ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

CELL-PENETRATING NUCLEIC ACID-BINDING HYBRID PEPTIDE AS AN EFFICIENT GENE TRANSFER VECTOR

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PH.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

02. 08. 2024

Maliheh Manteghi

PREFACE AND ACKNOWLEDGEMENT

This journey would not have been possible without many people's love, support, and encouragement. First of all, I would like to express my special thanks to my thesis supervisor Professor Tanıl Kocagöz for his support and guidance since we met. I thank him for giving me the opportunity to study at Acibadem University and for believing in my potential. His mentorship has shaped my academic and professional path. I would also like to sincerely thank my co-advisor Professor Özge Can for his guidance throughout my career. His expertise and insights have played a fundamental role in my growth as a researcher. I thank my family—my wonderful father, mother, and sisters for always being there and for their endless love and encouragement. To my loving husband Mesut, for his support and patience. I thank him for standing by my side through all the ups and downs and every challenge and I would like to thank my 2-year-old son Alp, who has been a light to me with his cheerful spirit during this challenging journey, for reminding me of the simple joys in life and adding unlimited happiness to my days. I am extremely grateful to my friends and TK lab members whose friendship and cooperation made this experience enjoyable and enriching. Whether through discussions or shared laughter, their support has been a constant source of motivation.

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I am also proud to announce that the results of this study have been recognized for their novelty and patent application has been made. (Türk Patent No: 2024/000662). This achievement highlights the importance of our research and its potential impact on biotechnology. Our findings have been published in the paper ‘Peptosome: A Novel and Efficient Transfection Tool as an Alternative to Liposome’, in the "International Journal of Molecular Sciences" further emphasizing the importance and innovation of our work.

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LIST OF ABBREVIATIONS AND SYMBOLES

°C	Celsius Degree
µl	Microliter
AMP	Antimicrobial Peptide
Arg	Arginin
$CaCl_2$	Calcium Chloride
CO_2	Carbon Dioxide
CPP	Cell-Penetrating Peptides
Cys	Cysteine
DCM	Diisopropylcarbodiimide
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
g	Gram
GPA	Glycopeptide Antibiotics
Ile	Isoleucine
kDa	Kilodaltons
Leu	Leucine
LSU	Large Subunit
M	Molarite
mg	Milligram
MIC	Minimal Inhibitory Concentration
ml	Milliliter
mRNA	Messenger RNA
MS	Mass Spectrometry
NaCl	Sodium Chloride
PBS	Phosphate Buffered Saline
PG	Peptidoglycan
pH	Power of Hydrogen
Pro	Prolin
RNA	Ribonucleic Acid

SEM	Scanning Electron Microscope
SPPS	Solid Phase Peptide Synthesis
SSU	Small Subunit
TEM	Transmission Electron Microscope
TFA	Trifluoroacetic Acid
TIS	Triisopropylsilane
UPLC	Ultra Performance Liquid Chromatography
α	Alpha
Β	Beta
Θ	Theta

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ABSTRACT

Cell-Penetrating Nucleic Acid-Binding Hybrid Peptide as an Efficient Gene Transfer Vector

Gene therapy is one of the most promising techniques for treating genetic diseases and cancer. The current most important problem in gene therapy is gene delivery. Viral and non-viral vectors like liposomes, used for gene delivery, have many limitations. We have developed new hybrid peptides by combining cell-penetrating peptides (CPPs) with the DNA-binding domain of the human histone H4 protein. These small peptides bind to DNA molecules through their histone domain, leaving the CPP part free and available for cell binding and penetration, forming complexes that we named "peptosomes". We evaluated the transfection efficiency of several hybrid peptides by delivering a plasmid carrying the green fluorescent protein gene and following its expression by fluorescent microscopy. Among several hybrid peptides, TM3 achieved a gene delivery efficiency of 76%, compared to 52% for Lipofectamine 2000. TM3 peptosomes may become important gene delivery tools with several advantages over current gene delivery agents.

Keywords: Gene therapy, Gene delivery, Cell- penetrating peptide, Histone.

