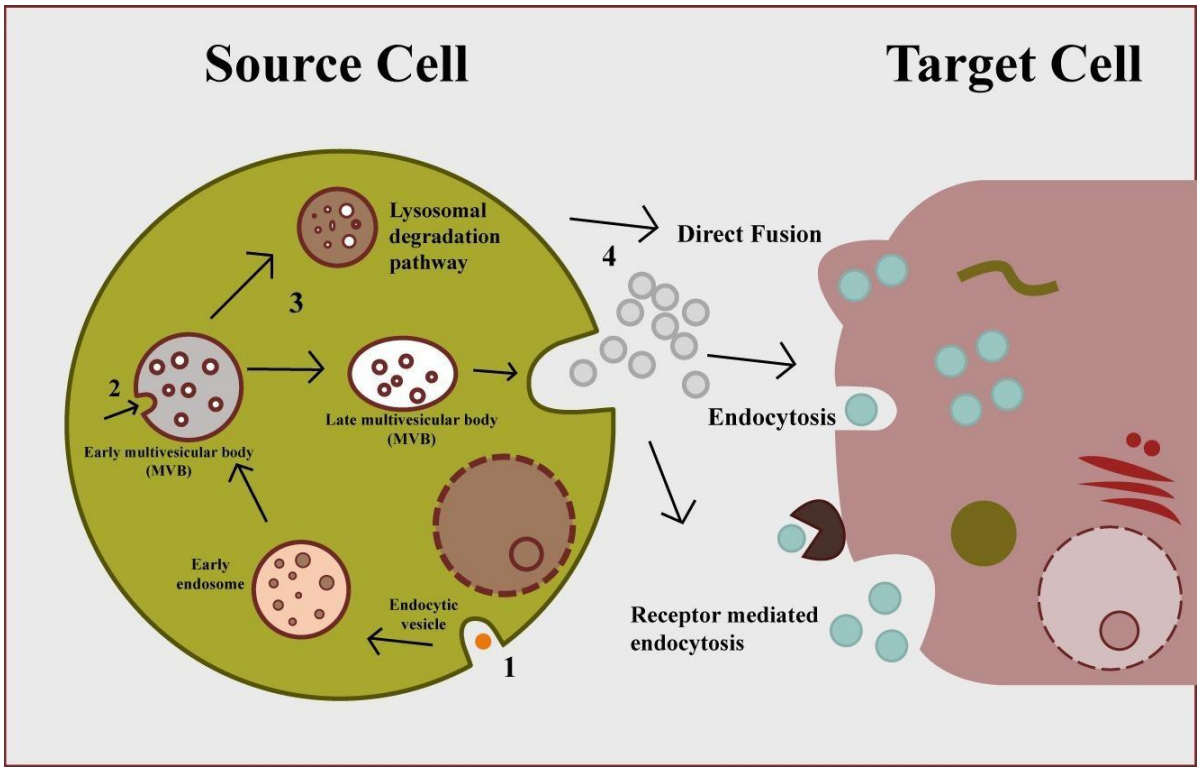

Figure 2.2. Exosome biogenesis, secretion, and cellular entry. *Early endosomes formed by plasma membrane invagination form multivesicular bodies via inward budding, which secrete exosomes through exocytosis. Secreted exosomes can be uptake by cells via three different mechanisms: direct fusion, endocytosis, and receptor-mediated endocytosis.*

2.9. Exosome Composition

Exosomes have a bilayer of lipids that form a nano-spherical membrane-type structure. Additionally, it is made up of several kinds of proteins and lipids **(Fig 2.3)** that come from the original cell from which the exosome is generated. ExoCarta, an exosome database, reports that approximately 8000 proteins and 194 lipids are currently known to be linked with exosomes (Vlassov et al., 2012). Exosome surfaces are composed of various proteins such as fusion proteins, transport proteins (annexins and flotillin), transmembrane proteins such as tetraspanins CDs protein (CD91, CD63, CD9), Lamp2b, antigen-presenting molecules, glycoproteins, adhesion molecules, phospholipases, and other lipid-related proteins (Théry et al., 2009). Additionally, lipids such as ceramides, cholesterol, phosphoglycerides, sphingolipids, and short and long chains of saturated fatty acids are abundant in exosomes

(Vickers & Remaley, 2012). Exosomes are often characterized according to their size or the expression of surface marker proteins. The most common exosome marker proteins found on the vesicle surface are tetraspanins such as CD9, CD63, and CD81. Tetraspanin antigens' surface location makes them good candidates for exosome identification and separation (Keerthikumar et al., 2016). Exosomes carry various cargoes, including proteins (heat shock protein, Ras-related protein, etc.), lipids, nucleic acid, and other cellular components. These cargoes are sorted and packaged directly into the exosomes. Exosome cargo depends on the type of cell they are packaged in as well as cellular conditions (Mahmood, 2022). Exosomes can deliver their cargo to a targeted intracellular region by crossing the plasma membrane. Since nucleic acids and proteins as their natural carriers, exosomes have been used as delivery vectors for these molecules (Yuan et al., 2017).

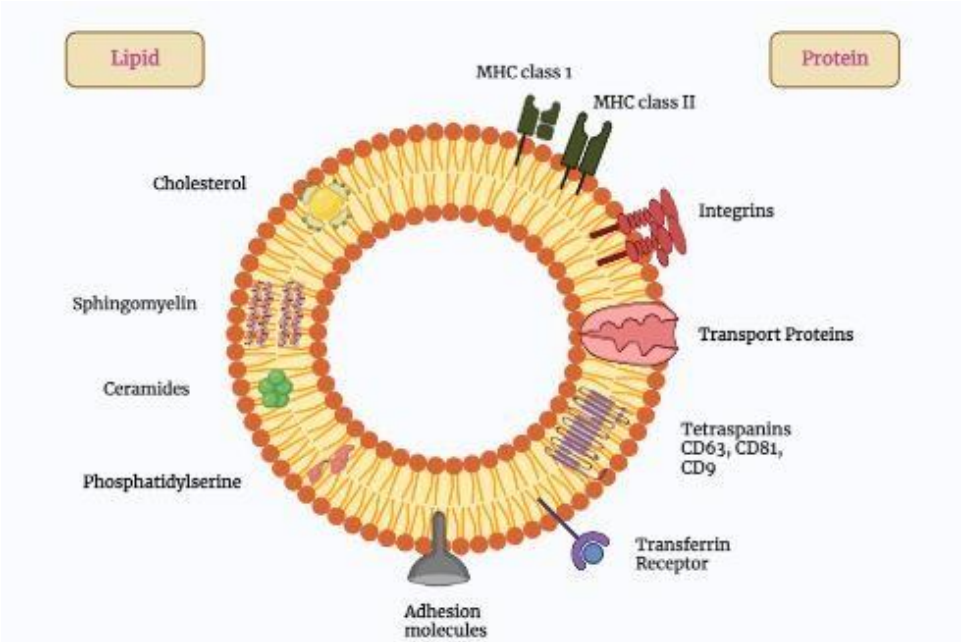

Figure 2.3. *Exosome Structure and Composition*

2.10. Exosome Cargo

Exosomes enclose a wide range of payloads, including proteins, nucleic acids, lipids, and other cellular components, as shown in Fig. 2.4. For precise delivery to particular cells or tissues, these payloads are carefully sorted and packed into exosomes (Anand et al., 2019). Exosomal protein cargo includes enzymes, growth factors, and signaling molecules capable of influencing cellular processes such as differentiation, proliferation, and migration. Exosomes also contain lipids that can influence membrane fluidity and cell signaling pathways. Additionally, exosomes carry different types of RNA molecules, which can affect gene expression and contribute to various physiological and pathological processes (Xie S et al., 2022). Exosome cargo composition varies according to cell type and conditions. Exosomes can deliver their cargo to a targeted intracellular region by crossing the plasma membrane. Since nucleic acids and proteins are their natural carriers, exosomes have been employed as transport carriers for these molecules (Yuan et al., 2017).

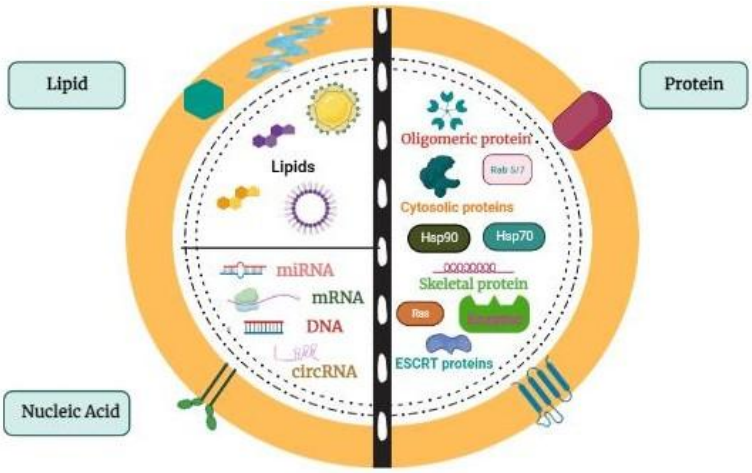

Figure 2.4. *Exosomes Cargo*

2.11. Exosomes Surface Modification for Targeted Delivery

Exosome characteristics by themselves are inadequate to provide targeted drug delivery and drug accumulation in damaged tissues. Moreover, several research suggest that exosomes require targeting approaches to enhance the targeted therapeutic delivery into recipient cells. Exosomes, however, are naturally occurring vehicles and can be easily surface altered. Exosomes' surface is important for their biodistribution, potential to target specific cells and therapeutic applications. The desired properties of exosomes can be achieved by modifying the surface, which enhances cell targeting (Man et al., 2020). Targeted delivery boosts the therapeutics' local concentration while reducing side effects. For promoting targeting delivery, exosomes can be engineered to express various targeting moieties on the surface of the exosome using direct modification methods such as physical modification and chemically changing exosomal surfaces or indirect methods by genetically engineering modification of exosome surface (Choi et al., 2021). Chemical modification includes non-covalent techniques as well as covalent techniques like click chemistry (Smyth et al., 2014). Another way to physically alter exosomes is to promote cellular or tissue targeting by hybridizing exosomes with chemically altered liposomes.

Chemical modification can also alter exosome surface, although this field of study is less developed. It involves direct chemical implantation of ligands onto the exosome surface, which is another way for exosome decorating. It enables the display of different ligands, both natural and synthetic, through covalent or non-covalent modification (Malekian et al., 2022). Covalent modification involves clicking chemistry and conjugation. Conjugation processes can covalently and firmly alter exosomal surface protein. In this process, covalent bonds help different molecules directly adhere to the surface of the exosomes. PEGylation is a common chemical conjugation process that uses covalent bonds to modify the surface of exosomes with branched polyethylene glycol (PEG) (Susa et al., 2019). However, due to the

complexity of the exosomal surface, conjugation reactions may be less effective and usually lack site-specific control. This modification might also compromise the exosome structure and function. Furthermore, “click chemistry” is appropriate for the bioconjugation of molecules on the surface of exosome membranes (Wang et al., 2015). Additionally, Click chemistry uses a covalent bond between an alkyne and an azide residue to generate solid triazole linkage, which can be used to bind targeting ligands to exosome surfaces in a variety of aqueous buffers such as water, alcohols, and DMSO (Villata et al., 2020). Triazole linkage reacts with alkyl and azide chemical groups more quickly than with conventional cross-linking and this results in improved control of the conjugation reaction at the target site (Smyth et al., 2014). Exosome lipid bilayers can also be modified to incorporate lipids or amphoteric molecules, enabling their hydrophilic components to be visible on the outside. This technique, mediated by lipid-self-assembly, may also make exosomes more toxic. However, one of the disadvantages of using covalent bonds is that they are very stable but require harmful chemicals to induce them, raising concerns about using covalent modification approaches in therapeutics. The con-covalent technique involves receptor-ligand binding, electrostatic interaction, and hydrophobic insertion (Armstrong et al., 2017).

The most popular means of displaying homing peptides or ligands on the exosomal surface is genetic engineering. Through genetic engineering, the gene sequence of ligands or homing peptides is fused with transmembrane protein (Marker protein) that is present on the surface of exosomes. It involves the following steps: First, the ligand’s coding sequence is inserted between the signal peptide and the N-terminus of the transmembrane protein permitting the desired protein to adhere to the exosome surface. After this, the sequence was cloned into a desired plasmid (Mentkowski et al., 2018). Exosomes carry different transmembrane proteins, such as CD9, CD63, PTGFRN, and Lamp2b, that can be used for exosome surface-engineering (Li et al., 2022). Second, donor cells or cell lines transfected with a plasmid,

expressing the fusion proteins produce engineered exosomes containing targeting ligands on their surface. Last, engineered exosomes are loaded with desired functional RNAs and therapeutic drugs to deliver into the target cell by endocytosis and cargo released into the cytoplasm. This method works well for surface protein and peptide display, but it can only be used to target sequences that can be genetically encoded (Liang et al., 2021). Both strategies such as genetic or chemical modification, have been adopted successfully despite their drawbacks (Liang et al., 2021).

