Characterization of extracellular vescicles - polymeric nanoparticles complexes aimed for gene delivery purposes.

Master's Degree Thesis in Bio-Nanotechnology

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Chapter 1

1.1 Abstract

Gene delivery holds immense promise in advancing therapeutic interventions by enabling precise regulation of cellular functions. This study aims to characterize the complexes formed by polymeric nanoparticles encapsulated in extracellular vesicles (EVs) and assess their potential for targeted gene delivery to dendritic cells (DCs). DCs, as professional antigen-presenting cells, play a crucial role in initiating and modulating immune responses, making them attractive targets for therapeutic interventions. The focus of this work lies in the comprehensive characterization of polymeric nanoparticle-encapsulated in extracellular vesicles complexes, including their shape, dimensions, toxicity, and encapsulation efficacy. By assessing these crucial parameters, we gain insights into the physical properties and safety profile of the complexes, which are essential for successful translation into clinical applications. While the scope of this work was primarily focused on the characterization of these complexes, the findings contribute to the foundational knowledge required for the development of future gene delivery platforms. The results obtained from this study provide a valuable baseline for further investigations, paving the way for the optimization and refinement of these delivery systems for enhanced therapeutic efficacy.

Chapter 2

Introduction

2.1 Tumors – General Overview

2.1.1 What are tumors?

Tumors, also known as neoplasms, are abnormal growths of cells that can develop in any part of the body and their behavior and treatment depend on the type and location of the tumor. In general, tumors arise due to changes or mutations in the DNA of cells, which can lead to uncontrolled growth and division. There are two main types of tumors: benign and malignant. Benign tumors are usually slow-growing and do not invade nearby tissues or spread to other parts of the body. They can often be removed with surgery and may not require further treatments. Malignant tumors, on the other hand, are fast-growing and can invade nearby tissues and organs, as well as spread to other parts of the body through the bloodstream or lymphatic system. This process is known as metastasis and is a hallmark of cancer [1]. Metastasis is the process by which cancer cells spread from the primary tumor to other parts of the body through the bloodstream or lymphatic system. This is a complex process that involves many different steps, including invasion of nearby tissues, intravasation (entry into the bloodstream or lymphatic vessels), circulation through the body, extravasation (exit from the bloodstream or lymphatic vessels), and colonization of new sites [2]. Metastasis is one of the major causes of mortality in cancer patients, as it can lead to the development of secondary tumors (metastases) in vital organs such as the lungs, liver, and brain. Metastatic tumors are often more difficult to treat than primary tumors, and the prognosis for patients with metastatic cancer is generally poorer than for those with localized disease [3]. The specific mechanisms by which cancer cells metastasize can vary depending on the type of cancer and the organs involved. For example, breast cancer cells tend to metastasize to the bones, liver, and lungs, while colon cancer cells tend to metastasize to the liver and

lungs. However, there are some common features of the metastatic process that are shared by many different types of cancer. Metastasis can be facilitated by several different factors, including genetic mutations, immune system suppression, and changes in the tumor microenvironment. For example, cancer cells may secrete factors that promote the growth of new blood vessels (angiogenesis), which can provide a pathway for cancer cells to enter the bloodstream or lymphatic system [4].

2.1.2 Types of Tumors

There are many different types of cancer, each with their own specific characteristics and treatments. Some of the most common types of cancer, based on the type of cells from which they arise, include:

- **Carcinomas**: Carcinomas are the most common type of cancer and arise from epithelial cells that line the surface of organs and tissues. They can occur in many different organs, including the lungs, breast, prostate, colon, and skin. Carcinomas can be further classified based on the type of epithelial cell they arise from, such as squamous cell carcinomas and adenocarcinomas.

- **Sarcomas**: Sarcomas arise from mesenchymal cells, which are the cells that make up connective tissues such as bone, muscle, and cartilage. They are relatively rare compared to carcinomas and can occur in many different parts of the body.

- **Lymphomas**: Lymphomas arise from lymphoid cells, which are the cells that make up the lymphatic system. They can occur in many different parts of the body, including the lymph nodes, spleen, and bone marrow. Lymphomas can be further classified based on the type of lymphoid cell they arise from, such as B-cell lymphomas and T-cell lymphomas.

- **Leukemias**: Leukemias arise from blood-forming cells in the bone marrow and can cause abnormal production of blood cells. They can affect the circulation of blood throughout the body and can be classified based on the type of blood cell affected, such as myeloid leukemias and lymphoid leukemias [5].

2.1.3 Statistical Overview

Cancer is a major public health issue, with a significant impact on individuals, families, and societies worldwide. According to the World Health Organization (WHO), cancer is the second leading cause of death globally, accounting for an

estimated 10 million deaths in 2020. This represents approximately one in six deaths worldwide. The incidence and mortality rates of cancer vary widely depending on factors such as age, gender, lifestyle factors, and geographic location. In high-income countries, the incidence of cancer is generally higher than in low- and middle-income countries. However, mortality rates are generally higher in low- and middle-income countries, due in part to a lack of access to effective cancer prevention, screening, and treatment services. In terms of specific types of cancer, lung cancer is the most diagnosed cancer worldwide, accounting for 11.7% of all new cancer cases in 2020 [6]. Breast cancer is the second most common, accounting for 11.6% of all new cases, followed by colorectal cancer (10.0%) and prostate cancer (7.3%). Cancer can affect people of all ages, but the risk increases with age. In 2020, approximately 70% of all cancer deaths occurred in people aged 60 years or older. However, some types of cancer, such as certain types of leukemia, are more common in children and young adults. It is important to note that while the incidence and mortality rates of cancer remain high, advances in cancer prevention, screening, and treatment have led to improvements in survival rates and quality of life for many cancer patients. According to the American Cancer Society, the 5-year survival rate for all cancers combined in the United States has increased from 49% in the mid-1970s to 69% in recent years.

2.1.4 Classic Therapies

Cancer treatment typically involves a combination of different therapies, depending on the type and stage of cancer. Some common cancer treatments include:

- **Surgery**: is used to remove the tumor and surrounding tissue. Surgery is often the first treatment option for many types of cancer, particularly those that are localized and have not spread to other parts of the body. Radiation therapy: uses high-energy radiation to kill cancer cells. It can be used alone or in combination with other treatments, such as surgery or chemotherapy.

- **Chemotherapy**: involves the use of drugs to kill cancer cells. These drugs can be administered orally or intravenously, and this therapy is often used in combination with other treatments.

- **Hormone therapy**, involves the use of drugs that block or reduce the levels of certain hormones in the body, which can help slow or stop the growth of hormone-sensitive cancers such as breast and prostate cancer.

- **Stem cell transplant**, involves the transplantation of healthy stem cells into the body to replace damaged or destroyed stem cells in the bone marrow. Stem cell transplant is often used to treat certain types of blood

cancers, such as leukemia and lymphoma.

It is important to note that cancer treatment can have side effects, and the specific side effects depend on the type and combination of treatments used. However, advances in cancer treatment have led to improvements in survival rates and quality of life for many cancer patients.

2.1.5 Novel Therapies

In recent years, several new promising therapies for cancer have been developing and have shown significant potential in improving patient outcomes. These include:

- **Immunotherapy**: This is a type of therapy that uses the body's own immune system to fight cancer. Immunotherapeutic drugs, such as checkpoint inhibitors, work by blocking proteins that prevent the immune system from attacking cancer cells. This approach has shown good results in treating certain types of cancer, such as melanoma and lung cancer [7].

- **Targeted therapy**: This is a type of therapy that targets specific molecules or proteins that are involved in the growth and spread of cancer cells. Targeted therapy drugs, such as tyrosine kinase inhibitors, have been developed for a range of different cancers, including lung cancer, breast cancer, and leukemia [8].

- **CAR T-cell therapy**: This is a type of therapy that involves genetically modifying a patient's own T cells to recognize and attack cancer cells. CAR T-cell therapy has shown promising results in treating certain types of blood cancers, such as leukemia and lymphoma [9].

- **Precision medicine**: This is an approach to cancer treatment that uses genetic testing to identify specific mutations or changes in a patient's DNA that are driving the growth of their cancer. This information can then be used to tailor treatment to the individual patient, with the aim of improving outcomes and reducing side effects.

While these therapies offer great promise, there are still challenges that need to be addressed to make them widely available and accessible to all patients. These include issues around cost and affordability, as well as the need for more research to better understand how these therapies work and how they can be optimized for different patient populations.

2.2 Dendritic Cells

2.2.1 Introduction to Dendritic Cells

Dendritic cells (DCs) are a subset of professional antigen-presenting cells (APCs) that play a crucial role in the initiation and regulation of the adaptive immune response. DCs are characterized by their distinctive morphology, that includes branching cytoplasmic extensions called dendrites. These structures enable DCs to efficiently capture and process antigens from their surroundings, and to present them to T cells in lymphoid organs in a manner that is critical for the induction of both CD4+ and CD8+ T cell responses [10]. DCs are also key regulators of immune tolerance, with the ability to induce T cell anergy or deletion in the absence of appropriate co-stimulation. Moreover, DCs play an important role in linking the innate and adaptive immune systems, through their ability to sense and respond to pathogens and danger signals via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). This allows DCs to rapidly activate immune responses against invading pathogens, while also promoting the resolution of inflammation and the restoration of tissue homeostasis. DCs exhibit remarkable functional diversity, with distinct subsets of DCs characterized by different patterns of surface marker expression, anatomical location, and functional properties [11]. Overall, dendritic cells are a critical component of the immune system, playing a key role in both the induction of protective immune responses and the maintenance of immune tolerance. A deeper understanding of the biology and function of DCs is essential for the development of novel immunotherapeutic strategies for the treatment of infectious diseases, cancer, and autoimmune disorders [12].

2.2.2 Role of Dendritic Cells in Tumors

DCs have emerged as important players in the anti-tumor immune response, with the ability to both stimulate and suppress tumor-specific T cell responses. In particular, DCs can promote tumor immunity by presenting tumor antigens to T cells and activating them to mount an effective anti-tumor response [13]. However, DCs can also be exploited by tumors to promote immune evasion, by inducing T cell anergy or tolerance through the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) or transforming growth factor-β (TGF-β). Additionally, tumors can directly impair the function of DCs by inducing their apoptosis or inhibiting their maturation and migration. Therefore, strategies aimed at enhancing DC function and promoting DC-

mediated anti-tumor immunity hold promise as potential immunotherapeutic approaches for cancer treatment. For this purpose, in recent studies, several DC-based vaccines have been developed that target tumor-specific antigens, and early clinical trials have shown promising results in terms of safety and efficacy. Furthermore, strategies aimed at enhancing DC function, such as the use of TLR agonists or cytokine therapies, have been shown to improve the anti-tumor response in preclinical models and may have potential for clinical translation. However, the clinical efficacy of DC-based immunotherapies has been limited by several factors, including the immunosuppressive tumor microenvironment and the limited availability of autologous DCs for ex vivo manipulation. Nonetheless, researchers are putting a lot of effort in order to optimize DC-based immunotherapies [14].

2.3 Polymeric Nanoparticles

2.3.1 History of Polymeric Nanoparticles

Polymeric nanoparticles have a rich history that spans several decades. The development of polymeric nanoparticles as drug delivery systems can be traced back to the pioneering work of researchers in the late 1970s and early 1980s. In the early years, polymeric nanoparticles were primarily prepared using emulsion polymerization or nanoprecipitation techniques. The first polymeric nanoparticles created were primarily used to deliver hydrophobic drugs, as they can efficiently encapsulate and protect these drugs from degradation. One of the first examples of this drug delivery systems is represented by poly(lactic acid) (PLA) nanoparticles that were used to deliver the anticancer drug doxorubicin in animal models. Over the years, significant progress has been made in the synthesis, characterization, and applications of polymeric nanoparticles. Various polymers, such as poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), and chitosan, have been extensively explored for the fabrication of polymeric nanoparticles. Polymeric nanoparticles have found diverse applications in drug delivery, including oral, parenteral, and topical routes of administration. They have been utilized for the delivery of small molecule drugs, peptides, proteins, and nucleic acids, offering advantages such as improved drug stability, controlled release, and targeted delivery. The ability to encapsulate both hydrophilic and hydrophobic drugs within the polymeric matrix has further expanded their utility in various therapeutic areas, for example for the treatment of cancer, cardiovascular diseases, and other conditions. With ongoing research and development, polymeric nanoparticles are expected to become an increasingly important platform for drug delivery and personalized medicine in the future [15][16].

2.3.2 General Overview

Polymeric nanoparticles have emerged as promising drug delivery systems due to their ability to encapsulate a wide range of drugs, protect them from degradation, and deliver them to the desired site of action with high specificity and efficiency. These nanoparticles are made from a variety of biodegradable and biocompatible polymers such as poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), poly(beta-amino ester) (PBAE) and chitosan. The properties of polymeric nanoparticles such as their size, surface charge, and hydrophobicity can be tailored to achieve optimal drug loading, release profile, and biodistribution. Additionally, the use of polymeric nanoparticles can potentially overcome the limitations of conventional drug delivery systems such as poor solubility, low bioavailability, and off-target effects.
These nanoparticles are characterized by several common properties that make them attractive for drug delivery applications. The following properties are the most interesting and useful for research purposes:

- High surface area to volume ratio, which allows for the efficient loading of drugs, peptides, or other therapeutic agents.

- They possess tunable surface properties, such as surface charge, that can be tailored to achieve optimal stability, biodistribution, and cellular uptake.

- Size can be controlled within a narrow range, typically between 10-200 nm, which makes them ideal for targeted drug delivery to specific tissues or cells.

- They can be designed to protect the encapsulated therapeutic agent from enzymatic degradation, thereby improving its stability and bioavailability.

- Low toxicity and are generally well-tolerated in vivo, which makes them safe for use in humans.

Overall, these properties make polymeric nanoparticles a versatile and promising platform for drug delivery applications in various therapeutic areas.