ÖZET

Etkili Bir Gen Aktarım Vektörü Olarak Hücreye Nüfuz Eden Nükleik Asit Bağlayıcı Hibrit Peptit

Gen terapisi, genetik hastalıkların ve kanserin tedavisi için en umut verici tekniklerden biridir. Gen terapisindeki mevcut en önemli sorun gen iletimidir. Gen iletimi için kullanılan lipozomlar gibi viral ve viral olmayan vektörlerin birçok sınırlaması vardır. Bu çalışmada, hücreye nüfuz eden peptitleri (CPP'ler) insan histon H4 proteininin DNA bağlayıcı bölgesiyle birleştirerek yeni hibrit peptitler geliştirilmiştir. Bu küçük peptitler, DNA moleküllerine histone bölgeleri aracılığıyla bağlanarak CPP kısmını serbest bırakır ve hücre bağlanması ve penetrasyonu için kullanılabilir hale getirerek "peptozomlar" adını verdiğimiz kompleksler oluşturur. Hibrid peptitlerin transfeksiyon etkinliği, yeşil floresan protein geni taşıyan bir plazmid kullanılarak ve floresan mikroskobu ile ekspresyonu izlenerek değerlendirilmiştir. Hibrit peptidler arasında TM3 peptitinin, Lipofectamine 2000'e göre %52'ye kıyasla %76'lık bir gen iletim verimlilik sunduğu gözlemlenmiştir. Çalışmanın sonuçlarına göre, TM3 peptozomlarının, mevcut gen iletim ajanlarına göre çeşitli avantajlara sahip önemli gen iletim araçları haline gelebileceği düşünülmektedir.

Anahtar Sözcükler: Gen terapisi, Gen aktarımı, Hücreye nüfuz eden peptit, Histon.

1 INTRODUCTION

1.1 Gene Delivery

1.1.1 Definition and significance of gene delivery

Genetic diseases have long affected humans. After years of research, the genetic basis of many of these diseases has been established. Consequently, over the past two decades, gene therapy, which works by inserting a gene into a patient's cells, has become an important strategy for preventing or treating genetic disorders and cancer (1, 2). Gene delivery refers to introducing exogenous genetic material into target cells to modulate their function, correct genetic defects, or induce therapeutic effects. This concept lies at the heart of gene therapy, a promising approach for treating various diseases, including genetic disorders, cancer, and infectious diseases (Figure 1).

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has greatly advanced editing genes. This was a major move towards treating genetic diseases (3). Since Cas9 is a gene-editing enzyme of bacterial origin and is not present in human cells, CRISPR/Cas9 gene modification requires sending a gene into the cell that can express Cas9 with a guide RNA specific for the region to be modified in the genome. Therefore, the guide RNA sequence and the gene for Cas9 are cloned on a plasmid which is subsequently delivered into the cells. This necessitates the transfer of a larger DNA into the cells (4). However, among several challenges that need to be solved for efficient gene therapy, gene delivery remains the leading problem (5). To overcome this problem, several approaches for gene delivery have been developed to efficiently introduce foreign genetic material and ensure its stability within host cells.

In certain genetic disorders, gene delivery can permanently correct the underlying genetic defect, offering the prospect of long-term therapeutic benefits. Gene delivery platforms can accommodate a wide range of therapeutic cargoes, including genes

encoding therapeutic proteins, RNA molecules for gene silencing, or gene-editing tools for precise genome modifications.

Gene therapy holds promise for addressing diseases that are currently incurable or inadequately managed by conventional therapies, providing new hope for patients with rare genetic disorders or cancers (6-9).

1.1.2 Importance of gene delivery

For years, scientists have tried to deliver nucleic acids into cells to study the mechanisms lying behind certain diseases, find out what is causing that specific disease, and propose a treatment for the patients. Therefore, it can be said that intracellular delivery has been a crucial step for biological studies that focus on delivering drugs or nucleic acids, as in DNA or RNA, to the patients to exhibit therapeutical effects or recover the damage caused by the disease mechanism. Via intracellular delivery, the cargoes are carried to the inside of the cell. A cargo is, in this means, anything that is transferred from one location to another, by a specific protein that carries it. The size, form, structure, and chemical nature of cargoes of interest for cellular uptake differ tremendously, and consequently, it is possible to categorize different cargo types based on these variable features, delivery challenges, and intracellular applications which brings out the discovery of assorted delivery methods of cargoes intracellularly. For example, small molecule cargoes, peptides, proteins, nucleic acids, plasmid DNA, etc. can be delivered inside the cells as cargoes. The intracellular delivery of nucleic acids and proteins is a key step in the development of cell-based therapies and this process is called transfection.