2.12. Genetically Modified Exosomes

In various preclinical studies, neurotropic virus-derived peptides such as RVG (Rabies Viral Glycopeptide) were employed to promote brain targeting of exosomes. In one study, RVG was expressed at the exosomal membrane and fused with Lamp2b, an exosomal membrane protein, to transfer siRNA-loaded exosomes to the brain. These modified exosomes successfully delivered siRNA to microglia, neurons, and oligodendrocytes in mouse brains. (Alvarez-Erviti et al., 2011). In another study, the same approach was used to effectively transport miR-124 to the infected location using a modified exosome that had the RVG fused to the exosomal protein lysosome-associated membrane glycoprotein 2b (lamp2b). Systematic treatment of RVG-exosomes containing miR-124 promoted cortical neural precursors to acquire neural identification and protected against ischemia injury via high cortical neurogenesis (Yang et al., 2017). In preclinical studies, engineered exosomes with the ability to target the brain demonstrated positive outcomes for CNS delivery.

In another recent study, miR-26a is delivered to liver cancer cells that express scavenger receptor class B type 1 (SR-B1) using modified exosomes. Exosomes are created by combining the CD63 and Apo-A1 proteins, allowing selective binding to SR-B1 on HepG2 cells. These exosomes, which are loaded with miR-26a, increase miR-26a levels, limiting cell

migration, preventing proliferation, and inhibiting cell cycle proteins (Liang et al., 2018). Wang et al. (Wang et al., 2018) used molecular cloning and lentivirus vector to develop an exosome-enriched membrane protein (Lamp2b) that is linked with the ischemic myocardium-targeting peptide CSTSMLKAC. To enhance the therapeutic advantages of drugs or microRNAs delivered to ischemic cardiac tissues, MSC-derived exosomes that target the ischemic myocardium may serve as delivery vehicles. Limoni et al. (Limoni et al., 2019) conducted a study in which they modified HEK293T cells to produce exosomes with HER2-targeting DARPin G3 proteins on their surfaces. When these modified exosomes were delivered to SKBR3 cells, they efficiently bind to the HER2/Neu receptors and downregulated the production of a certain gene (TPD52 gene), which prevented the proliferation of tumor cells like breast cancer cells.

2.14. Exosome Isolation and Characterization

Different exosome isolation strategies have been developed based on exosome size, shape, density, and membrane proteins Centrifugation (differential or density gradient), precipitation, immuno-capture, size exclusion chromatography (SEC), and ultrafiltration are some of the most extensively used techniques for EV separation (Lai et al., 2022). A traditional gold standard method called ultracentrifugation uses high-speed centrifugation to separate exosomes from larger particles relative to their densities (Gurunathan et al., 2019). Size-Exclusion Chromatography (SEC) uses size-based separation to purify exosomes, resulting in highly pure samples. Immunocapture-based techniques use antibodies that are unique to exosome surface markers, enabling the extraction of exosomes with high selectivity. Furthermore, precipitation methods, such as polyethylene glycol (PEG) or commercial reagents, precipitate exosomes, which offer simplicity and speed. Due to their simplicity of use, commercial isolation kits have also gained popularity (Chen et al., 2022).

Following isolation, an extensive choice of characterization techniques can be used to verify the identification and purity of the isolated vesicles. One of the most widely used methods is electron microscopy, which includes scanning electron microscopy (SEM) and transmission electron microscopy. These methods enable the visualization of exosomes at the nanoscale, providing comprehensive structural details and illustrating their spherical morphology (Kurian et al., 2021). Exosome concentration and size distribution can be found in a sample using methods like nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS). Western blotting is one immunoblotting technique that is used to identify particular protein markers and confirm the existence of exosomes. Tetraspanins (CD63, CD9, and CD81) are common exosomal markers that can be detected with antibodies (Kuncewicz et al., 2022).

Blood cancers and autoimmune diseases provide difficult challenges in the field of medicine, frequently requiring precision delivery of therapeutics to immune cells such as T cells, B cells, natural killer cells, and monocytes. Exosome genetic engineering with HSV glycoprotein D is a novel and promising technique for targeted medication delivery in the treatment of blood disorders and autoimmune illnesses. This innovative approach combines the natural properties of exosomes with precise targeting, offering the potential for improved therapeutic outcomes while minimizing adverse effects, and will change the way these conditions are managed, ultimately enhancing the quality of life for patients who are affected by these conditions.

CHAPTER 3

Methodology

3.1. Peptide Sequence Retrieval

For developing targeted exosomes, the HSV Glycoprotein D sequence that binds to the HVEM receptor was retrieved from a computational analysis study by Kuncewicz et al.(Kuncewicz et al., 2022). gD peptide is the 36 amino acid sequence peptides.

3.2. In-Silico Targeted Sequence Construction

One of the most important steps in the creation of a new exosome drug delivery system is the design of a targeted sequence. For in-silico sequence design, we utilized SnapGene software. To do this, we fused the exosomal transmembrane protein CD63 with the HSV gD peptide sequence, linked by a flexible GS linker to produce a sequence that is specifically designed for targeted delivery of drugs. The gD peptide sequence was reverse-translated into double-stranded DNA on SnapGene® (*SnapGene | Software for Everyday Molecular Biology*, n.d.).

Several important elements were considered during the construction process to ensure the engineered sequence's functionality and efficacy:

3.2.1. Kozak Sequence - Initiating Translation

The Kozak sequence was incorporated into the sequence. This contains the start codon (usually the AUG) and acts as a translation initiator. It ensures that the ribosome accurately identifies the start of translation, allowing for the development of the target protein.

3.2.2. Flexible Linker - Bridging Two Sequences

To attach the HSV gD peptide sequence to the CD63 protein, a flexible linker (GGGGSGGGGGGGGS) was used. This linker is essential for maintaining the fused protein's structural integrity and functioning, enabling appropriate folding and function.

3.2.3. His Tag - Protein Identification

A histidine (6xHis) tag was added to the design at the N-terminus via GS linker (GGGS), to help in the identification and purification of the target protein.

3.2.4. Stop Codon - Termination of Translation

The sequence design incorporated a stop codon (typically UAA, UAG, or UGA). This stop codon indicates the end of protein translation and ensures that the newly created protein is fully synthesized and functioning.

3.3. Selection of an Optimal Vector

Choosing the right vector to insert a sequence for mammalian expression is an important step in molecular biology and biotechnology research. During the selection process, special care was taken to verify that the vector chosen had all the necessary characteristics stated below.

- Origin of Replication
- Selectable Marker
- Multiple Cloning Sites (MCS)
- Mammalian expression promotor
- Enhancer Elements
- Expression System Compatibility

The pTwist CMV Puro vector was chosen to express the HSV gD sequence within mammalian cells. This human cytomegalovirus (CMV) promoter-driven vector has an ampicillin resistance cassette for growth and maintenance in E. coli and is intended for high

levels of transient expression in mammalian cells. An SV40 poly-adenylation signal 3' of the numerous cloning sites terminates transcription. Select vectors also carry mammalian selectable markers such as puromycin resistance genes for mammalian cell selection.

3.4. In silico cloning

In silico cloning was performed using the SnapGene program, where the HSV sequence was introduced into the pTwist CMV Puro vector between the BamHI and EcoRI insertion sites. After the in-silico cloning was successful, the modified vector containing the desired sequence was ordered from Twist Bioscience.

3.4. Plasmid recovery

The plasmid initially came in lyophilized form with a 2000 ng concentration. To recover a final plasmid concentration of 2000 ng, 25 µL of nuclease-free water (NF water) was added, which has a concentration of 80 ng/µL.

3.5. Electrocompetent cells preparation of DH5α

To make DH5 competent cells, inoculate a single colony from a glycerol stock onto an LB agar plate, then incubate it for the entire night at 37°C to produce isolated colonies. On the second day, isolated colonies from the agar plate were inoculated into 5-10 mL of sterile LB broth and incubation was done overnight at 37°C with agitation. This overnight culture serves as an inoculum for the main culture. The next day after incubation, 1 ml of overnight or mother culture was taken out and subcultured in another flask with 50 ml of fresh nutrient broth. The culture was incubated at 37°C with shaking for two to three hours, or until the OD600 reached around 0.4 to 0.5. Following that, the bacterial culture was transferred into falcon tubes that had already been cooled, and it was incubated on ice for 20 minutes. A 10-minute centrifugation was performed at 4°C and 5000 rpm on the bacterial culture contained in the flacons. The pellet was resuspended in 20 mL of ice-cold, 0.1M CaCl2, and incubated

for 30 minutes on ice after the supernatant was discarded. At 4°C and 5000 rpm, centrifugation was repeated for 10 minutes. After discarding the supernatant, the particle was redissolved in 5ml of ice-cold 0.1M CaCl2. The resuspended pellet was aliquoted as 50ul in pre-chilled microcentrifuge tubes. These competent cells were utilized for transformation immediately, while the remaining cells were kept at -80°C.

3.6. Transformation of pTwist CMV Puro vector in DH5α by Heat Shock Method

Heat-shock treatment was used to transform the ligated vector i.e., pTwist CMV Puro vector with insert HSV gD, into the DH5 competent cells. Competent cells were placed on ice to thaw. In a microcentrifuge tube, 1-5 uL of the ligated plasmid (pTwist CMV Puro plasmid) DNA was added to the 50ul competent cells. To mix the contents, gently tap or flick the tube. Do not vortex. The sample was kept on ice for 30 minutes. After incubation on ice, the sample was placed in a 42oC water bath for 90 seconds. Following two minutes on ice, the cells were incubated for one hour at 200 rpm and 37 °C in a shaking incubator to promote outgrowth. A 100l-200ul transformation mixture was spread on the plates with ampicillin as an antibiotic selection marker, and the plates were incubated at 37oC for 12-16 hours.

3.7. Colony PCR Confirmation of Single Transformation

Colony PCR was done to confirm whether a plasmid containing the desired HSV gD gene was present within a bacterial colony. For this process, a pair of forward primers and reverse primers were designed to amplify the target DNA sequence inside the vector. The following criteria were taken when designing primers:

- GC content between 40-60%.
- The forward and reverse primers' Melting Temperature (Tm) values are within 5°C
- Primer length ranges from 18-25 bp.

OligoCalc was used to check the secondary structures, self-annealing, or self-complementarity of primers. Primer 3 and OligoAnalyzer were also used to check the Tm, GC content, and possible hairpin formation of primers. The primer sequences are listed below.

3.7.1. Primers of colony PCR

Sr. No	Name	Sequence
1	Forward Primer	5’ ACGCACTTGTAGATGCAAG 3’
2	Reverse Primer	5’ CCACCTCCACCAACATGA 3’

3.7.2. Steps of Colony PCR

For colony PCR, a single bacterial colony was picked from a culture plate using a pipette tip and put in a sterilized microcentrifuge tube containing a 20 ul volume of sterile water or NF water. The tube was spun in the micro spin. After spinning, the tube containing the colony was heated in a thermocycler at about 95°C for 10 minutes. This helps in the lysis of bacterial cells and the release of DNA. Serve as a DNA template. In a fresh PCR tube, a 25 uL PCR reaction mix was prepared using 12.5 ul of DreamTaq Green PCR master mix, 1 uL of forward and reverse primers, and 2 uL of ready DNA template. The remaining volume was adjusted to 25ul by adding NF water.

The following temperatures were used for PCR amplification: The initial denaturation stage of the PCR amplification was carried out at 95 °C for 10 minutes. This was followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing at 58 °C for 45 seconds, extension at 72 °C for 45 seconds, and finishing with a final extension step at 72 °C for 10 minutes. Gel

electrophoresis was used to determine whether colony PCR amplification was successful. Upon completion of the PCR, the amplified DNA fragment was seen by running the PCR result on a 1.5% agarose gel.

3.7.3. Gel Electrophoresis

To make a 1.5% gel, 0.75 grams of agarose powder was weighed out and added to 50 mL of newly made 1X TAE buffer (Tris-acetate-EDTA), which has been prepared by diluting a 50X TAE stock with distilled water. The mixture was heated to boiling until it was clear, indicating agarose dissolution. Ethidium bromide, a DNA dye, was added to the cooled agarose-TAE solution and thoroughly mixed. A comb was used to make wells in the gel for putting DNA samples in the gel casting tray. After pouring the agarose gel, it was allowed to solidify. The comb was gradually removed from the gel after it had been set and placed in a gel tank containing a 1X TAE buffer. 15uL of the PCR reaction product was loaded into the gel's wells. To determine the sizes of the DNA fragments, a 50bp DNA ladder was also loaded in the gel. The gel was seen under UV light after electrophoresis.

3.8. Extraction and storage of plasmid

A single colony of transformed bacteria was inoculated into a culture of LB broth with 200ug/ml of ampicillin antibiotic. The flasks were cultured overnight for 16 hours in a shaking incubator at 37°C. Plasmids were extracted from the overnight bacterial culture using a plasmid DNA extraction kit. The same culture was utilized to prepare plasmid glycerol stocks.