2.3.3 Advantages and Disadvantages of Polymeric Nanoparticles

Polymeric nanoparticles have been widely studied for drug delivery applications due to their many advantages, including high drug loading capacity, sustained release, and improved pharmacokinetics. They also offer the potential for targeted drug delivery and the ability to cross biological barriers, such as the blood-brain barrier (BBB). Furthermore, polymeric nanoparticles can be modified with surface functional groups to enhance cellular uptake and reduce clearance by the reticuloendothelial system (RES). However, several challenges associated with the use of polymeric nanoparticles remain, including the potential for toxicity and immune responses, difficulty in scaling up production, and the need for precise control over particle size and distribution. Additionally, the stability of polymeric nanoparticles during storage and in biological environments is an ongoing concern. To address these issues, recent research has focused on developing new formulations and fabrication techniques for polymeric nanoparticles, such as the use of natural polymers and stimuli-responsive materials.

2.3.4 Nanoparticles Loaded with Peptides

The drug delivery of peptides plays a crucial role in targeted drug delivery strategies due to the unique characteristics and therapeutic potential of peptides. Peptides are short chains of amino acids that exhibit high specificity and affinity for their target receptors or biomolecules, making them promising candidates for targeted therapies due to their high specificity, versatility, and biocompatibility. Peptide-based drugs have shown efficacy in various therapeutic areas, including cancer, cardiovascular diseases, and metabolic disorders. However, the clinical application of peptides is often limited by their rapid degradation, poor bioavailability, and short half-life in vivo. Therefore, efficient, and targeted delivery systems are required to enhance the stability, circulation time, and site-specific accumulation of therapeutic peptides. Nanoparticles loaded with peptides have gained significant attention in recent years for their potential applications in targeted drug delivery and cancer therapy. Peptides, due to their high selectivity and affinity towards specific targets, can be utilized for the targeted delivery of drugs to specific cells and tissues. Nanoparticles, on the other hand, can protect the peptides from enzymatic degradation, prolong their circulation time in the body, and enhance their cellular uptake. In recent studies, various types of nanoparticles such as liposomes, polymeric nanoparticles, and dendrimers have been loaded

with peptides for targeted drug delivery. For example, liposomal formulations of the anticancer peptide p28 have been shown to selectively target tumor cells and induce apoptosis in cancer cells. Similarly, polymeric nanoparticles loaded with the antimicrobial peptide nisin have been demonstrated to effectively kill bacterial pathogens and prevent biofilm formation. Overall, nanoparticle-based delivery of peptides has immense potential in the field of drug delivery and offers promising opportunities for the development of novel therapeutic strategies.

2.4 Extracellular Vesicles

2.4.1 History of Extracellular Vesicles

The initial studies and discovery of extracellular vesicles (EVs) mark a significant milestone in the field of intercellular communication. The investigation of EVs began with observations of particles present in cell culture media as early as the 1960s. However, it was not until the 1980s and 1990s that these particles were recognized as biologically relevant entities. Studies utilizing electron microscopy provided crucial insights into the existence of EVs, revealing their diverse morphologies and sizes. The term "exosomes" was coined to describe the EVs derived from the endosomal pathway and released upon fusion of multivesicular bodies with the plasma membrane. Subsequent studies elucidated the role of EVs in the transfer of bioactive molecules, such as proteins and RNAs, between cells. The field gained momentum with the development of isolation and characterization techniques for EVs, including ultracentrifugation, density gradient centrifugation, and more recently, various nanoparticle-based approaches. These pioneering studies paved the way for the recognition of EVs as vital mediators of intercellular communication, driving subsequent research into their biogenesis, cargo sorting mechanisms, and functional roles in health and disease.

2.4.2 Function and Characteristics of Extracellular Vesicles

EVs are small membrane-bound structures released by various cell types that play a crucial role in intercellular communication and the exchange of biomolecules they emerged as essential mediators of intercellular communication, enabling cells to exchange information and molecules over short and long distances. These vesicles are released by virtually all cell types and can be found in diverse bodily fluids such as blood, urine, and saliva. EVs exhibit a remarkable heterogeneity in terms of their composition, size, and

cargo, reflecting the cell of origin and the specific biological context. They are classified into different subtypes based on their biogenesis and size:

- **Exosomes**: they are small EVs with a size range of 30-150 nm that are derived from the endosomal pathway. They are formed through the inward budding of multivesicular bodies (MVBs) within the cell, resulting in the formation of intraluminal vesicles (ILVs) that are released upon fusion of MVBs with the plasma membrane. Exosomes are characterized by the presence of specific markers, including tetraspanins (e.g., CD9, CD63, CD81), Alix, and TSG101. They contain a complex cargo of proteins, nucleic acids (such as miRNAs, mRNAs, and non-coding RNAs), lipids, and metabolites. Exosomes are known for their stability, as they are protected by a lipid bilayer, and they can efficiently traffic between cells, facilitating intercellular communication.

- **Microvesicles**: also known as microparticles or ectosomes, are larger EVs ranging from 100 nm to 1 μm in size. They are formed by the direct outward budding or shedding of the plasma membrane. Microvesicles express surface markers such as CD40, CD54, and Annexin V. They contain a diverse cargo, including proteins, lipids, and nucleic acids. Compared to exosomes, microvesicles have a higher heterogeneity in terms of size and cargo composition. They can be released constitutively or in response to various stimuli, such as cellular activation or stress.

- **Apoptotic bodies**: Apoptotic bodies are larger EVs ranging from 1-5 μm in size that are released during programmed cell death (apoptosis). They are generated as a result of apoptotic cell fragmentation and exhibit surface exposure of phosphatidylserine. Apoptotic bodies carry cellular components, including DNA fragments, histones, and organelles, which can be taken up by phagocytic cells for clearance. These vesicles play a role in maintaining tissue homeostasis and the regulation of immune responses.

  Between this, exosomes are the most extensively studied subtype among EVs. In general, EVs possess a lipid bilayer membrane that protects their cargo from degradation, rendering them capable of transferring proteins, lipids, and nucleic acids such as microRNAs and mRNAs. This cargo can reflect the molecular state of the originating cells, offering valuable insights into disease progression, biomarker discovery, and therapeutic potential. EVs are involved in numerous physiological and pathological processes, including immune modulation, tumor progression, and tissue regeneration. Furthermore, EVs have demonstrated potential as diagnostic and prognostic biomarkers, offering insights into disease states and therapeutic responses. The functional implications of EVs have sparked great interest in their potential diagnostic, prognostic, and therapeutic applications, making them an area of intense research.

Despite their ubiquitous presence and functional significance, the precise mechanisms underlying EV biogenesis, cargo sorting, and targeting remain subjects of intense investigation [17].

2.4.3 Extracellular Vesicles in Tumors

Recent studies conducted in the past few years have provided significant advancements in understanding the intricate involvement of EVs in tumor biology. Tumor-derived EVs, including exosomes and microvesicles, have emerged as important mediators of intercellular communication within the tumor microenvironment. These EVs carry a diverse cargo, consisting of proteins, nucleic acids, lipids, and metabolites, which can be transferred to recipient cells, modulating various aspects of tumor progression. EVs have been shown to promote tumor growth and angiogenesis by delivering oncogenic factors to neighboring cells and inducing a pro-angiogenic phenotype in endothelial cells. Additionally, EVs play a crucial role in the metastatic process by facilitating the formation of pre-metastatic niches, promoting tumor cell invasion, and preparing distant organs for colonization. Moreover, tumor derived EVs contribute to immune evasion by suppressing anti-tumor immune responses and facilitating the generation of an immunosuppressive microenvironment. Furthermore, recent advancements have highlighted the potential of EVs as biomarkers for cancer diagnosis, prognosis, and therapeutic response prediction. Ongoing research aims to decipher the complex mechanisms underlying EV-mediated tumor progression and explore their therapeutic potential in cancer treatment.

2.4.4 Extracellular Vesicles in Research

EVs have emerged as valuable research tools in various scientific fields due to their unique characteristics and potential applications. EVs, encompassing exosomes, microvesicles, and apoptotic bodies, can be isolated from different biological sources, including cell culture supernatants, biofluids, and tissues. These vesicles offer a glimpse into the molecular composition and functional attributes of the parent cells, making them valuable resources for understanding cellular communication, disease mechanisms, and biomarker discovery. Researchers utilize EVs to investigate the cargo they carry, including proteins, nucleic acids, and lipids, as these molecules can provide insights into disease progression, cellular signaling pathways, and therapeutic targets. Moreover, EVs can serve as non-invasive biomarkers for diagnosing and monitoring diseases, such as cancer, neurodegenerative disorders, and infectious diseases. Furthermore, EVs hold promise as delivery vehicles for therapeutic cargoes, including drugs, nucleic acids, and nanoparticles, offering targeted and controlled release strategies. With ongoing advancements in EV isolation,

characterization techniques, and functional studies, their utility as versatile research tools continue to expand, fostering advancements in various scientific disciplines.

2.4.5 Extracellular Vesicles for Drug Delivery and Gene Therapy

EVs have gained significant attention as potential carriers for drugs and genetic material due to their inherent properties, including their ability to protect cargo, target specific cells, and traverse biological barriers. EVs can be harnessed as natural delivery vehicles for therapeutic agents, offering advantages such as enhanced stability, prolonged circulation time, and reduced toxicity compared to conventional drug delivery systems. By encapsulating drugs within EVs, these vesicles can shield the cargo from enzymatic degradation and immune recognition, facilitating efficient delivery to target cells. Moreover, EVs can be engineered to display specific targeting molecules on their surface, enabling selective uptake by recipient cells and tissues. This targeted delivery approach holds promise for personalized medicine and precision therapeutics. Additionally, EVs have been explored as carriers of genetic material, such as small interfering RNAs (siRNAs), microRNAs (miRNAs), and plasmids, for gene therapy applications. EV-mediated delivery of genetic material offers advantages such as improved stability, protection from degradation, and potential for cell-specific targeting, allowing precise modulation of gene expression in recipient cells. However, challenges remain in optimizing cargo loading, scalability, and safety aspects of EV-based drug and gene delivery systems. Continued research and technological advancements are necessary to harness the full potential of EVs as carriers for therapeutic agents and genetic material.

2.5 Extracellular Vesicles – Polymeric Nanoparticles Interaction

2.5.1 History of Extracellular Vesicles – Nanoparticles Complexes

The utilization of nanoparticles encapsulated in EVs has emerged as a promising avenue of investigation in various scientific fields. The history of research in this area can be traced back to seminal studies conducted by researchers such as Smyth et al. (2014) and Kooijmans et al. (2016). These

studies shed light on the potential of utilizing EVs as natural nanocarriers, capable of protecting and delivering therapeutic cargo to target cells. Since then, the field has witnessed a substantial growth, with researchers exploring different strategies for the encapsulation of nanoparticles within EVs, such as direct loading, membrane fusion, and exogenous membrane modification. Overall, the history of research in nanoparticles encapsulated in EVs has paved the way for a new generation of targeted drug delivery systems with significant potential for clinical applications in fields like cancer therapy, regenerative medicine, and diagnostics.

2.5.2 Interaction Between Extracellular Vesicles and Nanoparticles

The investigation of the interaction between EVs and polymeric nanoparticles has garnered significant attention in recent years due to its potential implications in various biomedical applications. Several studies have shed light on this intricate interplay, revealing the diverse mechanisms underlying the interaction between EVs and polymeric nanoparticles. For instance, Wang et al. (2018) reported that polymeric nanoparticles can adsorb onto the surface of EVs, forming a corona-like structure that can influence the stability and biological fate of both entities. Moreover, the presence of polymeric nanoparticles can modulate the surface properties of EVs, altering their biodistribution and cellular uptake. In addition, functionalization of polymeric nanoparticles with ligands or targeting moieties can facilitate the specific binding of EVs to desired recipient cells. These findings underscore the intricate nature of the interaction between EVs and polymeric nanoparticles and highlight the importance of understanding this interaction for the rational design and optimization of EV-based therapeutic platforms.

2.5.3 Encapsulation Characteristics

The encapsulation of nanoparticles inside EVs combines the unique characteristics of both EVs and nanoparticles to develop advanced therapeutic delivery systems. Several studies have demonstrated successful encapsulation of various types of nanoparticles, including metal and metal oxide-based nanoparticles, liposomes, and polymer-based nanoparticles, within EVs. This encapsulation process can be achieved through various methods, such as passive loading, active loading, or membrane fusion. Passive loading involves the co-incubation of nanoparticles and EVs, allowing the nanoparticles to naturally enter the vesicles due to their physicochemical properties. Active loading, on the other hand, utilizes techniques like electroporation or sonication to facilitate the internalization of nanoparticles into EVs. The encapsulation of nanoparticles within EVs offers several advantages. Firstly, it provides

protection to the nanoparticles from enzymatic degradation and clearance by the immune system, enhancing their stability and prolonging their circulation time. Secondly, the presence of EV membranes can improve the biocompatibility and reduce the immunogenicity of nanoparticles, making them more suitable for in vivo applications. Furthermore, the natural targeting ability of EVs allows for the specific delivery of nanoparticles to desired recipient cells or tissues. This targeted delivery can be further enhanced by modifying the surface of EVs or nanoparticles with specific ligands or antibodies. Overall, the encapsulation of nanoparticles within EVs holds great potential for applications in drug delivery, imaging, and regenerative medicine.

2.5.4 Encapsulation Techniques

There are several techniques available for encapsulating nanoparticles within EVs, each offering different advantages and considerations. The following are the most used ones:

- **Passive loading**: This method involves co-incubating EVs and nanoparticles together in a solution, allowing the nanoparticles to naturally enter the vesicles through diffusion or adsorption. Passive loading takes advantage of the physicochemical properties of both EVs and nanoparticles to promote encapsulation. It is a simple and straightforward technique but may result in lower encapsulation efficiency.

- **Active loading**: Active loading techniques are employed to enhance the encapsulation efficiency of nanoparticles within EVs. One commonly used method is electroporation, where short electric pulses are applied to create temporary pores in the EV membrane, facilitating the entry of nanoparticles into the vesicles. Another technique is sonication, where ultrasonic waves are used to disrupt the EV membrane and promote the internalization of nanoparticles. These methods can achieve higher encapsulation efficiency, but optimization is necessary to maintain the integrity and functionality of EVs.