Gene delivery vectors are essential tools in the field of molecular biology and gene therapy, facilitating the transfer of genetic material into target cells. These vectors serve as vehicles for introducing therapeutic genes, gene editing tools, or other nucleic acid-based materials into cells, offering promising avenues for treating various genetic disorders, cancers, and infectious diseases.

Among several challenges that need to be solved for efficient gene therapy, gene delivery remains the leading problem (5). To overcome this problem, several approaches for gene delivery have been developed to efficiently introduce foreign genetic material and ensure its stability within host cells. To ensure effective delivery, the system must not damage cells or induce an inflammatory response in the organism, and the gene of interest must cross multiple barriers during the process (10). In this regard, the foreign gene must be transported inside the cell nucleus to induce gene expression. The gene must remain stable once inside the cell. The stability of a genetic element means that it can maintain certain characteristic behaviors for long periods without accumulating mutations inside the cell, without interacting with other structures in the host genome, and without undergoing structural, genetic, or epigenetic changes. The gene delivery system must provide appropriate conditions to protect the integrity of the gene (11).

Scientists use different gene delivery tools including viral vectors and non-viral systems such as mechanical or chemical methods. Viral gene delivery is achieved by using vi-ruses in which disease-causing genes are deleted and the gene needed to treat a genetic disease is added. Retrovirus, adenovirus, and adeno-associated viruses have been widely used because of their high efficiency in gene transfer (12). Retroviral vectors are produced in host cells that contain viral genes needed for virus replication, which are deleted from the virus to inhibit their replication in the cells to which they deliver the gene for treatment. However, the integrase gene is kept in the viral vector genome, to produce an integrase enzyme that integrates the desired gene into the human cell genome by recombination using long terminal repeat (LTR) sequences. This enables long-term expression of the gene and transfers the gene to daughter cells when the cell divides. However, since the site of integration cannot be controlled, there is always concern about insertional mutagenesis which can inactivate a tumor suppressor gene or create an oncogene that will transform the cell into a cancer cell (13). The use of viral vectors is associated with potential safety concerns, such as the possibility of immune response, as well as the limitations of viral vectors (6). Inserting genetic material into the host genome can sometimes disrupt normal gene function, potentially leading to adverse effects or diseases (14).

Another alternative approach for introducing genes involves employing natural or synthetic substances, commonly referred to as non-viral vectors. These vectors create complexes composed of DNA, proteins, polymers, or lipids, organized into particles with the ability to effectively transport genes into cells.