3.8.1. Plasmid Miniprep

Following the manufacturer's instructions, the GeneJET Plasmid DNA Purification Kit was utilized. The bacterial cells were pelleted by centrifuging 15 mL of the overnight culture for two minutes at 12,000 x g in microcentrifuge tubes. After carefully discarding the

supernatant, the cell pellet was resuspended in 250 uL of Resuspension Buffer (Buffer S1) from the kit. Pipette up and down slowly to ensure proper resuspension. 250 uL of Lysis Solution (Buffer S2), which causes cell lysis, was added to the resuspended cells and mixed by an upside-down tube 4-6 times or gently vortexing until the resulting solution turned viscous and slightly translucent. The tubes were placed on ice for no longer than 3 minutes. 350 uL of solution III was added to the tubes for the precipitation of chromosomal DNA and denatured proteins and mixed by inverting them 5 to 6 times. Due to white precipitates of chromosomal DNA and proteins, the liquid's appearance at this point turned cloudy. After a refrigerated centrifuge was used to centrifuge the microcentrifuge tubes for 15 minutes at 4°C and 13,000 rpm, the microcentrifuge tubes were centrifuged for 15 minutes at 4°C at 13000 rpm in a refrigerated centrifuge. The clear supernatant, which contained plasmid DNA, denatured protein debris, and chromosomal DNA, settled in the form of a pellet. The supernatant was carefully collected using a micropipette and transferred to a GeneJET purification column included in the kit. The white precipitate was not disturbed or transferred. At 10,000 x g for 1 minute, the column was centrifuged to bind the DNA to it.

After discarding the flow-through, the column was reinserted into the same collection tube. The column had been filled with 500 uL of Wash Solution (Buffer S4) and centrifuged at 10,000 x g for 1 minute. The washing step was carried out once again with 500 uL of Wash Solution (Buffer S4). The column was inserted in a fresh 1.5 mL microcentrifuge tube. Elution Buffer (Buffer S5) in a volume of 50-100 uL was poured directly over the column membrane. The column was kept in place for 15 minutes to allow the DNA to attach to the buffer. To elute the purified plasmid DNA into the microcentrifuge tube, the centrifuge was run at maximum speed for 1 minute. The plasmid DNA that had been purified was stored at -20°C, and the column was disposed of.

3.9. Plasmid DNA quantification and agarose gel electrophoresis

The quantification of plasmid DNA was measured using a Nanodrop spectrophotometer. Following the guidelines in the Colibri microvolume spectrophotometer user manual, 1 uL of the plasmid DNA sample was placed on the spectrophotometer's microvolume pedestal. The absorbance of the DNA sample was measured at 260 nm to determine its concentration. 0.7% agarose gel electrophoresis was also performed to confirm Plasmid DNA and the entire protocol for this procedure can be found in Section 3.7.3. 10ul Plasmid DNA samples were mixed with 3ul of 6X loading dye and loaded in the gel's wells. A 50 bp ladder was used as a reference. The gel was reviewed using a UV illuminator.

3.10. PCR confirmation of extracted plasmid

A PCR confirmation technique was used to verify the presence and integrity of the isolated plasmid DNA. Following the PCR steps detailed in section 3.7.2, extracted plasmid DNA was used as the template for PCR reactions and was added to a PCR reaction mix including DreamTaq Green PCR master mix, specific primers, and NF water. The PCR thermal cycler program, which included denaturation, annealing, and extension phases, was set up as described in section 3.7.2. After that, the PCR results were analyzed by running 1.5% agarose gel electrophoresis to validate both their size and the presence of amplified DNA fragments.

Cell Culture:

3.11. Maintenance of HEK293 Cell Line

HEK293 cell line was obtained from Professor Dr. Sobia Manzoor (ASAD, NUST, Islamabad, Pakistan). Cells were cultured as adherent monolayers in tissue-treated T25 flasks in Dulbecco's Modified Eagle's media (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin from Thermo Fisher Scientific (Fair Lawn, NJ). The cells were grown in a sterile cell culture incubator at 37°C in 5% CO2. Depending on the medium's pH, the

medium was replaced two to three times a week, and cells were allowed to develop to 70 to 90 confluency.

3.12. Preparation of Transfection in HEK293 Cells Modified Exosome

When HEK293 cells cultured in a T25 flask reached 70-90% confluency, the process of passaging was initiated. The cells were trypsinized with 1X trypsin-EDTA and incubated for 5 min at 37C to detach them from the flask's surface. Cells were then neutralized with complete growth media to stop trypsin action and provide a nutrition-rich environment. After neutralization, the cell suspension was collected into a 1.5ml centrifuge tube and centrifuged for around 5 minutes at a moderate speed (e.g., 800 g) to collect the detached cells into a pellet. After that, they were resuspended in a 1 ml media for counting. Cell counting was done to precisely calculate the cell density, ensuring correct seeding into a new culture flask at the required density.

3.12.1. Cell Counting

Trypan blue is a common stain used for cell counting and cell viability. Cell counting with trypan blue staining is based on the dye's differential exclusion by viable and non-viable cells. Viable cells have intact cell walls that exclude trypan blue, keeping them unstained and transparent under a microscope. In contrast, non-viable cells with ruptured or weakened membranes allow trypan blue to permeate and dye them blue. The assay was carried out by loading a hemocytometer with 10ul of cells from cell suspension mixed with 10ul Trypan blue solution. A loaded hemocytometer was placed on an inverted light microscope. Viable (unstained) and non-viable (stained) cells were counted within counting grids under a microscope to determine overall cell density and viability. After that, the average cell count, and dilution factor were calculated to determine the cell concentration.

Number of viable cells/mL = $\frac{\text{Number Live Cell Counted}}{\text{Number of Large Corner Square Counted}}$ X Dilution Factor X 2

After calculating the total viable cell concentration, cell plating calculation was carried out by using a formula.

Cell plating = $\frac{\text{Needed Cells}}{\text{Total Calculated Cells}}$ X 1000

3.12.2. Human Plasma Containing Media Preparation:

To provide nutrients, fetal bovine serum (FBS) is frequently added to the culture media in cell culture experiments, However, it should be noted that FBS contains a large number of exosomes, which might cause interference and contamination challenges. Human serum was used in place of FBS to enhance the accuracy and specificity of isolated exosomes from the cells of interest.

3.12.3. Transfection

The day before transfection, cells were seeded into two separate T25 flasks at a density of 0.7 x 106 cells: one for the control group and one for the experimental group. Before transfection, the plated cells were allowed to grow until they were 70-90% confluent. The Lipofectamine 3000 procedure for DNA transfection, as described in the manufacturer's instructions, was used to insert plasmid DNA into the cells. Cells were then incubated with the transfection mixture for six hours at 37°C and 5% CO2 in a cell culture incubator. After incubation, the transfection mixture was gently aspirated and replaced with fresh, pre-warmed complete cell culture media containing puromycin. Puromycin was used as a selection marker to confirm the success of transfection. The next day, both the control and experimental groups were given puromycin at a concentration of 5 ug/uL. The cells were then cultured for 48 hours in a puromycin-containing solution in an incubator set at 37°C with 5% CO2. During the incubation time, the cells were carefully monitored to examine the survival

and proliferation of transfected cells in contrast to untransfected control cells, which served as a sign of successful transfection.

3.13. Transcriptional Level HSV gD Gene Expression Study.

To analyze the transcription level of HSV gD and CD63, total RNA was extracted from control and transfected cells with the trizol method. We next quantified the extracted RNA using a Nanodrop spectrophotometer. After quantification, reverse transcription was performed using the FIREScript RT cDNA Synthesis, which was made especially for RT-PCR. The following protocol was used per the manufacturer's instructions.

In each tube, prepare the following RNA/primer mixture:

Template RNA	2ul
Random hexamers (100uM)	1ul
Nuclease-free H2O	Up to 16ul
Total	16ul

After five minutes of incubation at 65°C, the samples were placed on ice for a minimum of one minute. The reaction master mixture has been prepared. For each reaction:

10x RT Reaction Buffer with DTT	2ul
dNTP MIX (20 mM of each)	0.5ul
FIREScript RT	1ul
RiboGrip RNase Inhibitor (40 U/µl)	0.5ul
Total	20ul

The reaction mixture was subsequently added to the RNA/primer mixture, gently stirred, and left at room temperature for 2 minutes. For cDNA synthesis, use the following program:

Primer annealing	42°C	60 min
Reverse transcription	80°C	5 min
Enzyme inactivation	80°C	7 min

Following the successful synthesis of cDNA, quantitative RT-PCR was used to assess transcription levels.

3.13.1. Quantitative Real-time PCR (qRT-PCR)

Specific forward and reverse primers for the target gene HSV gD and CD63 were designed using Snapgene software, and which were ordered from Macrogen. A housekeeping gene, B actin, was used to normalize the expression of HSV gD and CD63. The primer sequences for each gene, as well as the B actin, were as follows:

HSV gD	Forward Primer	5’ GTACGCACTTGTAGATGCAAGC 3’
	Reverse Primer	5’ CGGCGGGTCACATAATTGATC 3’
CD63	Forward Primer	5’ GTGCCTGTGCGGTAGGTTTA 3’
	Reverse Primer	5’ CCGGCGGGTCACATAATTGATC 3’
B-Actin	Forward Primer	5’ CACAGAGCCTCGCCTTTGC 3’
	Reverse Primer	5’ CCATCACGCCCTGGTGC 3’

The reaction was carried out in triplicate for each gene of interest in the test samples and the control. In each PCR tube, the following real-time PCR reaction mixture was prepared.

25 ml SYBR Green Mix (2x)	10ul
cDNA Template	2ul
Forward primer	1ul
Reverse Primer	1ul
Nuclease-free Water	6ul
Total	**20ul**

Set up cycle parameters according to general guidelines and put the PCR tubes in the real-time PCR machine. Following PCR completion, the real-time PCR data was analyzed by determining gene expression levels using the comparative Ct method (Ct) with the reference gene.

3.14. Exosome Isolation

Exosomes were isolated from cell culture media using a procedure that involved four successive cycles of centrifugation. and lipofectamine 4000 treatment. The transfected cells were harvested and grown in T25 flasks for an additional 48 hours. Following that, exosomes were purified from the control and transfected cell culture media using the polyethylene glycol 4000 method. The published protocol (Weng et al., 2016) (Shieh et al., 2022) was modified and optimized for the obtained result. The modified protocol is:

1. To make the exosome-enriched solution, 5 ml of cell culture medium/supernatant was extracted from adherent cells, and it was then put into a 15 ml Falcon tube. In order to remove undesirable components, the medium undergoes a series of centrifugation processes.

2. First, the media had been centrifuged at 500 g for around 30 minutes at 4°C to remove cell debris. After carefully removing the cell-containing pellet, the remaining supernatant was centrifuged at 5000 x g for 20 minutes at 4°C in order to get rid of large debris.
3. The supernatant was then passed through a 0.22 m syringe filter (Pall Life Sciences, Port Washington, NY, USA) to exclude particles bigger than 0.22 μm.
4. Following that, a 30-minute centrifugation at 10,000–20,000 x g and 4°C was performed on the filtrate. After discarding the pellet, the supernatant was once more filtered through a 0.22 μm filter to eliminate larger particles including apoptotic bodies and microvesicles. The filtered supernatant was kept at 4°C until exosome isolation.
5. To enrich exosomes, PEG 4000 (40 g; Sigma-Aldrich, St. Louis, MO, USA) was probe sonicated in 100 mL of ddH2O to make a 40% PEG 6000 solution. 1 mL of prefiltered supernatant was mixed with 250ul of 40% PEG solution in 1.5ml microcentrifuge tubes to obtain 8% PEG-medium solution.
6. The mixture was vortexed for 30 seconds before being incubated at 4°C overnight. The enriched exosomes were further pelleted by spinning the samples for 20 minutes at 10,000-16,000 xg.
7. The supernatant had been carefully discarded, and the resulting pellets were marked and resuspended in 0.22 m filtered PBS before being kept at -80°C.

3.15. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a robust imaging technique for morphological and size-based detection of exosomes with high-resolution pictures. A series of steps were taken to prepare exosome samples for SEM imaging. First, exosomes were diluted in 0.22nm filtered phosphate-buffered saline (PBS) for 1 hour. These exosomes were then individually labeled and fixed to glass cover slips with a 25% glutaraldehyde solution. After fixation, the cover slips were thoroughly washed three times with 0.22m filtered PBS to ensure that any

remaining fixative was removed. The slides were treated to gradual osmotic shock-based dehydration by rinsing them in 0.22um filtered ethanol solutions ranging from 10% to 100% for 5 minutes at a time. Following the ethanol treatment, the slides were rinsed twice with 100% ethanol and air-dried overnight in a laminar hood cabinet.

SEM analysis was performed on these prepared slides at the School of Chemical & Materials Engineering (SCME) using a TESCAN MIRA3 instrument. Several parameters were changed in order to characterize the morphology and size distribution of exosomes from processed samples.

3.16. Exosome protein lysis

The Radioimmunoprecipitation Assay (RIPA) procedure was utilized to lyse exosome proteins. RIPA is a commonly used technique for effective protein extraction and cell lysis. The precipitated exosomes were directly lysed in RIPA buffer with added protease and phosphatase inhibitors (Thermo Scientific), and the sample was incubated on ice for 1 h with vortexing followed by brief sonication. The cell lysate had been centrifuged at 16,000 g after sonication to eliminate any leftover cell debris. The supernatant was carefully taken out and put into a fresh tube. This is a protein lysate that contains solubilized exosome proteins. The lysate was either immediately used for downstream processes like Western blotting or kept under suitable conditions after protein quantification using the Bradford test or nanodrop spectrophotometer.