- **Extrusion**: This technique involves the extrusion of a mixture containing EVs and nanoparticles through small-sized pores or filters. The process of extrusion generates shear forces that facilitate the incorporation of nanoparticles into EVs. This technique can achieve efficient encapsulation and control the size distribution of the resulting EV-nanoparticle complexes.

- **Freeze-thaw cycles**: Freeze-thaw cycles are commonly employed to destabilize EV membranes and promote the entry of nanoparticles. The process involves freezing the mixture of EVs and nanoparticles at sub-zero temperatures and subsequently thawing it. The freeze-thaw cycles induce

stress on the EV membranes, allowing the nanoparticles to enter the vesicles.

- **Membrane fusion**: This technique involves the fusion of EV membranes with the membranes of nanoparticles, resulting in encapsulation. It can be achieved by modifying the surface of nanoparticles with fusogenic peptides or lipids that can induce membrane fusion with EVs. Membrane fusion techniques offer controlled encapsulation, allowing precise loading of nanoparticles into EVs.

- **pH-gradient loading**: This technique exploits the pH sensitivity of certain nanoparticles and EVs. It involves incubating EVs and nanoparticles at different pH levels, causing a pH gradient between the external environment and the vesicle interior. The nanoparticles can then enter the EVs driven by the pH gradient. This method has been demonstrated to be effective for encapsulating pH-sensitive nanoparticles within EVs.

- **Microfluidics-based techniques**: Microfluidics platforms offer precise control over fluid flow and can be used to generate nanoscale droplets containing EVs and nanoparticles. These droplets can undergo various processes such as mixing, merging, or squeezing, resulting in encapsulation of nanoparticles within EVs. Microfluidics-based techniques enable high-throughput encapsulation and can provide control over the size and composition of the encapsulated particles.

- **Exogenous loading:** In this technique, EVs are isolated from donor cells and subsequently loaded with nanoparticles through incubation with a solution containing nanoparticles. This method involves the direct uptake of nanoparticles by EVs, bypassing the need for membrane fusion or disruption. Exogenous loading offers simplicity and ease of use, but the encapsulation efficiency can vary depending on the nanoparticle properties and EV uptake capacity.

- **Genetic modification**: Genetic engineering approaches can be employed to modify the donor cells that produce EVs, enabling the synthesis of EVs with intrinsic affinity for specific nanoparticles. For example, cells can be engineered to express specific surface receptors or proteins that have a high affinity for the nanoparticles of interest. The resulting EVs derived from these modified cells can then naturally encapsulate the nanoparticles.

Each encapsulation technique has its advantages, limitations, and considerations. The choice of technique depends on factors such as the nature of nanoparticles, encapsulation efficiency requirements, preservation of EV characteristics, and the intended application. Researchers often explore and evaluate various techniques to identify the most suitable one for their specific needs.

2.5.5 Properties of the Complexes

Nanoparticles-EVs complexes exhibit a range of properties that make them highly promising for diverse biomedical applications. The protective nature of EV membranes shields the encapsulated nanoparticles from enzymatic degradation and immune clearance, ensuring their preservation during systemic delivery. Furthermore, the presence of EV membranes imparts improved biocompatibility and reduced immunogenicity to the encapsulated nanoparticles, making them well-suited for in vivo applications. These complexes also possess inherent targeting abilities due to the surface molecules present on EVs, allowing for specific delivery to desired cell types or tissues. Surface modification strategies, such as incorporating targeting ligands, can further enhance the specificity and efficiency of nanoparticle-EV complex targeting. Additionally, the encapsulated nanoparticles can retain their physicochemical properties and biological functionalities, enabling their efficient and controlled delivery to target sites. These properties collectively make nanoparticle-EV complexes versatile platforms for drug delivery, imaging, and therapeutics, holding great potential for advancements in precision medicine and personalized therapies.

Chapter 3

Materials and Methods

3.1 Materials

Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin and streptomycin were obtained from Gibco®. Bovine serum albumin (BSA), sodium acetate (AcONa), Tween-80 and PBS were purchased from Sigma-Aldrich®, Cyanine 5 NHS ether dye was purchased from Lumiprobe. Recombinant human interleukin 4 (rhIL-4), phorbol-12-myristate-13-acetate (PMA) were purchased from PeproTech®. Arginine and Histidine end-modified poly(β)-amino ester) (pBAE) polymer was synthesized by the Group of Materials Engineering, following a procedure described in their paper [18]. In brief, Poly(β-aminoester)s were synthesized using a two-step procedure. In the first step, an acrylate-terminated polymer was synthesized by reacting primary amines with diacrylates at a molar ratio of 1:1.2 (amine:diacrylate). Subsequently, the acrylate-terminated polymer was modified by end-capping with various amine- and thiol-bearing moieties to obtain pBAEs (poly(β-aminoester)s) with different properties.

3.2 THP-1 cell line

3.2.1 Cell colture (FBS supplemented)

THP-1 cell lines were maintained in RMPI-1640 medium supplemented with 10% (v/v) depleted fetal bovine serum (FBS), 100

units/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. The cells were cultured at 37 °C, under a 5% CO2/95% air atmosphere and passaged after 3 days of incubation.

3.2.2 Cell Colture (without FBS)

THP-1 cell lines were maintained in RMPI-1640 medium supplemented with 100 units/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. All cells were cultured at 37 °C, under a 5% CO2/95% air atmosphere and the passages were made with the following cycle:

- 1 day of incubation with RPMI (without FBS supplement)
- 2 days of incubation with RPMI (FBS supplemented)
- 1 day of incubation with RPMI (without FBS supplement)
- 3 days of incubation with RPMI (FBS supplemented)

3.2.3 Differentiation of THP-1 cell line into immature dendritic cells

Since THP-1 monocytic cells do not express adhesion properties, in order to perform confocal analysis, we decided to achieve differentiation following the protocol explained in Deng et al. work [19]. THP-1 cells were cultured for 4 days in the conditions explained in the previous paragraph (3.2.1) and the medium was supplemented with 100 ng/mL of recombinant human granulocyte macrophage colony-stimulating factor (rhIL-4) and 100 ng/mL of phorbol-12-myristate-13-acetate (PMA) in order to achieve differentiation.

3.3 Polymeric nanoparticles

3.3.1 Synthesis of pBAE nanoparticles

The nanoparticles were prepared using the protocols developed and extensively used by the GEMAT research group [18, 20]. In summary, a polymeric solution composed of a mixture of 60% C6CR3-pBAE (polymer R) and 40% of C6CH3-pBAE (polymer H) was created and mixed using a vortex. Meanwhile another solution of OM-pBAE and pPAX (a plasmid given to the research group by a collaborating member) was created combining equal volumes of pPAX (0.5 mg/mL) and OM-pBAE (12.5 mg/mL) in a sodium acetate (AcNa) solution previously prepared (pH 5.2, 12.5 mM). This second solution was then mixed with the polymeric solution and vigorously pipetted. The resulting mixture was incubated at room temperature for 15 minutes after which an equal volume of miliQ water was added.

3.3.2 Dynamic light scattering (DLS) of pBAE nanoparticles

As part of the characterization of the nanoparticles a Zetasizer Nano ZS with the Zetasizer Softwar (Malvern Instruments, Worcestershire, UK) was used in order to analyze hydrodynamic diameter, polydispersity index and surface charge (z-potential). For the measurement of hydrodynamic diameter and polydispersity index 25 μL of freshly prepare nanoparticles (prepared as explained in the previous paragraph) were diluted in 100 μL of miliQ water and placed in a DLS micro-cuvette. For the measurement of the z-potential the nanoparticles were diluted with a ratio of 1:100 in miliQ water in order to reach a total volume of 1 mL and placed in a Disposable Capillary Cell (DTS1060, Molvern Instruments, Worcestershire, UK) and analyzed by DLS.

3.3.3 Nanoparticle tracking analysis (NTA) of pBAE nanoparticles

Nanoparticle Tracking Analysis Nanosight NS300 (Malvern Panalytics, United Kingdom) was used to determine size distribution and sample concentration. The freshly prepared nanoparticles were diluted 1:100 in miliQ water reaching a final volume of 1 mL and ran with the automated syringe pump in the NTA.

3.3.4 Labeling of pBAE nanoparticles with fluorophore

To obtain the detectable fluorescence of the polymeric nanoparticles it was decided to label the R-polymer with Cyanine 5 NHS ester (Cy5) with the protocol used in the GEMAT group (Riera et al.). The first phase of the procedure consists in mixing 60 µL (1,6 µmol) of Cy5 to 35 µL (1,6 µmol) in 270 µL of DMSO (0,98 µmol) with the addition of 4 µL (29 µmol) of triethylamine (Et3N). The solution was then stirred, with a magnet bar, in a water bath at the controlled temperature of 25 °C for 20 hours. At the end of this phase, the solution was precipitated in a 7:3 (V/V) mixture of diethyl-ether: acetone. The resulting product was dried overnight and dissolved in DMSO to obtain a solution of 100 mg/mL. The fluorescent labeled polymer was then used at 1% (V/V) of the total amount of R-polymer) while synthesizing polymeric nanoparticles as explained in paragraph 3.2.1.

3.4 Extracellular vesicles

3.4.1 Supernatant collection, harvesting and purification of extracellular vesicles

The supernatant was collected during the passage of the THP-1 cell line (after 3 days of incubation) using a mild centrifuge process of the culture medium for 5 minutes at 300xg and 20°C. The collected supernatant was placed in a 50 mL Falcon and stored at -80°C. After collecting at least

100 mL of supernatant and let it thaw, an Avanti® centrifuge J-26 XPI with JA-14.50 rotor (Beckman Coulter Life Science, USA) was used to extract the extracellular vesicles with the following process:

- The supernatant was centrifugated at 10'000xg for 45 minutes at 4°C to eliminate cell debris.
- The resulting medium (containing the EVs) was centrifugate a second time at 35'000xg at 4°C for 70 minutes, forming a pellet.
- The pellet was resuspended 1/50 of the initial volume of PBS.
- The sample containing the extracellular vesicles was then stored at -80°C.

3.4.2 Nanoparticle tracking analysis of extracellular vesicles

Regarding extracellular vesicles, NTA was not only used to determine size distribution and sample concentration but also to determine their size. The sample containing the extracellular vesicles was thawed and diluted 1:100 in PBS reaching a final volume of 1 mL and ran with the automated syringe pump in the NTA.

3.4.3 Labelling of extracellular vesicles

The fluorophore used for the labeling of the extracellular vesicles was NBD-PE which is a phospholipid labeled on the head group with the NBD fluorophore that is able to place itself into the extracellular vesicle's phospholipidic bilayer by passive incubation. The ratio used for this labeling was 5 µL of NBD-PE (1mg/mL in PBS) for 100 µL of extracellular vesicles (with a concentration of approximately 3x1010 EV/mL). The mixed solution was incubated for 1 hour at 37°C under agitation. After the period of incubation, the solution was filtrated using a 50kDa cutoff Amicon®Ultra Centrifugal Filter Unit (Merck Millipore, USA) to eliminate the remaining free label.

3.4.4 Western Blot

A western blot analysis was performed by a colleague to evaluate the EVs protein content, this are the methods generously shared for the thesis purposes:

The concentration of proteins in the analyzed samples was assessed using the PierceTM BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA; 23225) according to the manufacturer's instructions. A western blot was performed to evaluate EVs protein content. Briefly, isolated EVs were lysed in reducing sample buffer [0.25 M Tris-HCl (pH 6.8), 40% glycerol, 8% SDS, 5% 2-mercaptoethanol and 0.04% bromophenol blue] or non-reducing sample buffer (without 2-mercaptoethanol) and boiled for 10 minutes at 65°C . Proteins were resolved by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) 10% or 15% (for TGS101 and CD63 or BSA and CD81, respectively), transferred to polyvinylidene fluoride membranes, blocked in 5% non-fat powdered milk in PBS-T (0.5% Tween-20) and probed with antibodies. Antibodies applied with reduced samples were purified anti-TSG101 antibody(Cat. 934301, Biolegend) and Bovine Serum Albumin Polyclonal Antibody (BSA; Cat. A11133, Invitrogen). CD81 Antibody (1.3.3.22; sc-7637, Santa Cruz Biotechnology) and CD63 Antibody (MX-49.129.5; sc-5275, Santa Cruz Biotechnology) were applied to non-reduced samples. For detection, goat pAB to MS igG (HRP; Mouse; GR3219929, Abcam), HRP Goat Anti-rat igG (minimal x-reactivity; Cat 405405, Biolegend), goat anti-Rabbit, Rat IgG (H+L) secondary (NB7160, Novus Biologicals) and Pierce® ECL Western Blotting–substrate (Thermo Fischer Scientific, Rockford, IL, USA) were used, while the membrane was analyzed with a Amersham™ImageQuant™800 biomolecular imager (Cytiva Life Sciences, USA).

3.5 Polymeric nanoparticles extracellular vesicles complexes

3.5.1 Internalization procedure

The method chosen for this work involved fast freezing cycles that enabled the disruption of the extracellular vesicles followed by a period of recovery in which, during the last phase, the nanoparticles were added. The full procedure is explained in the following steps:

- 20 mL of acetone were added in a 50 mL Beaker.
- Dry ice was added in the Beaker to reach the temperature, if all the dry ice thawed, we proceeded by adding more.
- A proper amount of extracellular vesicles was placed in an Eppendorf and frozen inside of the acetone and then left at room temperature until they thawed. This cycle was repeated 3 times.
- After the extracellular vesicles thawed for the third time, freshly prepared nanoparticles were added inside of the Eppendorf and gently mixed.

From now on these complexes will be called as NPs@EVs and the ratio (v/v) that was used is 1:2 which gave the best results in previous studies.

3.5.2 Nanoparticle tracking analysis of NPs@EVs and stability tests

The freshly prepared NPs@EVs complexes were analyzed on the NTA using the same protocol as the extracellular vesicles as explained in paragraph 3.4.2. In this work we also wanted to see if these complexes could be stored at different temperature and conditions:

- Incubation at 37°C, the complexes were incubated at 37°C and analyzed on the NTA at different time steps, in specific after 30 minutes, 1 hour, 2 hours, 3 hours.