3.17. SDS-PAGE

SDS-PAGE is a protein separation technique based on molecular weight. SDS-PAGE was performed using a 10% resolving gel, a 4% stacking gel, and the running buffer. The gel apparatus was assembled and sealed with a comb to make wells for sample loading, and the gels were polymerized. The sample was made by diluting 40ug of exosome protein lysate in

3x loading dye at a 2:1 ratio, then heat-denatured at 100°C for 5 minutes, cooled, and loaded into the stacking gel wells with a micro-pipette. For reference, a known protein marker for exosomes with molecular weights ranging from 12 kDa to 130 kDa was also loaded into the wells. This was followed by setting the gel apparatus in a Bio-Rad mini gel tank that had a running buffer in it. The gel was afterwards operated at 110 V for the initial two hours, or until the dye front touched the bottom of the gel. After that, the gels were run at 110 V for two hours, or until the dye front touched the bottom of the gel. The gels were destained to reveal the separated proteins after electrophoresis and colored with Coomassie Brilliant Blue. To quantify protein molecular weights and relative amounts, gel pictures were captured and processed with specialist software.

The SDS-PAGE gel formulation is detailed in Table 3.1, which includes the recipe for both the stacking and resolving gels, using the Bio-Rad Protocol for MiniGels.

Resolving Gel 10%	**7ml (1 gels)**
Autoclaved DH2O	4ml
Acrylamide/Bis-acrylamide (w/v: 30%/0.8%	3.3ml
Tris Hcl 1.5M (pH=8.8)	2.5ml
10% (w/v) SDS	100ul
Ammonium persulfate (APS) 10% (w/v)	50ul
TEMED	7ul
Stacking Gel 4%	**5ml (1 Gels)**

Autoclaved DH2O	1.3ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	500ul
Tris Hcl 1.5M (pH = 6.8)	625ul
10% (w/v) SDS	25ul
Ammonium persulfate (APS) 10% (w/v)	25ul
TEMED	5ul

3.18. Western Blotting and Immunodetection

Western blot (WB) is a biochemical technique used to detect specific proteins in a sample containing many proteins. This technique is also known as immunoblotting as antibodies are employed to detect specific proteins in complex protein samples. Target protein expression, EV-associated protein expression (e.g., CD9, CD81, CD63, Alix, Tsg101), and the absence of interfering protein expression (e.g., Calnexin, Albumin, Fibronectin) in extracted samples are all frequently verified using Western blot analysis. After exosome lysis, the proteins were separated using gel electrophoresis (usually SDS-PAGE), as was covered in section 3.17, and then they were placed onto a membrane, typically, polyvinylidine difluoride (PVDF), to be exposed by certain antibodies.

Every material and reagent used in western blotting was prepared in the lab, including the LDS loading buffer, blocking buffer, running buffer, 1x transfer buffer, and 1x TBST. Polyvinylidene difluoride (PVDF) membranes and Western protein standards have been provided by Elabscience (Wuhan, China).

3.18.1. Western Blot Transfer

A pre-cut PVDF membrane with a pore size of 0.45um was soaked in methanol for 15 minutes. Simultaneously, the filter paper sheets along with sponges were pre-soaked in transfer buffer to fill the transfer cassette. The stacking gel was separated when the gel was gently removed from the tank, and the gel was covered by a sandwich comprised of presoaked blotting membrane, filter paper, and blotting pads, as depicted in **Fig. 3.1.** A transfer buffer was then added to the transfer module and ran at a constant current at 400mA for one hour.

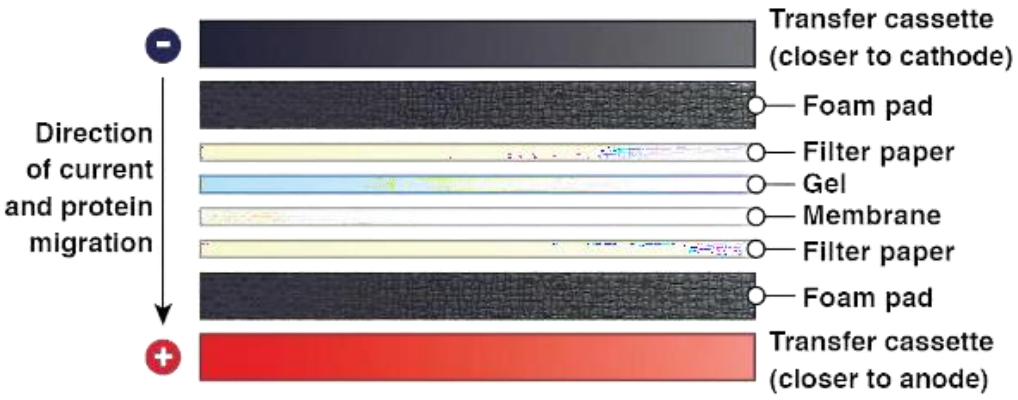

Figure 3.1. A transfer sandwich is set up for wet western blotting.

3.18.2. Immunodetection

After the transfer was complete, the membrane was taken out, and two 5-minute rinses in 20 milliliters of ddH20. It was then incubated for 1 hour with agitation in 10ml of 5% blocking buffer (5% BSA in TBS-T), followed by 30 minutes of washing in TBST at 6 x 5-minute intervals. Next, Rabbit anti-His-Tag pAb (AE068) was added, diluted at a ratio of 1:1500 in 10 ml of 5% blocking buffer, and incubated on a refrigerator shaker for an entire night at a temperature between 2 and 8 degrees Celsius.

Following the incubation of the primary antibody, the membrane was rinsed twice in TBST for 30 minutes. Next, it was incubated for 60 minutes at a ratio of 1:500 in a 2% blocking solution with Mouse Anti-Rabbit IgG(H+L) (peroxidase/HRP conjugated). After that, the membrane was washed every 10 minutes for thirty minutes, and it was then processed with Elabscience ® Excellent Chemiluminescent Substrate (ECL). Lastly, the membrane was processed on the ChemiDoc MP imaging system for a duration of time ranging from 1 to 60 minutes, depending on the signal strength.

CHAPTER 4

Results

4.1. Vector Selection

The selected vector, pTwist CMV Puro with the target sequence, was ordered from Twist Bioscience. It included all essential sequence components and met every requirement that was carefully considered while selecting the best vector for expressing the recombinant protein in mammalian cells. (Fig. 4.1).

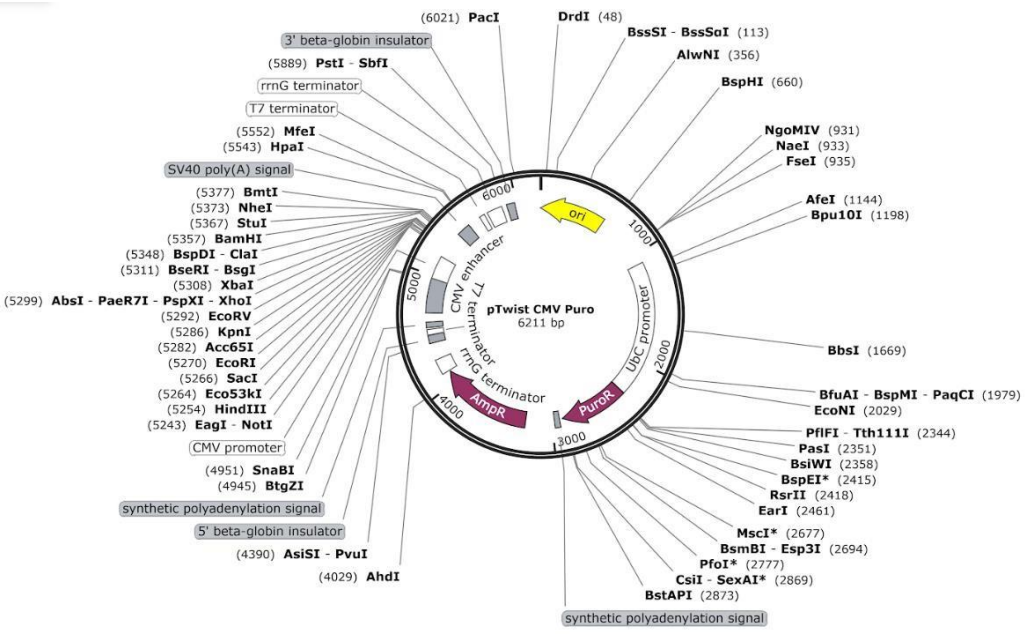

Figure 4.1. pTwist CMV puro.

The vector consists of all the necessary sequence elements.

4.2. In silico Target DNA Template Sequence Assembly

The HSV gD peptide sequence that binds to the HVEM receptor was retrieved from a previous computational analysis study. Then, a DNA sequence was constructed on Snapgene software using the peptide sequence as a foundation that was initially obtained. The first protein sequence containing HSV gD was reverse-translated into double-stranded DNA. Then the HSV gD DNA sequence was fused CD63 via a flexible linker to ensure proper alignment, and a histidine tag (his tag) was added at the N-terminus. Additional elements, including insertion sites, a Kozak sequence to start the translation, and a stop codon to signal the end of the translation, were added. As a result, a final 918-bp dsDNA template was formed, as illustrated in **Fig. 4.2.**

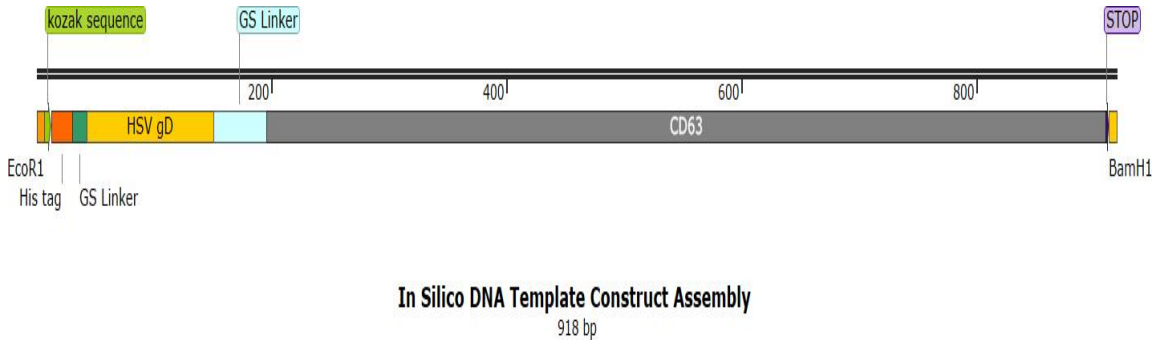

Figure 4.2. In Silico DNA Template Construct Assembly

For analysis of the protein properties of that sequence, Snapgene software was used to convert the DNA sequence into its corresponding amino acid sequence (peptide). A 316-residue-long peptide sequence with a calculated molecular weight of 33KDa was found shown in **Fig. 4.3**

	Whole Protein
Length	316 aa
Molecular Weight	33,016.73 Da
Extinction Coefficient (280 nm)	21,430 M^{-1} cm^{-1}
Absorbance (280 nm, 0.1%)	0.65
Isoelectric Point (pI)	6.63
Charge at pH 7.0	-5.30

Figure 4.3. Properties of the targeted peptide. *The peptide expressed on the exosome surface is 316 amino acids long with 32KDa weight.*

4.2.1. In Silico Cloning

The finalized target template was cloned in the pTwist CMV Puro using SnapGene software before ordering and proceeding with wet lab experiments, visually represented in **Fig. 4.4.** After in silico cloning was successful, the pTwist CMV Puro vector with target template was ordered from Twist Bioscience.

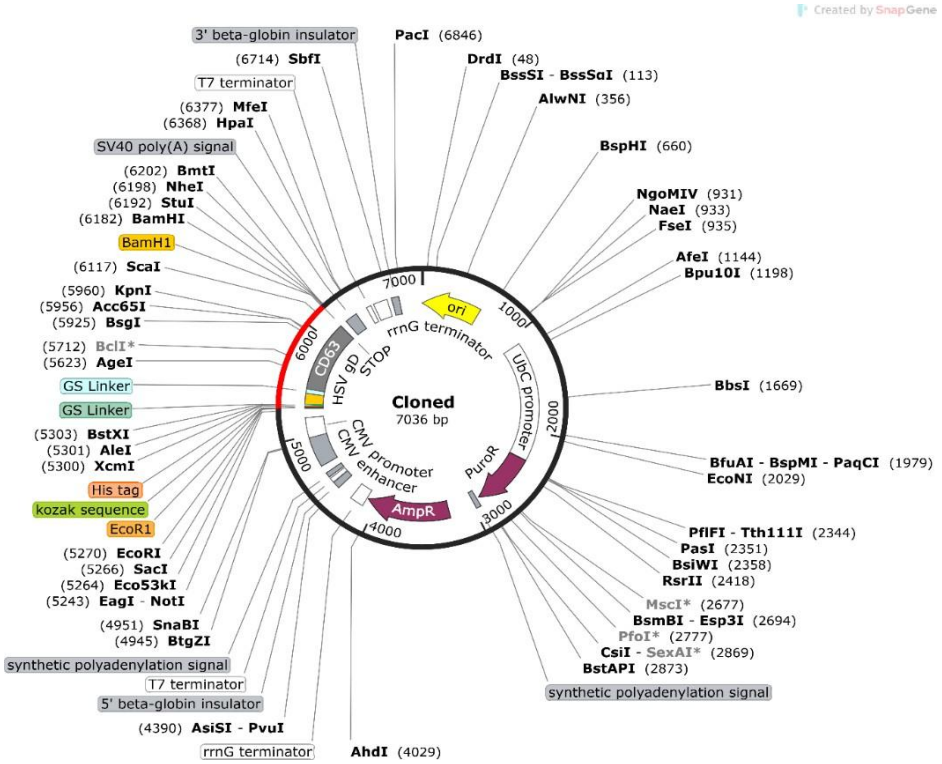

Figure 4.4. Cloned Target HSV gD Template in pTwist CMV puro

4.3. Propagation of pTwist CMV Puro Vector:

The pTwist CMV Puro vector received from Twist Bioscience was successfully converted into electrocompetent E. coli DH5 cells using the heat shock method. After transformation, the cells were plated on ampicillin-positive agar plates. In the results, colonies were seen on the ampicillin-positive plates as shown in **(Fig. 4.5)**, indicating a successful transformation. The experiment also included a negative control in which E. coli cells undergo the same transformation procedure without the addition of the plasmid. No colonies were found on the ampicillin-positive plates in the case of negative control. The lack of colonies in the negative control group confirms that the colonies seen in the experimental group were the consequence of a successful transformation with the pTwist CMV Puro vector.