- Incubation at room temperature (RT), the complexes were incubated at RT and analyzed on the NTA at different time steps, in specific after 30 minutes, 1 hour, 2 hours, 3 hours, and 1 day.

- Freezing at -4°C, the complexes were frozen at -4°C and analyzed on the NTA at different time steps, in specific after 30 minutes, 1 hour, 2 hours, 3 hours, and 1 day.

- Freezing at -80°C, the complexes were frozen at -80°C and analyzed on the NTA at different time steps, in specific after 1 day, 14 days, 30 days, 60 days.

- Lyophilization with HEPES 4% sucrose, the complexes were mixed with an equal volume of HEPES 4% sucrose and then placed into a lyophilizing machine and the resulting pellet was stored in the freezer (-4°C). In order to analyze them on the NTA the pellet was resuspended in 1 mL of PBS and analyzed at different time steps, in specific 14 day, 30 days.

- Lyophilization with HEPES 8% sucrose, the complexes were mixed with an equal volume of HEPES 4% sucrose and then placed into a lyophilizing machine and the resulting pellet was stored in the freezer (-4°C). In order to analyze them on the NTA the pellet was resuspended in 1 mL of PBS and analyzed at different time steps, in specific 14 day, 30 days.

3.5.3 Confocal microscope analysis

Confocal microscope (Leica DMi8) analysis was done in order to verify the colocalization of the nanoparticle and extracellular vesicles after the internalization process to verify if the protocol was successful. As reported in the previous paragraphs, the nanoparticles were labeled with 1% Cy5 and the extracellular vesicles were stained with NBD-PE. In order to run the analysis 10 µL of sample were placed on the glass, covered with the coverslip, and sealed with nail polish. In addition to the complexes, analysis was run for nanoparticles alone, extracellular alone and a mixture of them to use as a control. The images obtained from the confocal microscope were processed using the ImageJ/Fiji software (1.53v, Wayne Rasband, National Institutes of Health, USA).

3.5.4 Cytotoxicity test (MTT assay)

In order to check the toxicity of the complexes an MTT test was run. A 96 well transparent polystyrene plate (Greiner, Bio-One) was used for this analysis. A volume of 100 µL of THP-1 cells (with freshly changed medium, paragraph 3.2.1) was seeded in 45 different wells. Subsequently, the wells were loaded using the following scheme:

- Three triplicates with cells only

- Three triplicates with nanoparticles only, the concentrations used for every triplicate was (0,1 µg plasmid/well, 1 µg plasmid/well, 2,5 µg plasmid/well).

- Three triplicates with extracellular vesicles only, the concentrations used for every triplicate was (0,1 µg plasmid/well, 1 µg plasmid/well, 2,5 µg plasmid/well)

- Three triplicates with a mix of nanoparticles and extracellular vesicles (without the encapsulation process), the concentrations used for every triplicate was (0,1 µg plasmid/well, 1 µg plasmid/well, 2,5 µg plasmid/well)

- Three triplicates with NPs@EVs, the concentrations used for every triplicate was (0,1 µg plasmid/well, 1 µg plasmid/well, 2,5 µg plasmid/well.

This process was followed a period of incubation at 37 °C (5% CO2/95% air atmosphere) for 24 hours. After the incubation, medium was added to each well in order to reach a total volume of 200 µL for each well, in addition a volume of 50 µL of MTT reactant was added to have a final concentration of the same reactant of 1 mg/mL. Every well was mixed by pipetting, the mixture was then remove using mild centrifuge process (300xg, 20°C, 5 min); the remaining pellet was then resuspended with 100 µL of DMSO, mixed by pipetting and the plate was plate in the plate reader (Infinite 200Pro, Tecan, US) and analyzed using the Tecan i-control software for MTT analysis.

3.5.5 CryoTEM analysis

In order to have high resolution images of the samples and to verify colocalization of the nanoparticles and extracellular vesicles, it was decided to proceed with a cryoTEM analysis. The experiment took place in the Universitat Autonoma de Barcelona (UAB) and the preparation of the samples, the loading and the visualization have been conducted by a technician. For this experiment, NPs@EVs complexes but also nanoparticles alone and extracellular vesicles alone as controls and the steps in order to capture the image are the following:

- A droplet of the sample was into a grid that was later plunged into a liquid cryogen (nitrogen), this flash freezing prevents the formations of ice crystals, maintaining the integrity of the specimen.

- The frozen grid was then loaded in the microscope under low dose conditions in order not to damage the sample.

- After a latency period for the machine to get ready, the technician had to move to the grid to find the best images and capture them.

- The images obtained were processed using the ImageJ/Fiji software (1.53v, Wayne Rasband, National Institutes of Health, USA).

Chapter 4

Experimental Results

4.1 Differentiation of THP-1 cell line into immature dendritic cells.

Images 1 A/B/C are images taken from THP-1 cells after 4 days of incubation while images 2 A/B/C are immature dendritic cells (differentiated from THP-1 cells). The images were taken in different positions and with different shades of color. Based on the comparative analysis of the images provided, two main differences are evident:

- Concentration: the non-differentiated cells exhibit a considerably higher concentration compared to the differentiated cells.

- Morphology, the non-differentiated cells display a round shape, which further supports their suspension state. This uniformity in shape suggests a homogeneous population of cells that have not yet undergone significant structural changes. On the other hand, some of the differentiated cells exhibit an elongated or even a star-shaped morphology, which can be attributed as a characteristic feature commonly observed in adherent cells, such as immature dendritic cells. This can tentatively indicate that cells have started to differentiate, as well as have transited from a suspension state to an adherent state. This transformation is likely associated with changes in cell adhesion properties, as well as cytoskeletal reorganization.

  In conclusion, the images could provide evidence of cellular differentiation, given by the distinct differences in concentration and morphology between the non-differentiated and differentiated cells. The higher concentration and uniform round shape of the non-differentiated cells in suspension contrast

with the lower concentration and more elongated morphology of the tentatively differentiated cells. These observations should be further supported by additional phenotypic measurements but can contribute to a deeper understanding of the differentiation process and highlight the unique characteristics associated with each cell type.

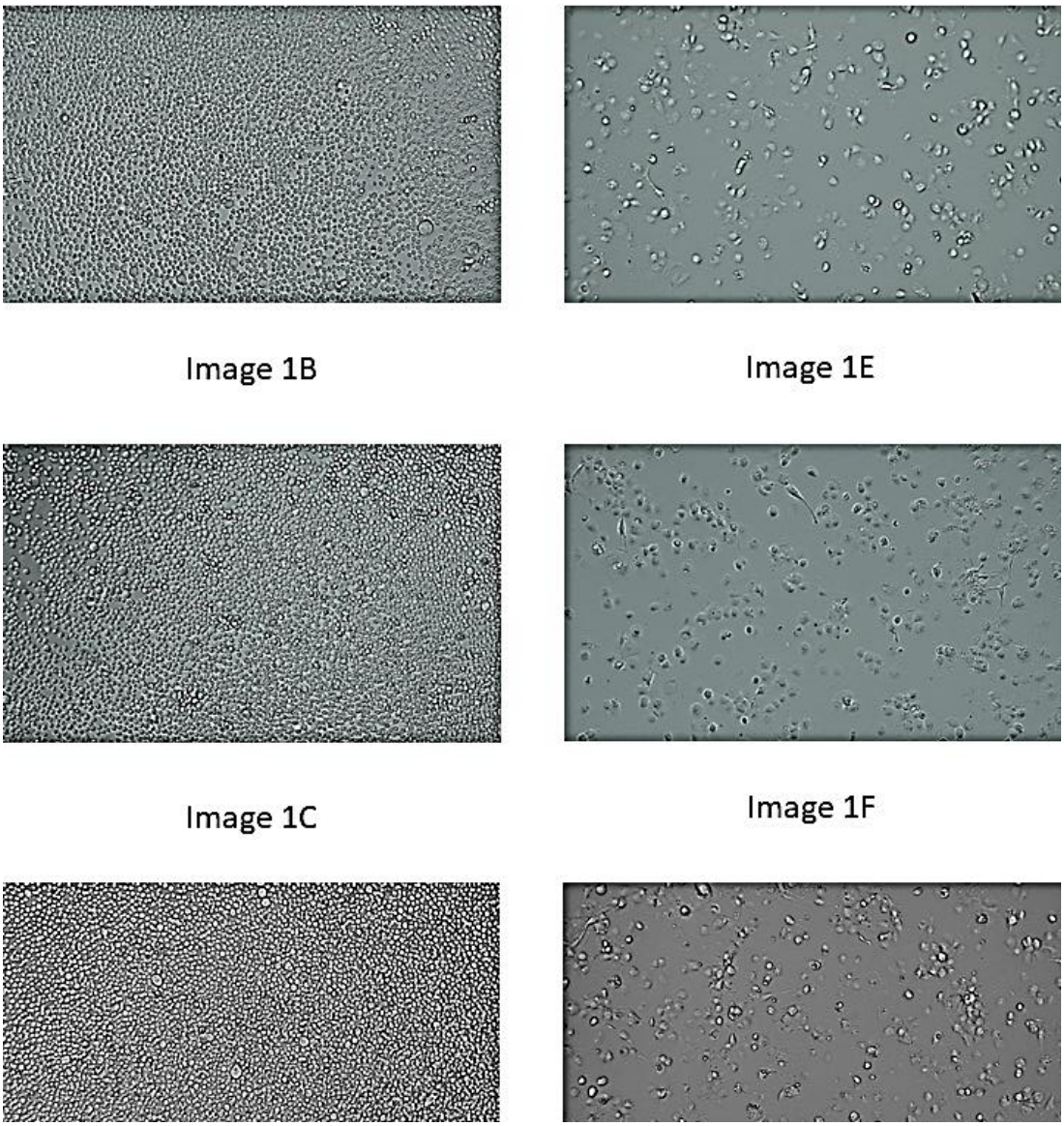

Figure 1 Comparative analysis of THP-1 cell lines (1A, 1B, 1C) and their derived imDcs (1D, 1E, 1F),

4.2 Characterization of pBAE nanoparticles

The results obtained from both Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) for the polymeric nanoparticles reveal a size range of 120-150 nm, with a size distribution varying between 105 and 130 nm. Additionally, the concentrations of the nanoparticles were found to be between 1*10^11 and 2*10^11 particles/mL. These findings provide valuable insights into

the characteristics of the nanoparticles under investigation. It is important to note that the obtained size range is an average value, and the presence of a size distribution among the nanoparticles should be considered. The use of both DLS and NTA techniques for size characterization is advantageous since DLS provides information about the hydrodynamic size of the nanoparticles in solution, while NTA allows for direct visualization and tracking of individual nanoparticles. The concentration range suggests a relatively high density of nanoparticles within the solution. Such concentrations can have significant implications for various applications, such as drug delivery, catalysis, and nanomedicine, where the interactions between nanoparticles and their surrounding environment play a crucial role. In addition, the polydispersity index (PDI) of the nanoparticle size distribution is in a range between 0,13 and 0,21 and provides insights into the uniformity or heterogeneity of the nanoparticle population. A PDI value closer to 0,1 indicates a narrow size distribution, suggesting a more homogeneous system, while a higher PDI value indicates a broader range of particle sizes.

4.3 Characterization of extracellular vesicles

The results obtained from the extracellular vesicles extracted from THP-1 cell lines using nanoparticle tracking analysis (NTA) revealed distinct characteristics based on the medium used. When using a medium supplemented with fetal bovine serum (FBS), the vesicles exhibited a nanoparticle size range between 125 and 145 nm. The concentrations of these vesicles ranged from 7 * 10^10 to 2 * 10^11 particles/mL, with a size distribution centered between 115 and 142 nm. In contrast, when the cells were cultured in a medium without FBS, the extracellular vesicles displayed a larger size range, with diameters ranging from 135 to 165 nm. The concentrations of these vesicles were lower, ranging from 3 x 10^10 to 7 x 10^10 particles/mL. The size distribution in this case spanned from 125 to 150 nm. These findings highlight the influence of the medium composition on the characteristics of extracellular vesicles released by THP-1 cells. Fetal bovine serum is commonly used as a supplement in cell culture media to provide essential nutrients and growth factors. Its presence appears to result in smaller-sized vesicles with higher concentrations, potentially indicating a more active release or altered vesicle biogenesis in the presence of FBS. On the other side, the medium without FBS led to the generation of larger vesicles with lower concentrations. This suggests that the absence of FBS might affect the release mechanisms or alter the cellular signaling pathways involved in vesicle production. In conclusion, the results demonstrate that the choice of culture medium, specifically the presence or absence of FBS, has a significant impact on the size, concentration, and size distribution of extracellular vesicles derived from THP-1 cells. These findings offer valuable insights into the dynamics of extracellular vesicle release and lay the

groundwork for further investigations, potentially contributing to the advancement of knowledge in the field of extracellular vesicle research.