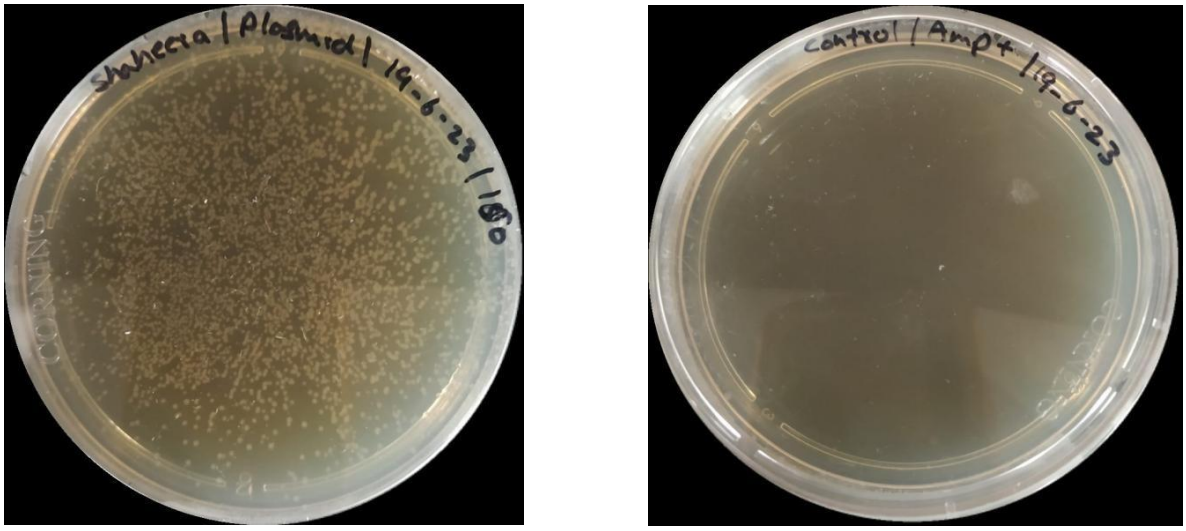

Figure 4.5. pTwist CMV puro positive colonies.

pTwist CMV puro positive colonies (left) and negative control cells transformed without plasmid (right) on ampicillin-positive plates.

4.4. Confirmation of Transformation HSV gD by colony PCR

After observing colonies on antibiotic plates that suggested successful single transformations, the presence of the required DNA fragment was confirmed using colony PCR, as described in section 3.7.2. Gel electrophoresis was used to further validate the colony PCR amplification verification.

The primers used for colony PCR had an estimated product size of 444 base pairs (bp). The gel electrophoresis results were analyzed, and it was found that the bands obtained matched the predicted size of 444bp **(Fig. 4.6)**, with a 100-base pair (bp) ladder serving as a reference. This data suggests that the colonies chosen for PCR had the desired DNA sequence, providing conclusive evidence of effective transformation and insertion of the targeted DNA fragment into the bacterial colonies.

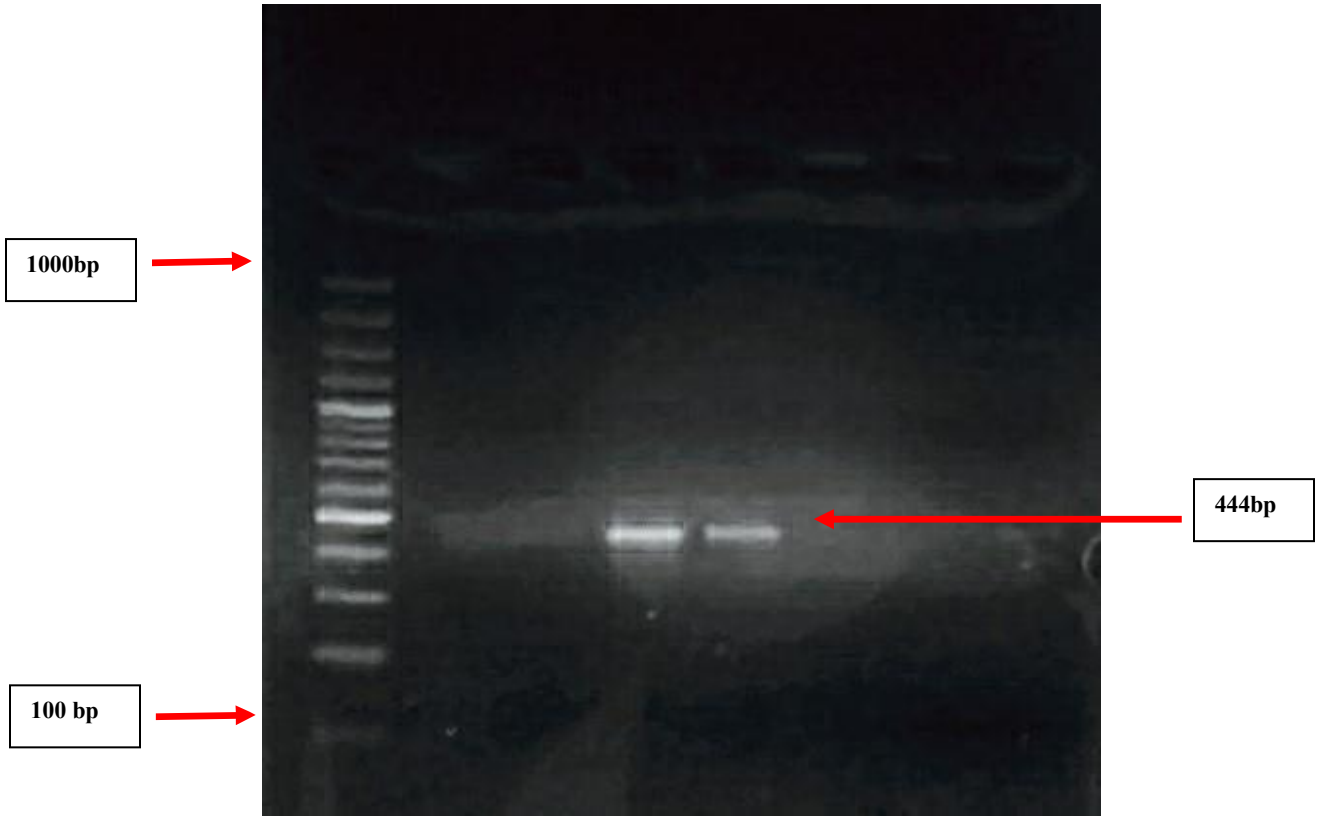

Figure 4.6. Colony PCR confirmation with external primers.

The PCR of pTwist CMV puro with external primers resulted in a 444 bp amplicon that can be visualized on 2% 1XTAE agarose gel with a 100 bp DNA marker.

4.5. Quality and Quantity assessment of plasmid after miniprep

GeneJET Plasmid DNA Purification Kit was used to successfully extract the plasmid from an overnight inoculum of transformed colonies in LB broth. Gel electrophoresis was then carried out, and the results were seen using a UV transilluminator **(Fig. 4.7).** When compared to a 1-kilobase pair (kbp) ladder, the gel electrophoresis results showed bands at a size of about

4000 base pairs (bp). These results confirm the effective isolation of the plasmid (which is roughly 6,211 kb in linear size) in it supercoil form, The supercoil plasmid (4000bp) appeared significantly smaller than its linear size (6,211 kb) due to its supercoiled shape.

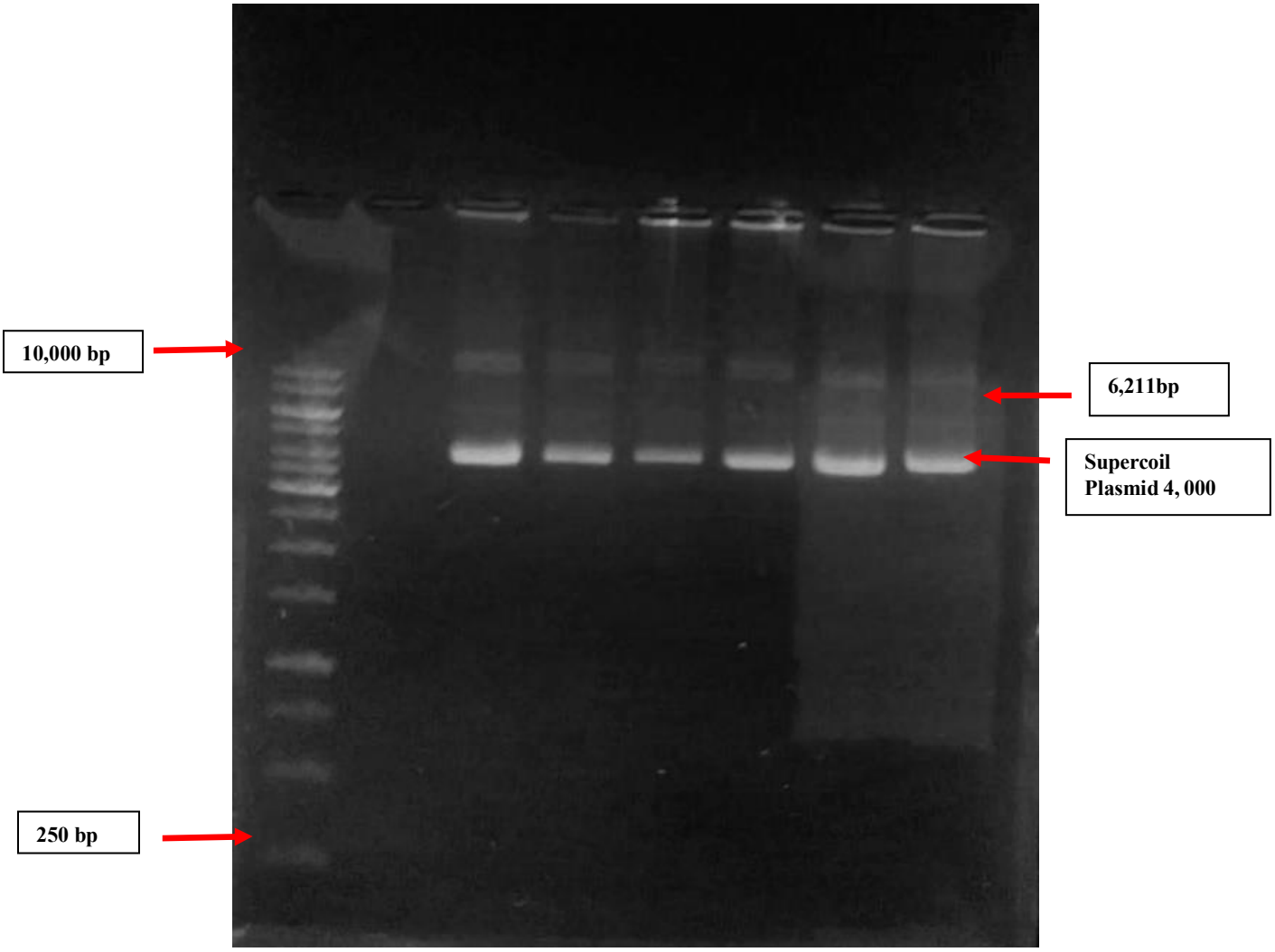

Figure 4.7. Gel electrophoresis of pTwist CMV Puro plasmid.

Supercoiled plasmid bands at ~4000bp with a 1kbp DNA marker on 0.7% TAE agarose gel.

The plasmid's quantity was determined by nanodrop spectrophotometric measurement in addition to gel electrophoresis. The results showed a considerable yield of plasmid DNA at a concentration of 151.59 nanograms per microliter (ng/L). Furthermore, the A260/A280 ratio, which was found to be 1.80 (**Fig. 4.8**), was used to evaluate the isolated plasmid's purity. This

ratio highlights the isolated plasmid's excellent purity, indicating low contamination with proteins or other contaminants.