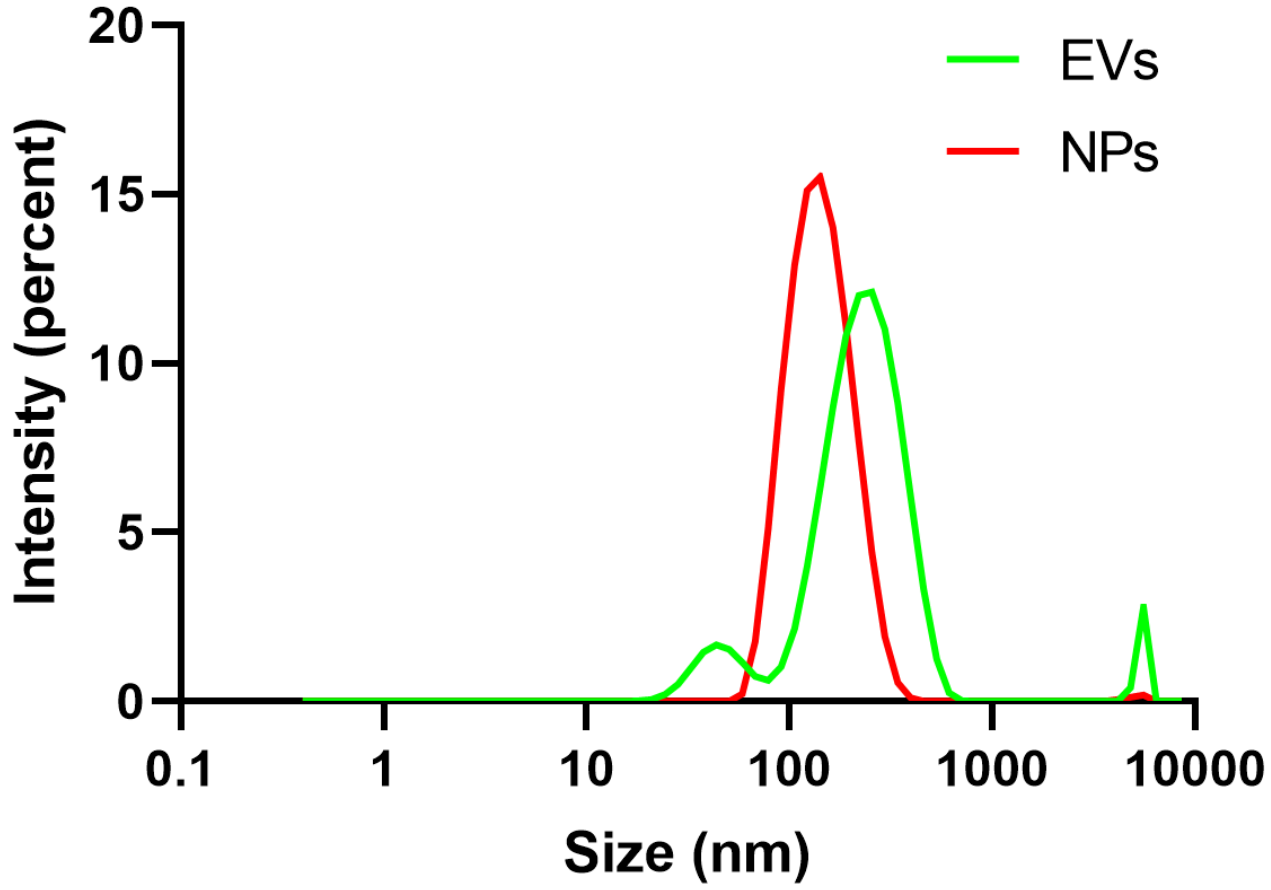

Figure 2A Comparative size and Intensity Analysis of extracellular vesicles (green) and nanoparticles (red) using DLS technique

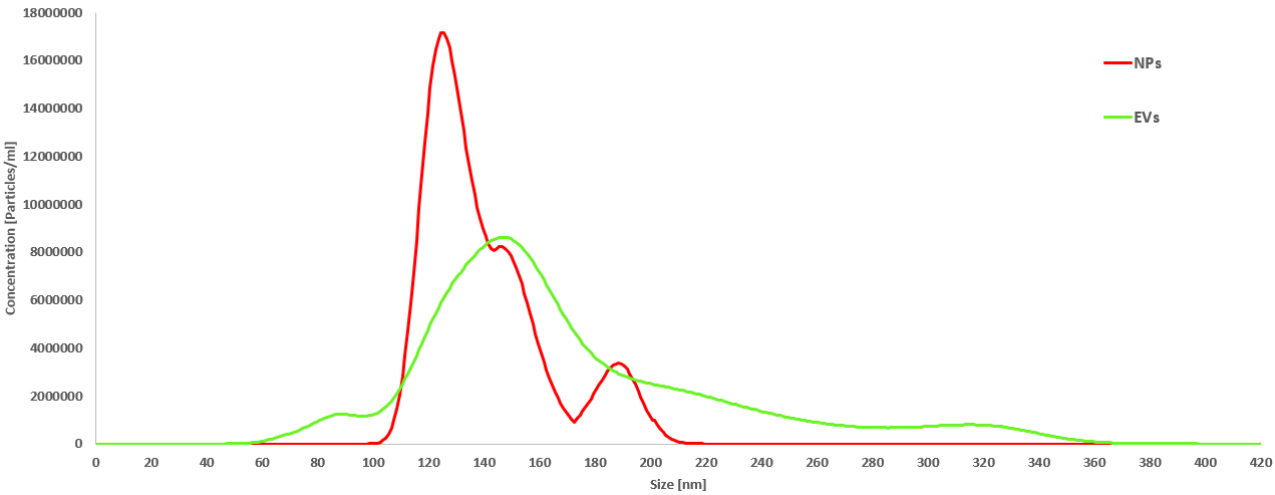

Figure 2B Comparative Size and Concentration Analysis of Extracellular Vesicles (green) and Nanoparticles (red) using NTA. Nanoparticles were freshly prepared and EVs were thawed from a stock.

4.4 Characterization of NPs@EVs (NTA analysis)

The results obtained from the NTA of NPs@EVs complexes are quite interesting. The size of the complexes falls within a relatively narrow range, ranging from 135 to 170 nm. The concentrations of the complexes were found to be between 6 x 10^10 and 2 x 10^11 particles/mL. These concentrations indicate a relatively high yield of complexes, which is favorable for potential applications. The size distribution of the complexes was observed to be between 125 and 155 nm. This narrow size distribution further confirms the successful encapsulation of polymeric nanoparticles within the EVs, as it indicates a uniformity in the size of the complexes. The size range and uniformity of the complexes could enhance their stability and ensure consistent therapeutic efficacy. In conclusion, the results of the NTA analysis on NPs@EVs reveal a consistent size range, a relatively high concentration, and a narrow size distribution. These findings support the potential of these complexes for targeted drug delivery applications.

4.5 Stability tests

4.5.1 Stability test – 37°C

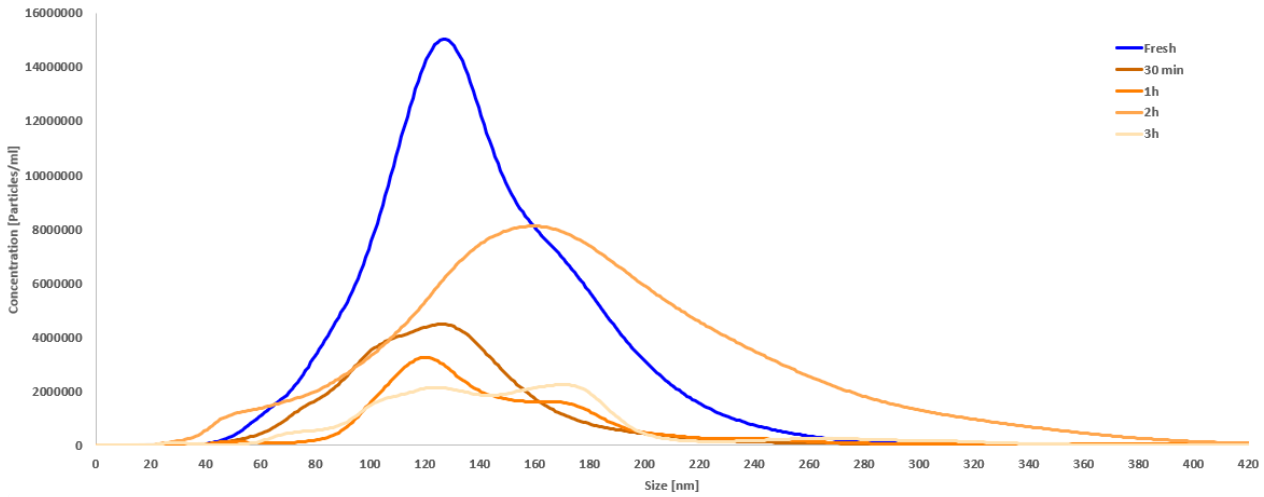

Figure 3A Comparative image, created with NTA analysis data, highlighting the difference between freshly prepared complexes and, the same complexes incubated at 37°C in different time steps that are depicted in a scale of orange, from the darkest (30 minutes) to the clearest (3 hours)

The stability of NPs@EVs was investigated through a series of stability trials. The complexes were incubated at 37°C and tested at four different time points: 30 minutes, 1 hour, 2 hours, and 3 hours. The initial complexes were prepared with a concentration of 1.2 × 10^11, a mean size of 140 nm, and a size distribution of 127 nm. Analysis of the stability data revealed notable changes in the concentration, mean size, and size distribution of the complexes over time. After 30 minutes of incubation, the complexes exhibited a relatively stable behavior, with a slight decrease in concentration to 3.4 × 10^10. The mean size remained consistent at 140 nm, indicating minimal aggregation or growth of the complexes. However, the size distribution increased to 138 nm, suggesting a broader range of sizes within the complex population. This finding implies a potential alteration in the structural integrity of the complexes, which could impact their functionality and application. Subsequently, at the 1-hour, 2-hour, and 3-hour time points, a substantial decrease in complex concentration was observed. This decline in concentration may indicate potential degradation or destabilization of the complexes over time. Additionally, the size distribution exhibited a loss of uniformity, suggesting a heterogeneous population of complexes with varying sizes. This variation in size

distribution may be indicative of complex aggregation or fragmentation, which can have implications for their intended biomedical applications. These results suggest that while the complexes initially exhibit a relatively stable behavior after 30 minutes of incubation, their long-term stability is compromised.

4.5.2 Stability test – Room Temperature

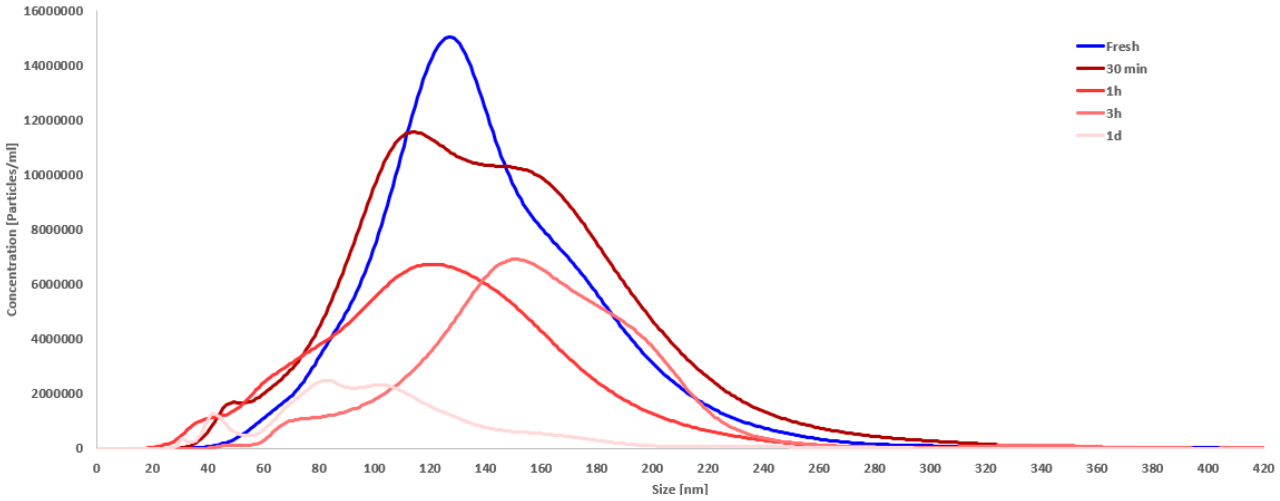

Figure 3B Comparative image, created with NTA analysis data, highlighting the difference between freshly prepared complexes and, the same complexes incubated room temperature in different time steps that are depicted in a scale of red, from the darkest (30 minutes) to the clearest (1 day)

The complexes were incubated at room temperature and tested at four different time points: 30 minutes, 1 hour, 3 hours, and 1 day. The initial complexes were prepared with a concentration of 1.2 × 10^11, a mean size of 140 nm, and a size distribution of 127 nm. Analysis of the stability data revealed important observations regarding the concentration, mean size, and size distribution of the complexes over time. After 30 minutes of incubation, the complexes exhibited a stable behavior, with a minor decrease in concentration to 1.18 × 10^11. The mean size showed a slight increase to 144 nm, while the size distribution decreased to 115 nm. These findings suggest that the complexes maintain their structural integrity and remain relatively stable during this initial incubation period. Subsequently, at the 1-hour and 3-hour time points, a noticeable decrease in complex concentration was observed, reaching approximately 7 × 10^10. Alongside the decrease in concentration, the mean size of the complexes decreased to 125 nm. However, the size distribution remained relatively consistent around 120 nm. These observations indicate a potential loss of complex stability over time at room temperature. The decline in concentration and

decrease in mean size suggest complex degradation or destabilization, which may affect their functional properties and potential applications. Furthermore, when the complexes were incubated for 1 day at room temperature, a significant decrease in concentration was observed. The size distribution also exhibited a wide range of sizes, suggesting a heterogeneous population of complexes. These findings indicate a substantial loss of complex stability and suggest that these complexes are not suitable for use after 1 day at room temperature. In conclusion, based on the results obtained from this preliminary study, it is evident that the stability of polymeric nanoparticle-encapsulated complexes in extracellular vesicles is influenced by the duration of incubation at room temperature. While these complexes demonstrate initial stability over a short incubation period of 30 minutes, longer incubation times of 1 hour and 3 hours result in a decline in concentration and changes in size characteristics. The observed instability after 1 day further emphasizes the importance of considering storage and handling conditions to ensure the optimal performance and utility of these complexes.