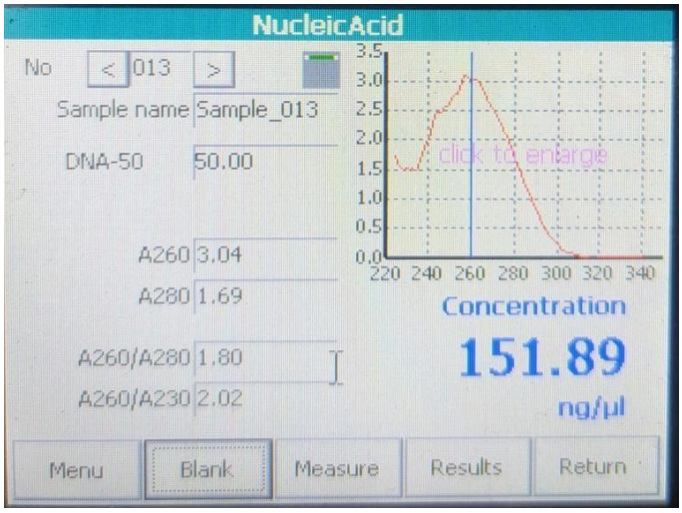

Figure 4.8 DNA quantification

Purity and concentration of plasmid. A good A260/A280 and A230/A260 ratio of 1.80 and 2.02 respectively on the nanodrop spectrophotometer indicates the purity of the plasmid.

4.6. Confirmation of plasmid

After the plasmid extraction, the presence of the plasmid was verified using PCR (Polymerase Chain Reaction) with external primers. When PCR reactions were resolved on a 2% agarose gel and seen with a transilluminator, the results showed identifiable bands with an approximate size of 444 base pairs (bp), as shown in **Fig. 4.9**. The presence of this accurate 444 bp band in the PCR result provides strong validation of the plasmid's successful purification and retention of the target DNA sequence.

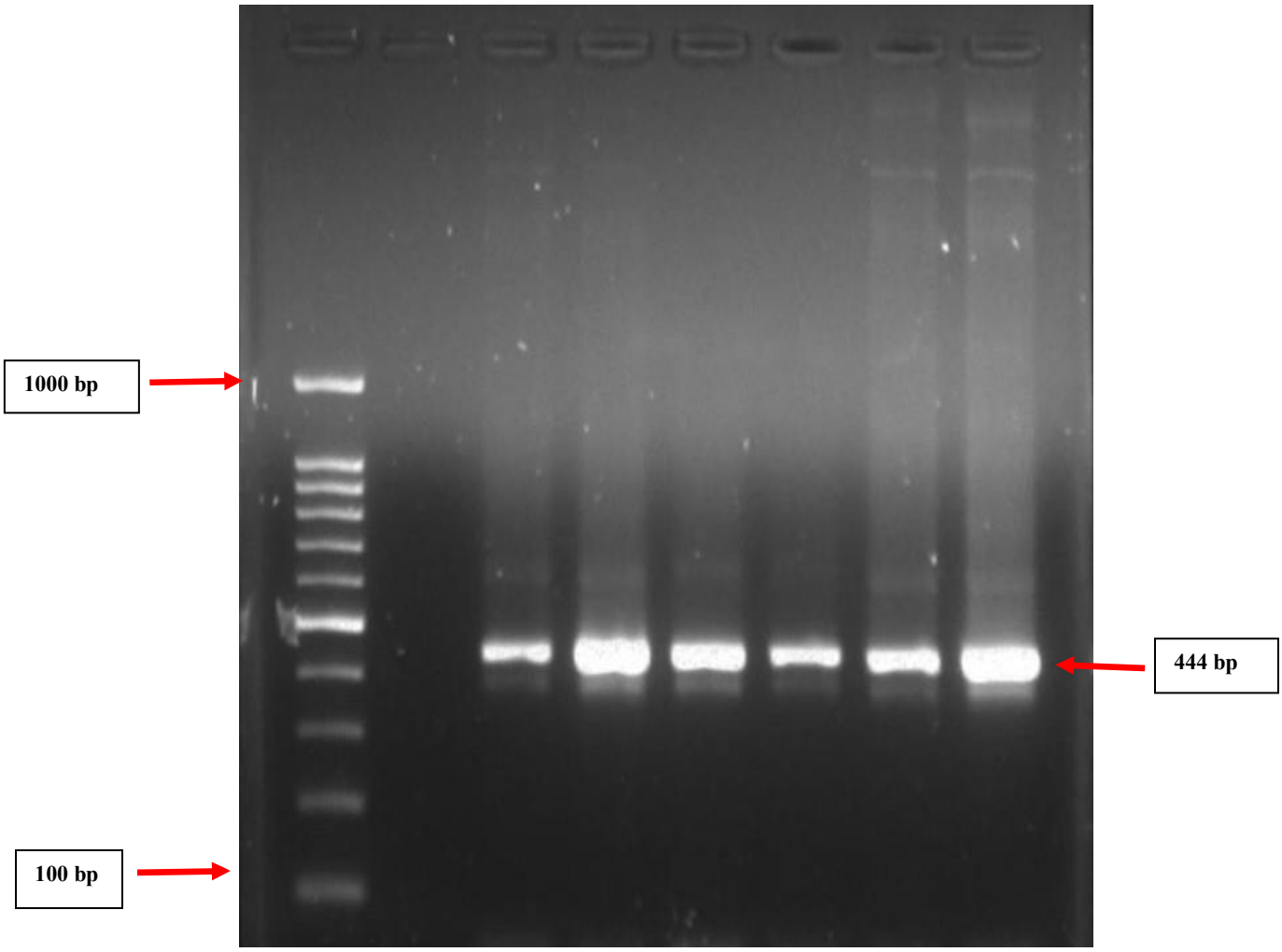

Figure 4.9 PCR confirmation with external primers.

The PCR of pTwist CMV puro with external primers resulted in a 444 bp amplicon that can be visualized on 2% 1XTAE agarose gel with a 100 bp DNA marker.

4.7. Validation of successful Transfection in HEK292 cell line

Lipofectamine 3000 was used as the transfection reagent to transfect cells with a plasmid carrying a puromycin resistance gene. Transfection was confirmed by placing the cells in puromycin selection, which ensured the survival and multiplication of the transfected cells. Following a 24-hour and 48-hour analysis period in puromycin selection media, the results depicted in **Fig. 4.10**, show the presence of surviving and proliferating cells in the experimental group that was exposed to puromycin after 24 and 48 hours. In contrast, untransfected control cells did not proliferate significantly under puromycin selection. This result demonstrates that the transfected cells successfully incorporated the plasmid DNA and displayed puromycin resistance, enabling them to grow rapidly in the presence of this selective agent is strong proof of the successful transfection procedure.

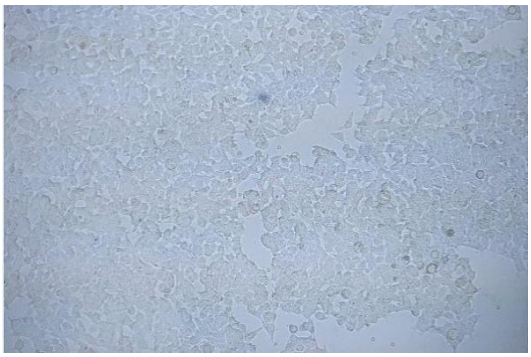

Figure. 4.10a. Control Cell after 24 hours of Transfection

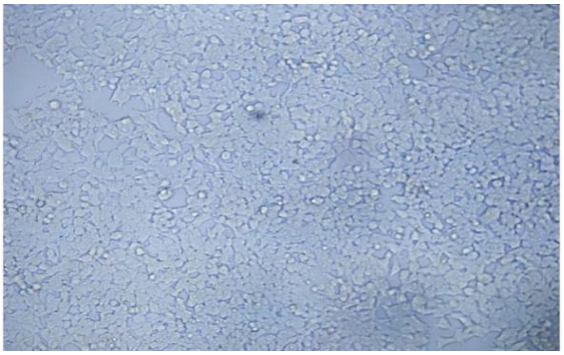

Figure. 4.10b. Transfected Cell after 24 hours of Transfection

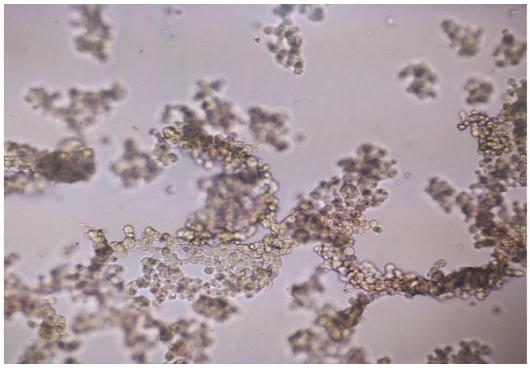

Figure. 4.10c. Control Cell after 24 hours of puromycin Treatment

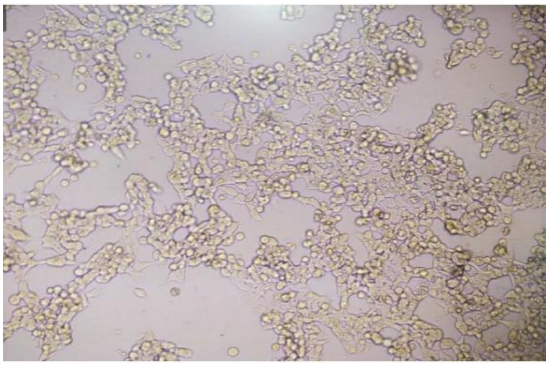

Figure. 4.10d. Transfected Cell after 24 hours Puromycin Treatment

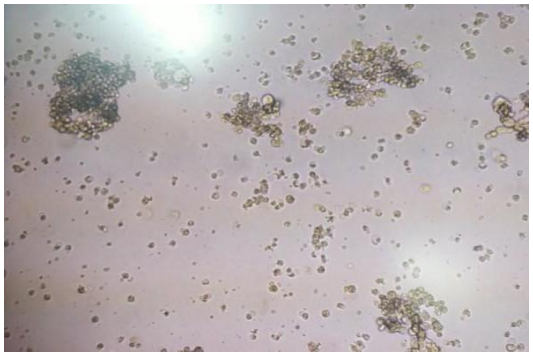

Figure. 4.10e. Control Cell after 48 hours of puromycin Treatment

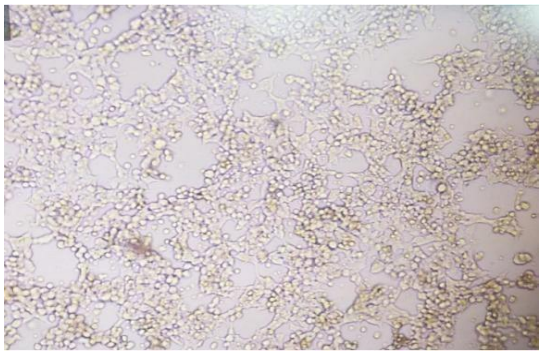

Figure 4.10f. Transfected Cell after 48 hours of Puromycin Treatment

Figure 4.10. Transfection in HEK 293 Cells. (a) Control cells were transfected without a plasmid, whereas **(b)** experimental cells, also called transfected cells, were transfected with a plasmid using Lipofectamine 3000 **(c)** After 24 hours of puromycin treatment, half of the control cell dies. **(d)** After 24 hours of puromycin treatment, the transfected cell survives due to the presence of the puromycin resistance gene. **(e)** Almost all control cells die after 48 hours of puromycin treatment, **(f)** Transfected cells continue to survive indicating the successful selection of transfected cells for further experiments.

Successfully transfected cells were seeded into a T25 flask and incubated for 48 hours, as shown in Figure 4.11a and b, for isolation of exosomes from these cells.

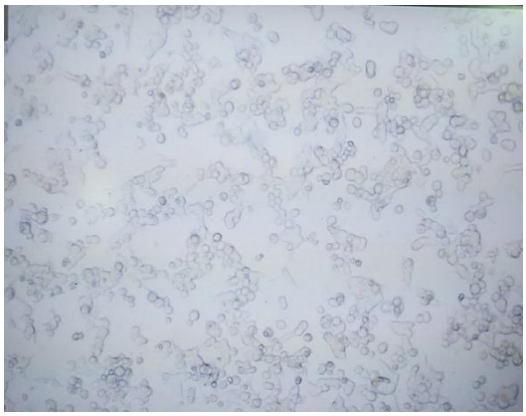

Figure 4.11a. Selected Transfected cell after 24 hours of seeding

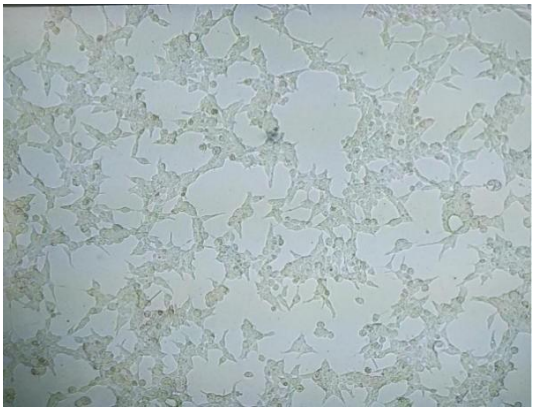

Figure 4.11b. Selected Transfected cell after 48 hours of seeding

4.8. Transcription Level Gene Expression Analysis

HSV gD and CD63 gene expression levels were determined by extracting RNA from both control and transfected cells. A Nanodrop spectrophotometer was used to quantify the separated RNA, and the results are presented in **Fig. 4.12a and 4.12b**, which show the RNA concentrations for the control and transfected cells, respectively. To verify RNA purity, RNA gel electrophoresis was also performed.