4.5.3 Stability test – -4°C

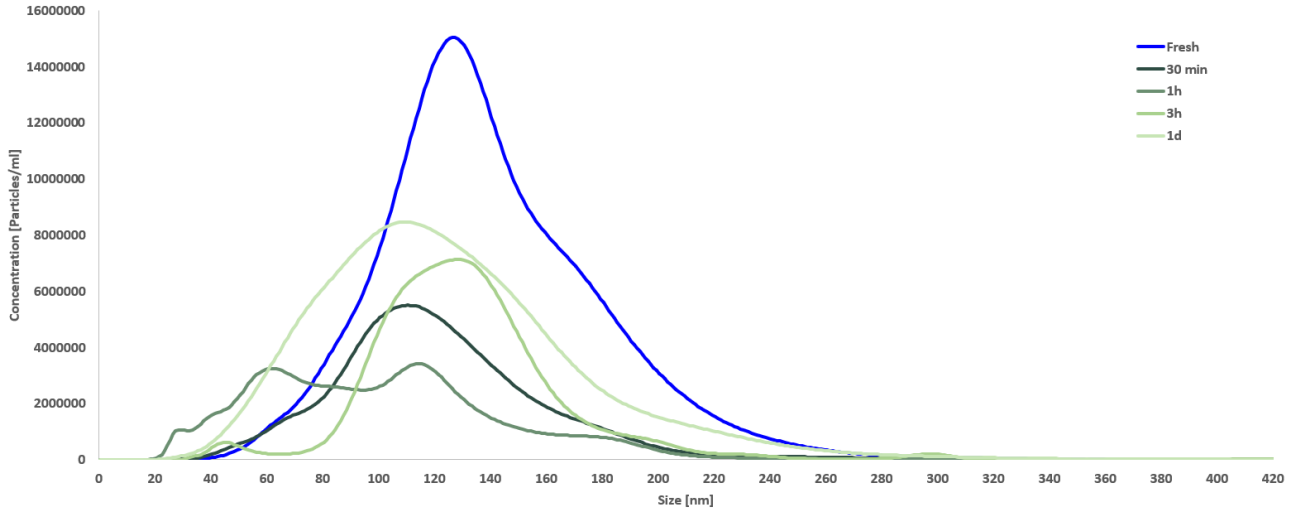

Figure 3C Comparative image, created with NTA analysis data, highlighting the difference between freshly prepared complexes and, the same complexes frozen at -4°C in different time steps that are depicted in a scale of green, from the darkest (30 minutes) to the clearest (1 day)

Stability trials were conducted on complexes consisting of polymeric nanoparticles encapsulated within extracellular vesicles (EVs) to evaluate their stability when stored at -4°C. The complexes were frozen at -4°C and tested at four different time points: 30 minutes, 1 hour, 3 hours, and 1 day. The initial complexes were prepared with a concentration of 1.2 × 10^11, a

mean size of 140 nm, and a size distribution of 127 nm. The analysis of the stability data revealed significant observations regarding the concentration, mean size, and size distribution of the complexes during the storage period. Surprisingly, even after just 30 minutes of storage at -4°C, a substantial decrease in concentration was observed, with the concentration dropping to 4.2 × 10^10. Furthermore, the mean size of the complexes decreased to 122 nm, and the size distribution decreased to 110 nm. These results indicate that storage at -4°C is not an effective method for preserving the stability of these complexes, even for a short period of time. In conclusion, the results obtained from this study demonstrate that storage at -4°C is not a viable method for preserving the stability of complexes made of polymeric nanoparticles encapsulated within extracellular vesicles. The considerable decrease in concentration, along with the changes in mean size and size distribution, indicate potential degradation of the complexes during this storage condition.

4.5.4 Stability test – -80°C

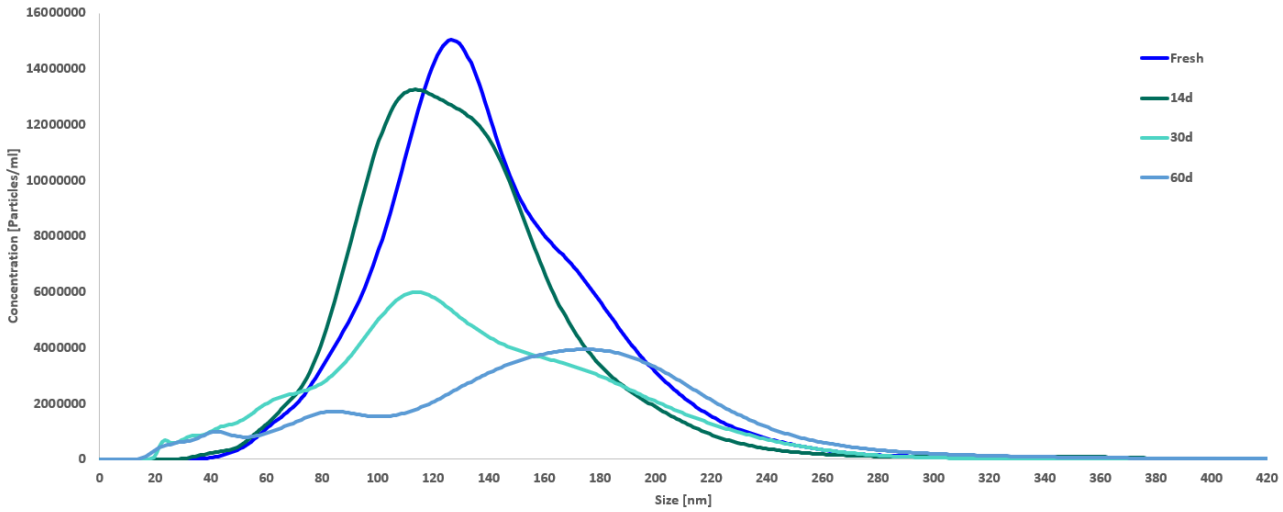

Figure 3D Comparative image, created with NTA analysis data, highlighting the difference between freshly prepared complexes and, the same complexes frozen at -80°C in different time steps that are depicted in a scale of aquamarine green, from the darkest (14 days) to the clearest (60 days)

Stability trials were conducted on complexes to assess their stability when stored at -80°C. The complexes were frozen at -80°C and tested at three different time points: 14 days, 30 days, and 60 days. The initial complexes were prepared with a concentration of 1.2 × 10^11, a mean size of 140 nm, and a size distribution of 127 nm. The analysis of the stability data revealed noteworthy observations regarding the concentration, mean size, and size distribution of the complexes during the freezing period. After 14 days of

storage at -80°C, the complexes remained stable, as evidenced by the concentration being maintained at 1.11 × 10^11. Additionally, the mean size of the complexes remained nearly unchanged at 132 nm, and the size distribution exhibited minimal variation, with a value of 120 nm. These results indicate that freezing at -80°C is an effective method for preserving the stability of these complexes over a period of 14 days. When extending the storage duration to 30 days, a decrease in concentration was observed, with the concentration dropping to 6.5 × 10^10. However, the mean size of the complexes remained stable at 135 nm, and the size distribution exhibited a slight decrease to 114 nm. These findings suggest that freezing at -80°C can maintain the stability of the complexes for up to 30 days, although there is a slight reduction in concentration. Upon reaching the 60-day storage mark, the concentration of the complexes remained consistent with the 30-day measurement. However, the size distribution displayed significant variation, and the mean size increased. These results indicate a loss of stability in the complexes after 60 days of storage, as evidenced by the altered size characteristics. It can be inferred that extended storage at -80°C beyond 30 days may lead to degradation or changes in the complex structure, rendering them unsuitable for further use. In conclusion, based on the results obtained from this study, freezing the complexes at -80°C offers a viable preservation method for NPs@EVs up to 30 days. Beyond this period, the stability of the complexes becomes compromised. These findings contribute to our understanding of the storage requirements for and highlight the importance of selecting appropriate storage durations for their intended applications.

4.5.5 Stability test – Lyophilization (HEPES 4% Sucrose)

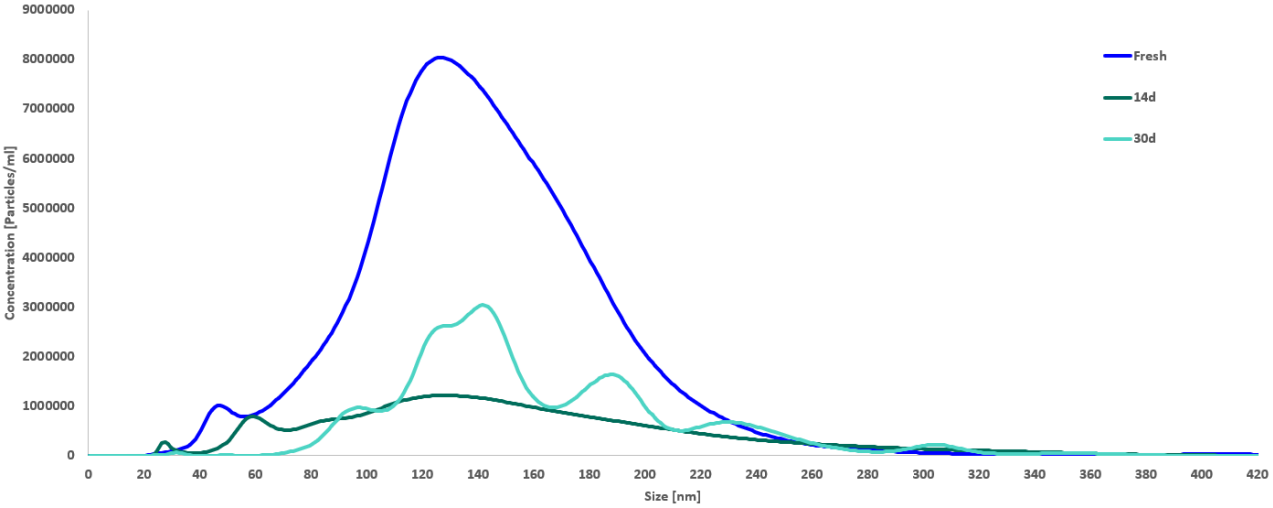

Figure 3E Comparative image, created with NTA analysis data, highlighting the difference between freshly prepared complexes and, the same complexes were freeze-dried with a process of lyophilization after mixing them with the same volume of HEPES 4% sucrose and the resuspended in PBS. The different time steps are depicted in a scale of aquamarine green, from the darkest (14 days) to the clearest (30 days)

Stability trials were conducted on complexes after lyophilization with an equal volume of HEPES solution containing 4% sucrose. The complexes were then tested at two different time points: 14 days and 30 days. The initial complexes were prepared with a concentration of 7.8 × 10^10, a mean size of 141 nm, and a size distribution of 128 nm. The analysis of the stability data revealed significant findings regarding the concentration and size distribution of the complexes following lyophilization and resuspension in 100 µL of PBS. After 14 days of storage, a substantial decrease in concentration was observed, indicating a loss of the complexes. This reduction in concentration was consistent with the results obtained after 30 days. Furthermore, the size distribution of the complexes became non-homogeneous, indicating potential alterations in the structural integrity of the complexes during the lyophilization process. These results indicate that the chosen storage option, involving lyophilization of the complexes mixed with HEPES solution containing 4% sucrose, is not suitable for preserving the stability of the complexes over an extended period. The significant decrease in concentration and the non-homogeneous size distribution suggests a loss of integrity and functionality of the complexes. Therefore, it is recommended not to consider this storage method for these complexes.

4.5.6 Stability test – Lyophilization (HEPES 8% Sucrose)

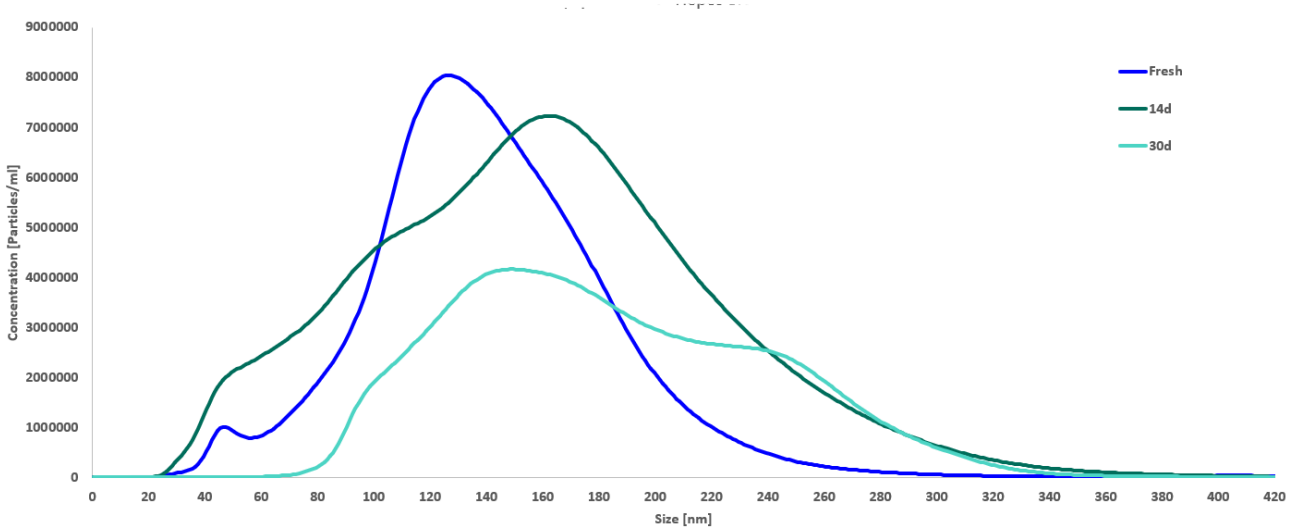

Figure 3F Comparative image, created with NTA analysis data, highlighting the difference between freshly prepared complexes and, the same complexes were freeze-dried with a process of lyophilization after mixing them with the same volume of HEPES 8% sucrose and the resuspended in PBS. The different time steps are depicted in a scale of aquamarine green, from the darkest (14 days) to the clearest (30 days)

Stability trials were conducted on complexes after lyophilization with an equal volume of HEPES solution containing 8% sucrose. The complexes were then tested at two different time points: 14 days and 30 days. The initial complexes were prepared with a concentration of 7.8 × 10^10, a mean size of 141 nm, and a size distribution of 128 nm. The analysis of the stability data revealed important findings concerning the concentration and size characteristics of the complexes following lyophilization and resuspension in 100 µL of PBS. After 14 days of storage, the concentration of the complexes remained relatively stable at 7.7 × 10^10, indicating that there was no significant loss of complexes during this period. However, the mean size of the complexes increased to 160 nm, and the size distribution expanded to 162 nm, suggesting changes in the structural properties of the complexes. Continuing the storage for 30 days led to a slight decrease in the concentration of the complexes, reaching 5.8 × 10^10. This decrease suggests a gradual loss of complexes over time. Furthermore, the mean size of the complexes further increased to 221.5 nm, and the size distribution expanded to 185 nm. These observations indicate that prolonged storage may lead to an increase in the size and heterogeneity of the complexes, which could impact their functionality and efficacy. Overall, while the concentration of the complexes remained relatively stable during the storage period, the observed increase in mean size and size distribution raises concerns regarding their long-term stability. The increase in size

could be attributed to aggregation or structural changes within the complexes, which may affect their performance and suitability for specific applications.

4.5.7 Stability test - Tables

Thee tables 1 and 2 were made to better visualize the informations about the concentrations and size distribution at different time steps and storage conditions.

Table 1. (The table shows concentration and size distribution at different time steps for different storage conditions (37°C, room temperature and -4°C.)

/	37°C	R.T.	-4°C
Starting Concentration [particles/mL]	1,2*10^11	1,2*10^11	1,2*10^11
Starting Size Distribution [nm]	127	127	127
Concentration 30 minutes [particles/mL]	3,4*10^10	1,18*10^11	4,2*10^10
Size Distribution 30 min [nm]	138	115	111
Concentration 1 hour [particles/mL]	2,3*10^10	6,9*10^10	3,4*10^10
Size Distribution 1 hour [nm]	120	120	114
Concentration 2 hours [particles/mL]	1,1*10^10	/	/
Size Distribution 2 hours [nm]	159	/	/
Concentration 3 hours [particles/mL]	1,2*10^10	6,2*10^10	4,5*10^10
Size Distribution 3 hours [nm]	170	151	128,5
Concentration 1 day [particles/mL]	/	1,6*10^10	8,7*10^10
Size Distribution 1 day [nm]	/	83	110

Table 2. (The table shows concentration and size distribution at different time steps for different storage conditions (-80°C, Lyophilization with HEPES (4% Sucrose) and Lyophilization with HEPES (8% Sucrose)))

/	-80°C	Lyo 4%	Lyo 8%
Starting Concentration [particles/mL]	1,2*10^11	7,8*10^10	7,8*10^10
Starting Size Distribution [nm]	127	128	128
Concentration 14 days [particles/mL]	1,1*10^11	1,72*10^10	1*10^11
Size Distribution 14 days [nm]	114	128	162
Concentration 30 days [particles/mL]	6,5*10^10	2,3*10^10	5,8*10^10
Size Distribution 30 days [nm]	114	142	186
Concentration 60 days [particles/mL]	5,3*10^10	/	/
Size Distribution 60 days [nm]	170	/	/

4.6 Confocal microscope analysis

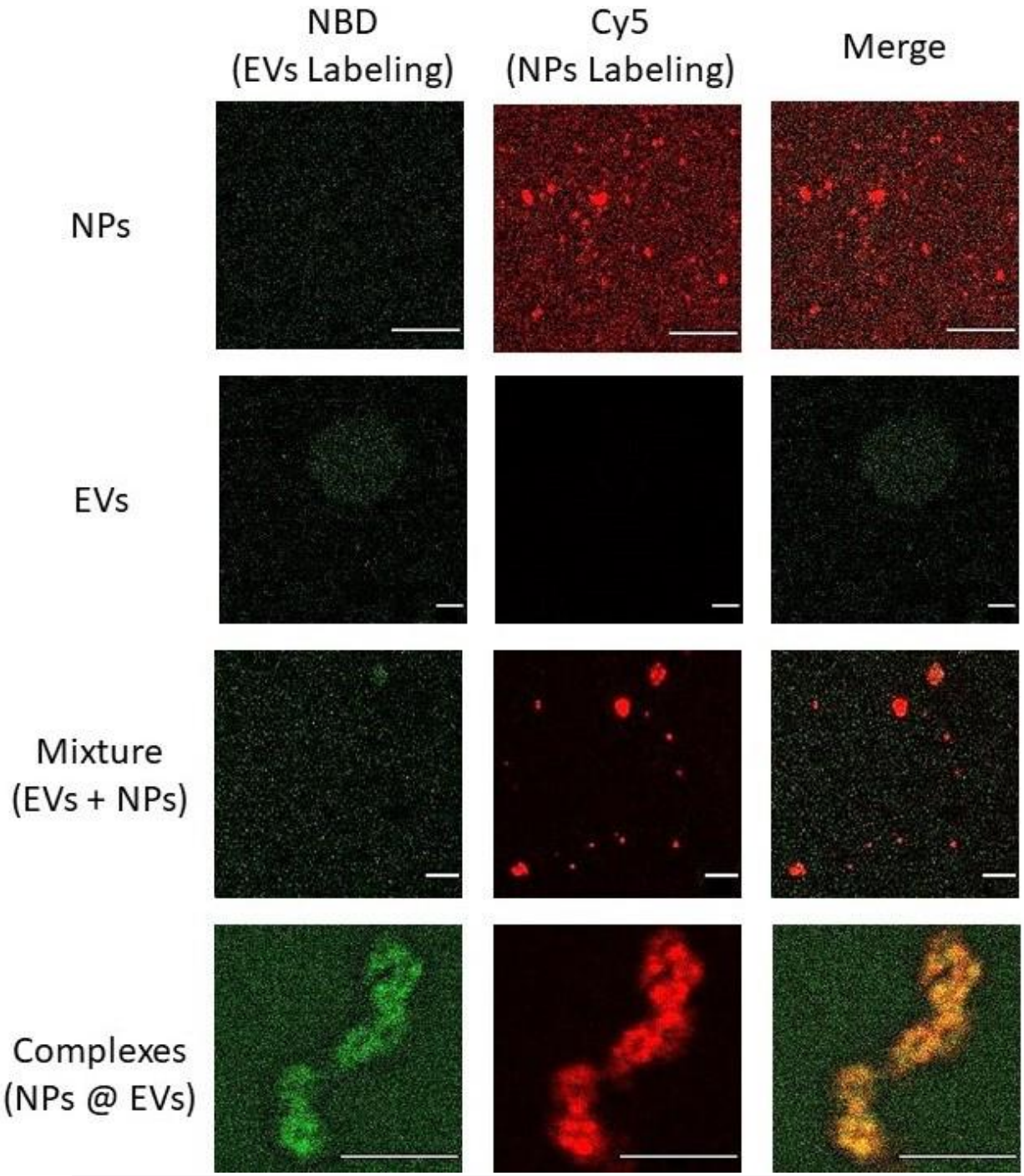

Figure 4 Confocal microscopy analysis of labeled extracellular vesicles (NBD-pe, green), nanoparticles (Cy5, red) and their complex formations in different control and experimental groups. The figure shows three rows representing control samples (nanoparticles, extracellular vesicles, and their mixture without encapsulation process) and a fourth row displaying the complexes. The first column represents NBD (green) channel, the second column shows Cy5 (red) channel, and the third column displays the merged channels to visualize the co-localization of the two labels.

A confocal microscope analysis was performed on complexes NPs@EVs. Control samples of extracellular vesicles alone (labeled with NBD-PE - green) and

nanoparticles alone (labeled with Cy5 - red) were also examined, along with a mixture of nanoparticles and extracellular vesicles (without the encapsulation process). It is important to note that the scale bar in the image correspond to 7 μm. Looking at the image the following observations can be made:

- Extracellular vesicles alone (NBD-PE - green channel): The green fluorescence signals indicate the presence of extracellular vesicles. These vesicles are visible as discrete entities, confirming the successful labeling with NBD-PE.

- Nanoparticles alone (Cy5 - red channel): The red fluorescence signals correspond to the presence of nanoparticles. The distinct red spots indicate the localization of nanoparticles within the sample, validating the successful labeling with Cy5.

- Mixture of nanoparticles and extracellular vesicles: The merged image shows that nanoparticles and extracellular vesicles are in close proximity to each other, but they are not directly overlapping. This observation suggests that there is interaction between the nanoparticles and extracellular vesicles, but it does not indicate an actual internalization of the nanoparticles into the vesicles. This finding provides evidence of potential surface association between the two entities.

- NPs@EVs complexes: The merged image of the complexes reveals an overlap between the green (NBD-PE-labeled EVs) and red (Cy5-labeled nanoparticles) fluorescence signals. This clear overlap confirms the successful aggregation of the nanoparticles with the extracellular vesicles.

These results provide insights into the interactions and encapsulation efficiency of polymeric nanoparticles within extracellular vesicles. The clear difference between the overlap observed in the complexes and the lack of complete overlap in the mixture of nanoparticles and extracellular vesicles prove that the encapsulation process was partially successful, with strong aggregation of the complexes which made them not idoneous for further cell culture. More test should be carried out to improve this conjugation system.

4.7 Cytotoxicity Test (MTT analysis)

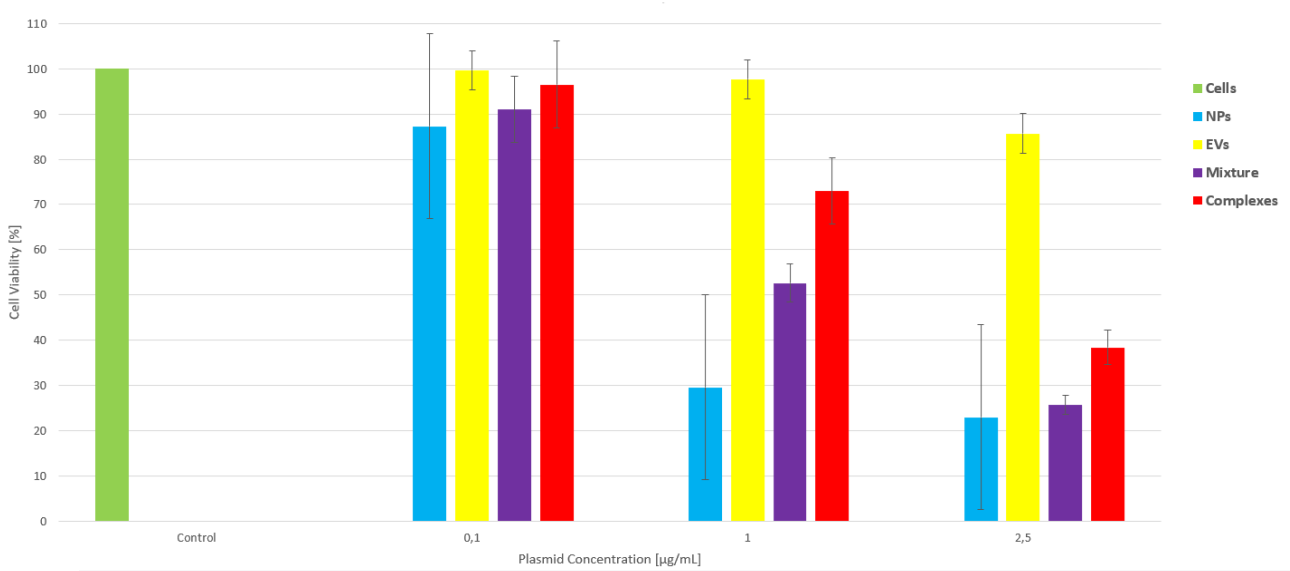

Figure 5 MTT cytotoxicity assay demonstrating the impact of different plasmid concentrations (0, 1 µg/mL, and 2.5 µg/mL) on cell viability. Each concentration is represented by bars indicating the vitality of cells in the presence of various samples: nanoparticles (blue), extracellular vesicles (yellow), mixture (purple), and complexes (red). The bar heights illustrate the relative cell viability for each corresponding sample.

A cytotoxicity analysis using the MTT assay was performed on complexes NPs@EVs. Control samples of extracellular vesicles alone, nanoparticles alone, and a mixture of nanoparticles and extracellular vesicles (without the encapsulation process) were also included. The cell viability results at three different plasmid concentrations (0.1 µg/mL, 1 µg/mL, and 2.5 µg/mL) are as follows, as can be seen from the image:

- Nanoparticles: at a concentration of 0.1 µg/mL, the cell viability decreased slightly to 87%. at concentrations of 1 µg/mL and 2.5 µg/mL, the cell viability dropped significantly to 30% and 23% respectively, indicating that the nanoparticles alone are toxic to cells at higher dosages.

- Extracellular vesicles: at concentrations of 0.1 µg/mL and 1 µg/mL, the cell viability remained high at 99.7% and 98.7% respectively. At a high concentration of 2.5 µg/mL, the cell viability decreased slightly to 85.7%, indicating that extracellular vesicles are generally non-toxic to cells even at higher dosages.

- Mixture of nanoparticles and extracellular vesicles: at a concentration of 0.1 µg/mL, the cell viability remained relatively good at 91%. At a concentration of 1 µg/mL, the cell viability dropped significantly to 52%, indicating higher toxicity. At a concentration of 2.5 µg/mL, the cell viability decreased to 25.6%, indicating significant toxicity.

- Complexes (NPs@EVs): at a concentration of 0.1 μg/mL, the cell viability was very good at 96.6%. At a concentration of 1 μg/mL, the cell viability decreased to 73%, indicating significant toxicity but still acceptable. At a concentration of 2.5 μg/mL, the cell viability further dropped to 38.4%, indicating high toxicity.

The cytotoxicity analysis reveals that the nanoparticles alone exhibit toxicity to cells, especially at higher concentrations. In contrast, extracellular vesicles alone show high cell viability even at higher dosages, indicating their non-toxic nature. The mixture of nanoparticles and extracellular vesicles shows reduced cell viability, however, when encapsulated within extracellular vesicles, the complexes demonstrate improved cell viability compared to the mixture. This last result can be attributed to the high aggregation level of the complexes, which barely interacts with cells and due to their large size, can not be internalized. The significant difference in cell viability between the complexes and the mixture is noteworthy. At a concentration of 1 μg/mL, the cell viability of the complexes is 20% higher than that of the mixture, and at 2.5 μg/mL, the viability difference exceeds 10%. This indicates that the encapsulation process reduces the toxicity of the complexes. The results highlight the need for further work to reduce the toxicity of the complexes, particularly at higher concentrations. However, the observed viability difference between the complexes and the mixture is an encouraging finding. It suggests that encapsulation plays a role in mitigating the toxicity of the nanoparticles. Further research is warranted to optimize the formulation and improve the biocompatibility of these complexes, paving the way for their potential use in targeted drug delivery and other biomedical applications.