(a)

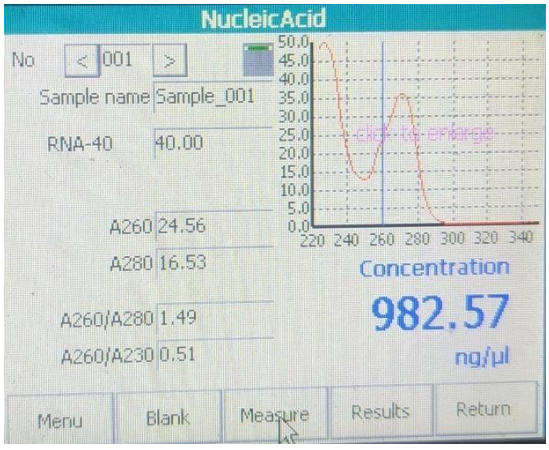

Figure 4.12a. Control Cells RNA quantification

(b)

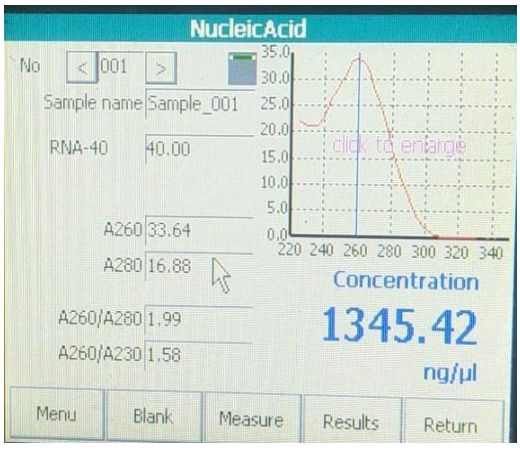

Figure 4.12b. Transfected Cells RNA quantification

Fig. 4.13a and 4.13b show the quantification of cDNAs after cDNA synthesis. RT-PCR was used to analyze the expression levels of HSV gD and CD63 in these cDNAs.

(a)

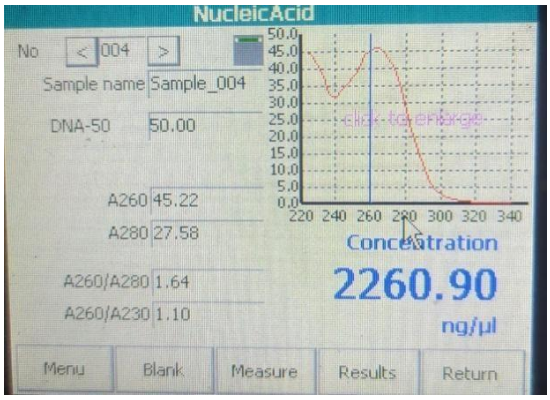

Figure 4.13a. Control Cells cDNA quantification

(b)

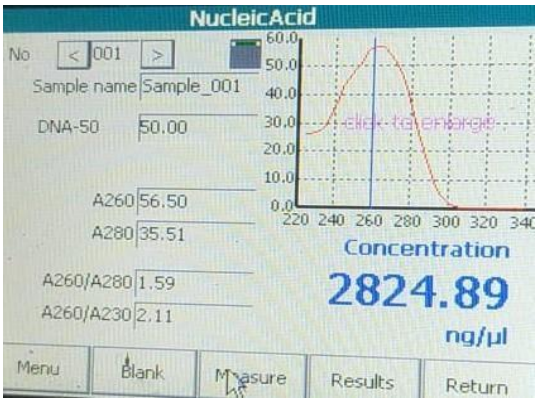

Figure 4.13b. Transfected Cells cDNA quantification

The RT-PCR analysis produced distinct results by using primers against the HSV and CD63 genes, as well as B-actin for normalization. The HSV gD gene was observed in transfected cells, presenting their existence. This gene, however, was noticeably absent in control cells, indicating a distinct expression profile. Similarly, the CD63 gene was detected in both control and transfected cells, with higher expression in transfected cells compared to controls. This discrepancy is attributed to the fact that control cells also possess the CD63 gene, while in transfected cells, additional CD63 is incorporated externally, resulting in elevated expression levels.

A quantitative study was carried out to determine the fold change in gene expression levels. Statistical analysis using a t-test provided p-values, showing the significance of observed differences. The plot was generated using GraphPad prism Software. When comparing transfected cells to control cells, the fold change in HSV gene expression was 296.9 and the associated p-value was P = 0.0015. Likewise, for the expression of the CD63 gene, the fold change was 13.76 and the corresponding p-value was P = 0.0071 as shown in **Fig. 4.14a and 4.14b.**

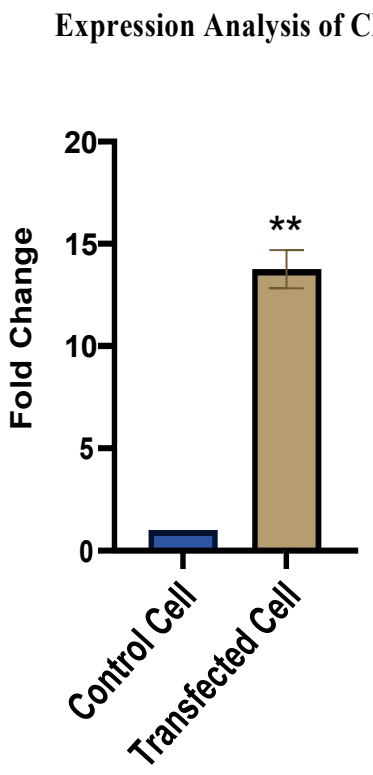

Figure 4.14a: Expression analysis of CD63 in control and transfected HEK293 cells. *Cells' total RNA was isolated. qRT PCR measured the expression of CD63. Statistical significance was calculated using a one-sample t-test (a p-value < 0.05 is considered statistically significant).*

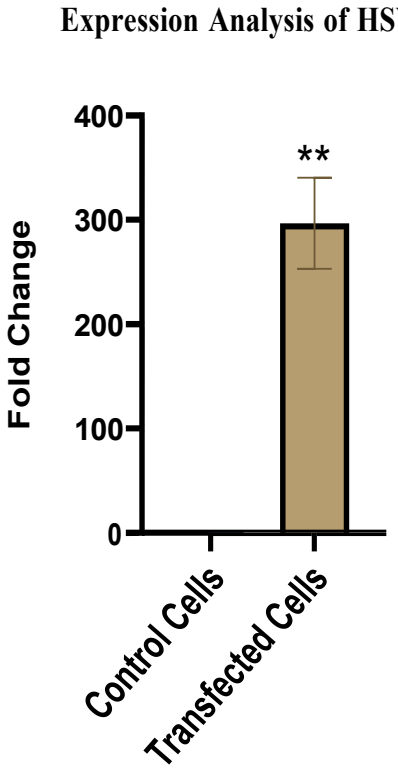

Figure 4.14b: Expression analysis of HSV gD in control and transfected HEK293 cells. *Cells' total RNA was isolated. qRT PCR measured the expression of HSV gD. Statistical significance was calculated using a one-sample t-test (a p-value < 0.05 is considered statistically significant).*

These results support the successful transfection and expression of the HSV and CD63 genes in the transfected cells.

4.9. Result of Exosome Isolation

By using an 8% PEG 4000 (Polyethylene Glycol 4000) precipitation protocol as explained in section 3.14, exosomes were successfully isolated from the transfected HEK293 cell supernatants while exosomes were isolated from control cell supernatants. This approach resulted in the isolation of concentrated exosomes from the samples. Following identical treatment of both supernatants with an 8% PEG 4000 solution and subsequent incubation, the exosomes aggregated and formed precipitates due to the high molecular weight of PEG,

while smaller particles and proteins remained in the solution. After the incubation with PEG 4000, centrifugation resulted in the formation of beige-colored exosome pellets, as shown in **Fig. 4.15a and 4.15b**. PEG 4000 was used to selectively extract exosomes from the supernatant. Despite the presence of various contaminants, the exosomes were successfully contained within the pellet and stored at -80oC. Then SEM was performed to identify the shape and size of isolated exosomes.

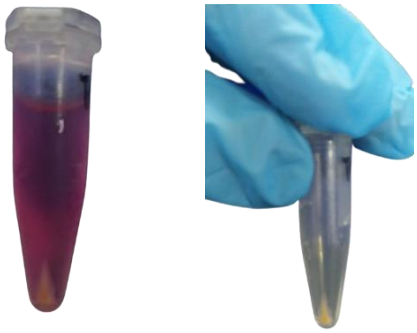

Figure 4.15a. Exosome Pellet Extracted from Transfected Cell

Figure 4.15b. Exosomes Pellet Extracted from Control Cells

4.10. Scanning Electron Microscopy (SEM)

SEM (Scanning Electron Microscopy) was used to assess the shape and size distribution of exosomes contained in a pellet. The prepared sample was put on a glass coverslip and coated with gold before being examined under a scanning electron microscope. The following results were seen in the micrographs shown in Figure **4.16 a, b, c, d, and e**, which indicated that Exosomes had a distinctive spherical form, with diameters ranging from around 30 nanometers to 150 nanometers and were observed throughout the slide. This demonstrates that the exosomes in the sample had a wide variety of sizes, most of which were within the range of sizes that are tiny vesicles. The spherical or cup-like form observed in the micrographs fits into the normal morphology of exosomes. The size difference is also consistent with the known heterogeneity of exosomes, which can be attributed to changes in

their biogenesis, payload, and cellular origin. The size distribution of exosomes was determined through histogram analysis as shown in **Fig. 4.17a and 4.17b.**

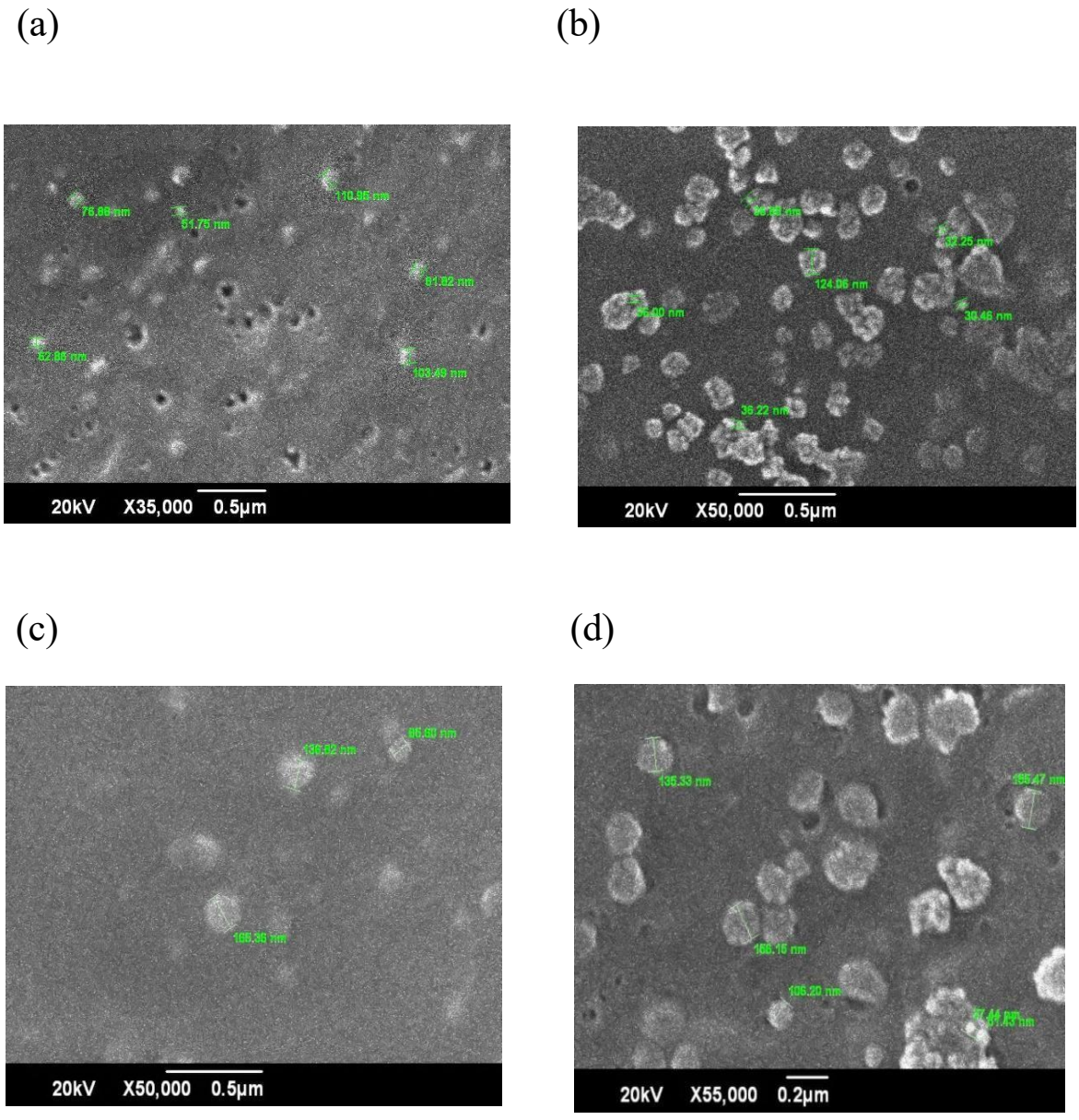

Figure 4.16. Scanning electron micrographs of exosomes from control and transfected cells at different Resolution
(a) and (b) micrographs showing exosomes extracted from the supernatant of transfected cells. Exosomes have been shown to range in size from 30 to 200 nm. (c) and (d) a micrograph of exosomes extracted from the supernatant of control cells. Exosomes have been found to range in diameter from 30 nm to 150 nm.

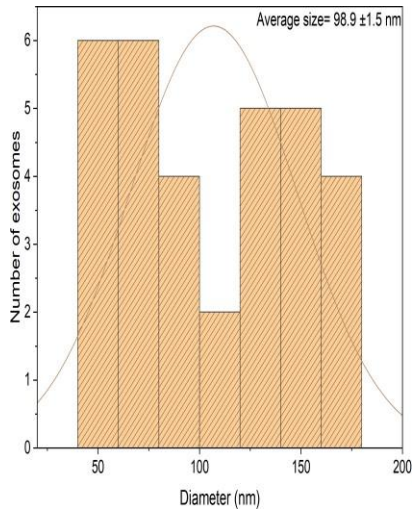

Figure 4.17a. Exosomes Size Histogram.
The histogram illustrates the size distribution of control exosomes, revealing an average size of 102.5 nm.

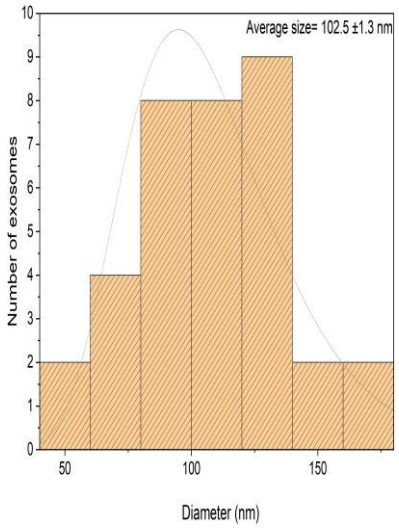

Figure 4.17b. Exosomes Size Histogram.
The histogram illustrates the size distribution of engineered exosomes, revealing an average size of 98.9nm

4.11. Exosome Protein Quantification

The concentration of exosome protein was determined using the Nanodrop spectrophotometer, which included absorbance measurements at 260 nm and a conversion factor derived from the molecular weight and extinction coefficient of the protein of interest in the sample. Using a target protein conversion factor, incorporating a 33 kDa molecular weight and a molar extinction coefficient of 21,430, revealed a target protein concentration was found to be 4.64ug/ml in **Fig.4.18a**. In Contrast, in **Fig.4.18b**, the protein concentration of control cells was negative. This difference occurred because the target protein was missing from the control cells, which led to a negative value even with the same conversion factor.

(a) (b)

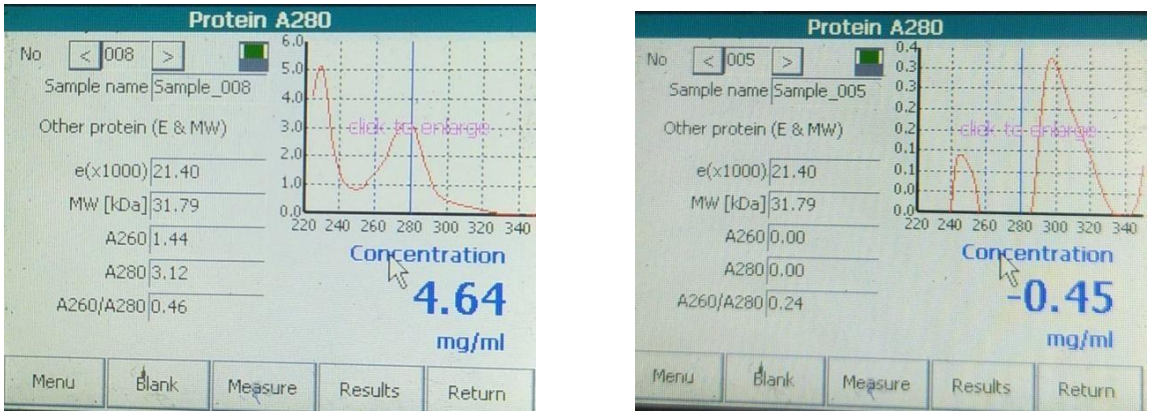

Figure 4.18. Protein Quantification. *(a) Transfected exosome protein quantification was determined to be 4.64mg/ml. (b) In contrast, the Control exosome protein concentration was negative, showing the absence of protein of interest in this structure*

4.12. Protein-based detection of HSV gD

SDS-PAGE gel electrophoresis successfully separated proteins according to their molecular weights for protein-based detection of the target protein. After electrophoresis, the gel was stained and distinct bands on the gel corresponding to proteins present in the sample were seen. The presence of the target protein in the samples was confirmed by the position of these bands, which matched the target protein's expected molecular weight.

This band's intensity was proportional to the amount of target protein in the engineered eoxosmes, facilitating semi-quantitative analysis. The gel was run alongside molecular weight protein markers (Biobasic 20-120kDa Mid-Range Protein Molecular Weight Marker, Prestained), which helped in determining the molecular weight of the observed bands. The results were in line with the His-tagged target protein's expected size of 33kDa in engineered exomes while no His-tagged target protein band was present in control exosomes, as shown in Fig.4.19, confirming the specificity of the detection approach.

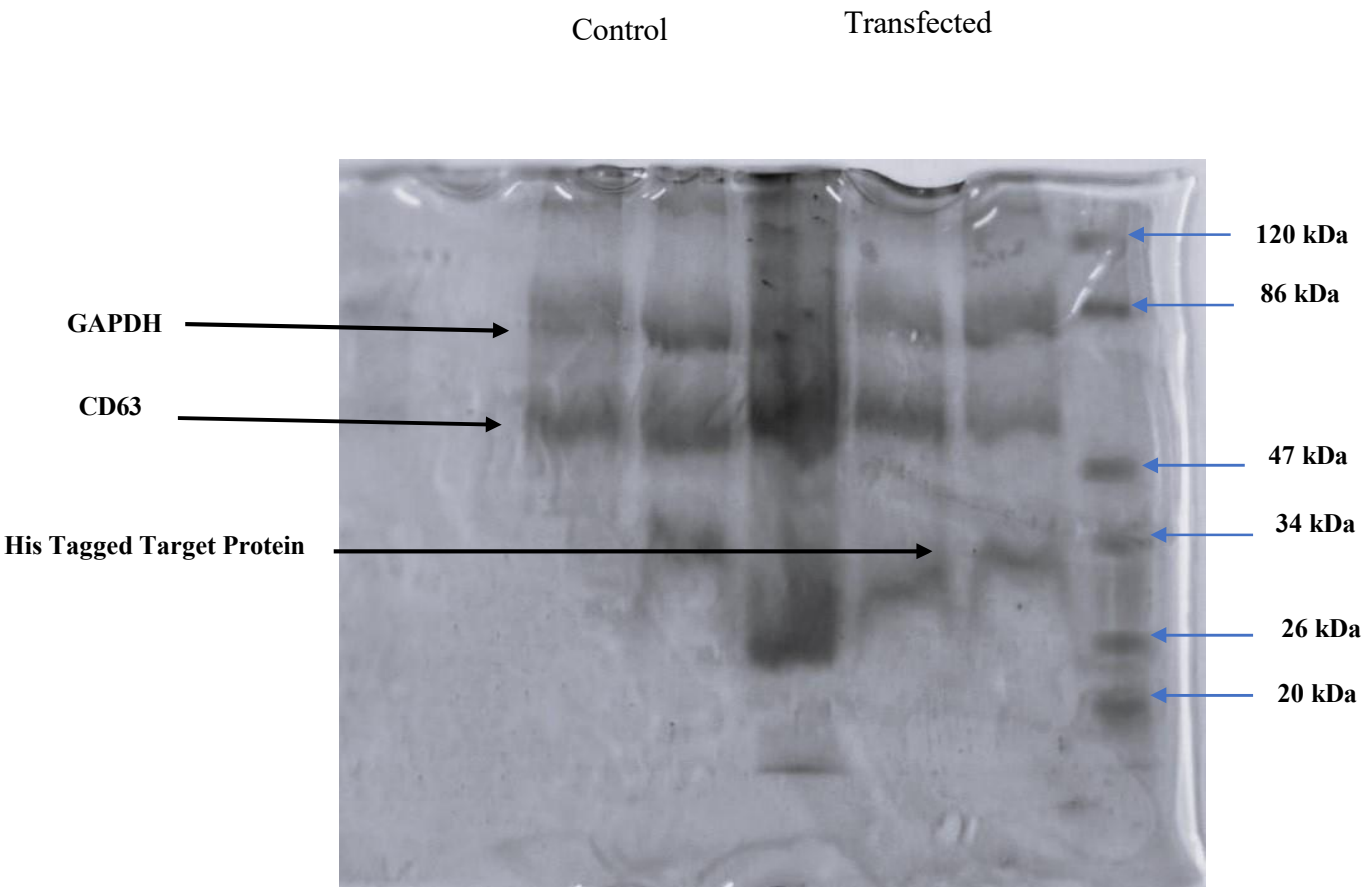

Figure 4.19. 10 % acrylamide SDS-PAGE gel of exosomes lysate. *His tagged Target Protein was found at 33 kDa molecular weight in engineered exosomes.*

Overall, the SDS-PAGE results showed that the target protein was successfully separated and detected, providing important details on its size and presence in the sample.

4.13. Western Blotting Analysis of Target Protein

Western blotting was used to look for the presence of the target protein within the exosome sample. A full experimental setup included the use of cell lysate, SDS-PAGE for gel electrophoresis, and then membrane transfer. After blocking, Rabbit anti-His-Tag pAb (AE068) was used as the primary antibody, and it was diluted at 1:1500 and incubated for the

entire night. The secondary antibody Mouse Anti-Rabbit IgG(H+L) (peroxidase/HRP conjugated) was then incubated for an hour at a dilution ratio of 1:500 after the subsequent washing stages. The detection was done using an Excellent Chemiluminescent Substrate (ECL) and a 2-minute exposure time, and imaging was performed on a ChemiDoc MP imaging system. A band of 33 kDa, which is the target protein, was identified.

This result shows a difference between the exosome sample containing the target protein and the control sample without the target protein. As shown in **Fig.4.20**, the Western blot results confirmed the presence of a 33 kDa band, showing that the targeted protein is present on the modified exosomes. In the control sample, however, no band was visible.

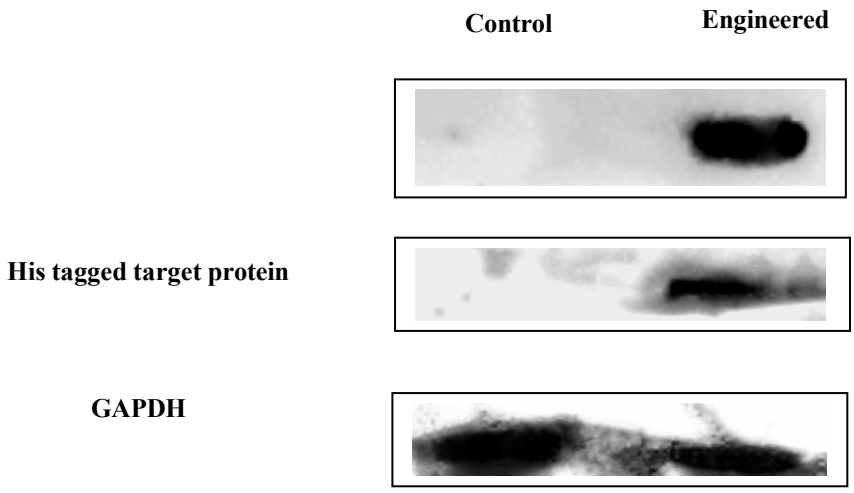

Figure.4.20. Western Blot of Exosomes
Western blot of exosomes from the culture supernatant of HEK293 cells that had been untreated (Control) and transfected with his tag-CD63-HSVgD vector (Engineered). Anti His-Tag pAb (AE068) was used as the primary antibody. The housekeeping gene GAPDH on *a Western blot against* Rabbit GAPDHS Rabbit pAb (A10471) *serves as an internal control.*

CHAPTER 5

Conclusion

In conclusion, this study advances the field of targeted drug delivery by investigating the potential of exosome-based systems, which provide a natural and effective alternative to synthetic counterparts. The engineered exosomes, which were specifically altered with HSV gD to selectively target HVEM, demonstrate their potential as novel delivery systems therapeutics into white blood cells, providing a new treatment option for many disorders. When these targeted exosomes are delivered systemically, surface-expressed gD HSV promotes their accumulation in target cells such as T cells, B cells, natural killer cells, and monocytes, where they attach to receptor HVEM. These targeted exosomes have the potential to be used as immunotherapeutic delivery vehicles for immunotherapy medicines, miRNA, mRNA, siRNA, and vaccines in the future. The effective engineering and characterization of these exosomes opens the door to more research and applications in the development of novel and targeted therapeutic approaches.

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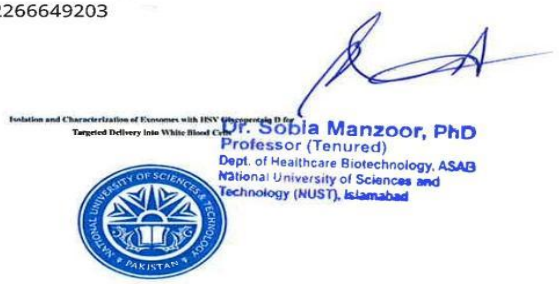

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