4.8 CryoTEM analysis

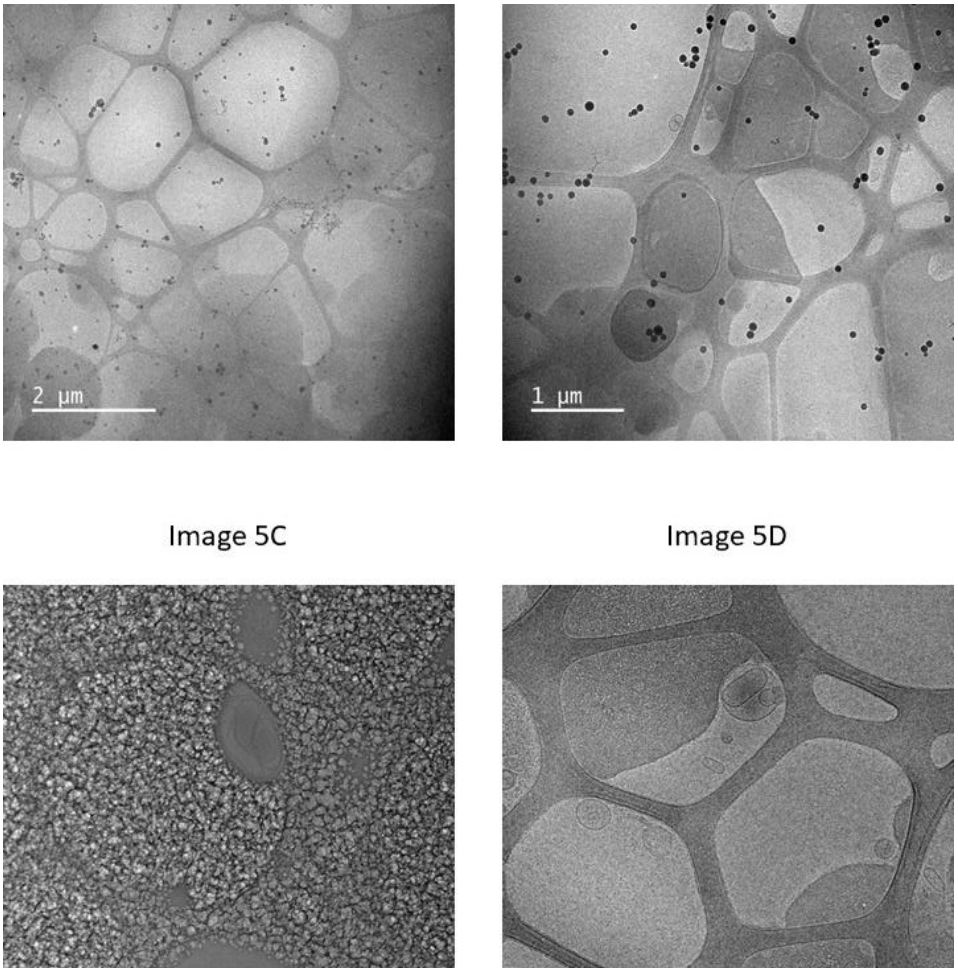

Figure 6 CryoTEM images showing (A and B) the complexes visualized as black dots. Images C and D show extracellular vesicles.

A cryoTEM analysis was performed on complexes NPs@EVs. The obtained images reveal distinct features that provide insights into the morphology and structure of the complexes and extracellular vesicles. In the cryoTEM images 6A and 6B, black dots are observed, which we suggest could represent the complexes of polymeric nanoparticles encapsulated within extracellular vesicles. These black dots indicate the presence of structures with a distinct morphology, suggesting successful encapsulation of the nanoparticles within the vesicles. The presence of these complexes further supports the efficacy of the encapsulation process and provides visual evidence of their formation. In addition to the black dots representing the complexes, separate images (6C and 6D) show the presence of extracellular vesicles. The observation of extracellular vesicles in the cryoTEM images validates the presence of these entities in the samples. These visual observations align with the expected morphology and structure of the complexes and vesicles.

Chapter 5

Conclusions

5.1 Future steps

This chapter outlines the potential future steps and experimental directions that can be undertaken to further explore and advance the field of complexes made with polymeric nanoparticles encapsulated in extracellular vesicles. Building upon the findings and insights gained from the current study, these proposed steps aim to refine and optimize the encapsulation process, investigate alternative encapsulation strategies, and explore the application of the complexes in the context of mRNA vaccines. By undertaking these future steps, we can deepen our understanding of the encapsulation efficacy, enhance the targeting capabilities, and evaluate the therapeutic potential of these complex systems. Moreover, the aim is to contribute to the advancement of nanomedicine and targeted drug delivery systems. The knowledge gained from these experiments will not only enhance our understanding of nanoparticle-EV complexes but also pave the way for their potential translation into clinical applications, particularly in the field of mRNA vaccines.

5.1.1 Verification and quantification of encapsulation efficacy

- FRET (Fluorescence Resonance Energy Transfer) Assay: This technique can be employed to measure the proximity and interaction between the nanoparticles and encapsulating extracellular vesicles. By using suitable fluorescence labels, FRET

can provide evidence of successful encapsulation and enable quantification of the encapsulation efficacy.

- PicoGreen Assay: This assay is commonly used to quantify nucleic acid content. It can be adapted to measure the encapsulated nucleic acid payload within the complexes, thus providing an indirect measurement of the encapsulation percentage. This assay can help refine and optimize the encapsulation process to achieve higher encapsulation efficiencies.

5.1.2 Targeted encapsulation within dendritic cell-derived extracellular vesicles

- Isolation of EVs from Dendritic Cells (DCs): DC-derived EVs possess specific membrane proteins and ligands that can facilitate targeted delivery to antigen-presenting cells. Isolating EVs from DCs and evaluating their encapsulation potential for nanoparticles would be a valuable stepbut n towards developing targeted delivery systems.

- Encapsulation of Nanoparticles within DC-Derived EVs: This experiment aims to explore the potential of DC-derived EVs as carriers for nanoparticles. By encapsulating the nanoparticles within EVs derived specifically from DCs, it is possible to leverage their inherent targeting capabilities and potentially enhance the delivery efficiency to immune cells.

5.1.3 Investigation with pOVA

- Evaluation of pOVA Encapsulation: As mRNA vaccines often employ plasmids similar to pOVA, investigating the encapsulation of pOVA within the complexes can provide insights into the suitability of the nanoparticle-EV system for mRNA vaccine delivery.

- In vitro Transfection Efficiency: Assessing the transfection efficiency of pOVA within target cells (e.g., antigen-presenting cells) can provide evidence of successful delivery and expression of the encapsulated genetic material.

5.1.4 In vivo testing

- Animal Model Studies: Conducting in vivo studies in suitable animal models can provide critical information regarding the behavior, biodistribution, and therapeutic potential of the nanoparticle-EV complexes. The evaluation of pharmacokinetics, tissue distribution, and therapeutic outcomes can contribute to the translation of this technology into clinical applications.

5.2 Final comments

The experiments conducted in this study have provided valuable insights into the characterization of complexes formed by polymeric nanoparticles encapsulated in extracellular vesicles. The initial assessment of the nanoparticles, extracellular vesicles, and the complexes themselves has yielded important information regarding their size, concentration, and physical properties. The characterization experiments have shed light on the size distribution and concentration of the nanoparticles within the complex systems, offering a preliminary understanding of their behavior. These findings not only contribute to the fundamental knowledge of the complex systems but also provide guidance for further investigations in terms of optimizing the synthesis and formulation processes. Furthermore, the experiments conducted to validate the successful encapsulation of nanoparticles within EVs have demonstrated the potential of this technique. Although the encapsulation process may not have been perfect, these preliminary experiments serve as a steppingstone for future studies aiming to enhance the encapsulation efficiency and stability of the complexes. Such advancements would pave the way for the development of novel drug delivery systems with enhanced efficacy and reduced side effects. Moreover, the assessment of cytotoxicity exhibited by the samples on THP-1 cell lines has yielded critical insights into the biological effects of the nanoparticle-EV complexes. These findings contribute to our understanding of the safety profile of the complexes and their potential implications for biomedical applications. Further investigations into the mechanisms underlying the observed cytotoxicity would provide a comprehensive understanding of the complex interplay between the complexes and cellular systems. In summary, this study has made significant strides in the characterization of complexes formed by nanoparticles encapsulated in extracellular vesicles. The experiments conducted have laid a strong foundation for future research endeavors, allowing for the refinement of encapsulation

techniques and the exploration of potential biomedical applications. As the field of nanomedicine continues to evolve, the knowledge gained from this study will contribute to the development of innovative therapeutic strategies and the translation of nanoparticle-EV complexes into clinical practice, ultimately benefiting patients worldwide.

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Bibliography

[1]. **American Cancer Society – What are tumors? (2021) - https://www.cancer.org/cancer/cancer-basics/what-are-tumors.html**

[2]. **Gupta, G. P., & Massagué, J. (2006). Cancer Metastasis: Building a Framework. In Cell (Vol. 127, Issue 4, pp. 679–695). Elsevier B.V. https://doi.org/10.1016/j.cell.2006.11.001**

[3]. **American Cancer Society – Metastatic Cancer (2021) - https://www.cancer.org/cancer/cancer-basics/metastatic-cancer.html**

[4]. **Valastyan, S., & Weinberg, R. A. (2011). Tumor metastasis: Molecular insights and evolving paradigms. In Cell (Vol. 147, Issue 2, pp. 275–292). Elsevier B.V. https://doi.org/10.1016/j.cell.2011.09.024**

[5]. **National Cancer Institute – (2022) - https://www.cancer.org/cancer/lymphoma.html**

[6]. **GLOBOCAN 2020**

[7]. **Sharma, P., Hu-Lieskovan, S., Wargo, J. A., & Ribas, A. (2017). Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. In Cell (Vol. 168, Issue 4, pp. 707–723). Cell Press. https://doi.org/10.1016/j.cell.2017.01.017**

[8]. **Roskoski, R. (2021). Properties of FDA-approved small molecule protein kinase inhibitors: A 2021 update. In Pharmacological Research (Vol. 165). Academic Press. https://doi.org/10.1016/j.phrs.2021.105463**

[9]. **June, C. H., O'connor, R. S., Kawalekar, O. U., Ghassemi, S., & Milone, M. C. (n.d.). CAR T cell immunotherapy for human cancer. http://science.sciencemag.org/**

[10]. **Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. In NATURE (Vol. 392).**

[11]. Collin, M., & Bigley, V. (2018). Human dendritic cell subsets: an update. In Immunology (Vol. 154, Issue 1, pp. 3–20). Blackwell Publishing Ltd. https://doi.org/10.1111/imm.12888

[12]. Merad, M., Sathe, P., Helft, J., Miller, J., & Mortha, A. (2013). The dendritic cell lineage: Ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. In Annual Review of Immunology (Vol. 31, pp. 563–604). https://doi.org/10.1146/annurev-immunol-020711-074950

[13]. Palucka, K., & Banchereau, J. (2013). Dendritic-Cell-Based Therapeutic Cancer Vaccines. In Immunity (Vol. 39, Issue 1, pp. 38–48). https://doi.org/10.1016/j.immuni.2013.07.004

[14]. Garris, C. S., Arlauckas, S. P., Kohler, R. H., Trefny, M. P., Garren, S., Piot, C., Engblom, C., Pfirschke, C., Siwicki, M., Gungabeesoon, J., Freeman, G. J., Warren, S. E., Ong, S. F., Browning, E., Twitty, C. G., Pierce, R. H., Le, M. H., Algazi, A. P., Daud, A. I., … Pittet, M. J. (2018). Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN-γ and IL-12. Immunity, 49(6), 1148-1161.e7. https://doi.org/10.1016/j.immuni.2018.09.024

[15]. Kreuter, J. (2007). Nanoparticles-a historical perspective. In International Journal of Pharmaceutics (Vol. 331, Issue 1, pp. 1–10). https://doi.org/10.1016/j.ijpharm.2006.10.021

[16]. Mehnert, W., & Mader, K. (2001). Solid lipid nanoparticles Production, characterization and applications. In Advanced Drug Delivery Reviews (Vol. 47). www.elsevier.com/locate/drugdeliv

[17]. Yáñez-Mó, M., Siljander, P. R. M., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-Da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., … de Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. In Journal of Extracellular Vesicles (Vol. 4, Issue 2015, pp. 1–60). Co-Action Publishing. https://doi.org/10.3402/jev.v4.27066

[18]. Segovia, N., Dosta, P., Cascante, A., Ramos, V., & Borrós, S. (2014). Oligopeptide-terminated poly(β-amino ester)s for highly efficient gene delivery and intracellular localization. Acta Biomaterialia, 10(5), 2147–2158. https://doi.org/10.1016/j.actbio.2013.12.054

[19]. Deng, Y., Govers, C., Beest, E. ter, van Dijk, A. J., Hettinga, K., & Wichers, H. J. (2021). A THP-1 Cell Line-Based Exploration of Immune Responses Toward Heat-Treated BLG. Frontiers in Nutrition, 7. https://doi.org/10.3389/fnut.2020.612397

[20]. Fornaguera, C., Guerra-Rebollo, M., Ángel Lázaro, M., Castells-Sala, C., Meca-Cortés, O., Ramos-Pérez, V., Cascante, A., Rubio, N., Blanco, J., & Borrós, S. (2018). mRNA Delivery System for Targeting Antigen-Presenting Cells In Vivo. Advanced Healthcare Materials, 7(17). https://doi.org/10.1002/adhm.201800335