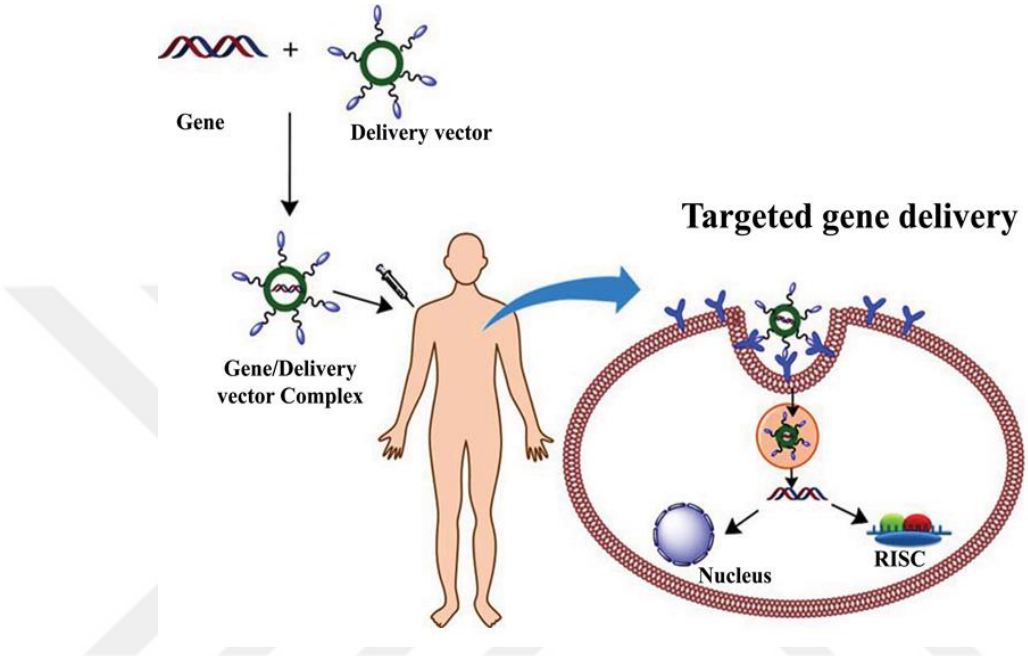

Figure 1. Gene delivery mechanism (3)

1.1.3 Categories of gene delivery vectors

Gene delivery vectors can be broadly categorized based on their origin, composition, and mode of delivery.

1.1.3.1 Viral vectors

Viral vectors are derived from viruses that have been genetically modified to carry therapeutic genes instead of their genetic material. These vectors can efficiently infect a wide range of host cells and often result in gene expression. Commonly used viral vectors include Retroviruses, Adenoviruses, Adeno-associated viruses (AAVs), and Herpes simplex viruses (HSV) (15, 16).

1.1.3.2 Non-viral vectors

Non-viral vectors include a diverse range of delivery systems that do not involve viral components. They are generally safer and easier to produce at scale compared to viral vectors. Non-viral vectors can be further divided into: Lipid-based vectors (liposomes, lipid nanoparticles), Polymer-based vectors, Naked DNA/RNA, and Cell-penetrating peptides (CPPs) (6, 17).

1.1.3.3 Hybrid vectors

Hybrid vectors combine elements of both viral and non-viral vectors to capitalize on the strengths of each system. These vectors may incorporate viral components for efficient cell entry and non-viral components to improve protection or targeting specificity (18, 19).

Each category of gene delivery vector has its advantages and limitations, and the choice of vector depends on factors such as the target cell type, desired level and duration of gene expression, safety considerations, and scalability for clinical applications.

1.1.4 Applications of gene delivery in medicine

Gene delivery holds immense promise in medicine, serving as a critical tool in numerous therapeutic applications from treating genetic disorders to fighting cancer and infectious diseases. In gene therapy, delivery systems, such as viral vectors and non-viral vectors, transport therapeutic genes into target cells, thereby correcting or modulating dysfunctional gene expression. This technology has shown remarkable efficacy in clinical trials for inherited disorders like cystic fibrosis and hemophilia, offering hope for patients with previously untreatable conditions. Moreover, in cancer therapy, gene delivery enables the introduction of genes encoding tumor-suppressive proteins or therapeutic molecules, increasing conventional treatments and enhancing their efficacy while minimizing unfavorable effects. Additionally, the interest in genome editing tools, such as CRISPR-Cas9, further expands the scope of gene

delivery by enabling precise manipulation of the genome to correct disease-causing mutations. All the multifaceted applications of gene delivery offer personalized and targeted therapeutic interventions to improve patient outcomes (6, 20, 21).

1.1.5 Challenges and limitations in gene delivery

Gene delivery presents numerous challenges and limitations that must be addressed to achieve efficient and safe therapeutic outcomes. One of the primary challenges is the development of delivery systems capable of overcoming biological barriers, such as cellular uptake and endosomal escape, while minimizing off-target effects and immunogenicity. Additionally, the size and complexity of genetic materials, including large genes or gene editing machinery like CRISPR-Cas9, pose barriers to delivery efficiency and stability. Furthermore, the choice of delivery vector, whether viral (e.g., adenovirus, lentivirus) or non-viral (e.g., liposomes, nanoparticles), entails trade-offs between transfection efficiency, safety, and scalability. Moreover, concerns regarding the long-term effects and potential insertional mutagenesis associated with viral vectors underscore the need for safer alternatives. Addressing these challenges requires interdisciplinary approaches integrating molecular biology, materials science, and biomedical engineering to develop next-generation gene delivery platforms (6, 22).

1.2 Viral Vectors in Gene Delivery

Viral vectors represent a basis in gene delivery, offering efficient and versatile platforms for introducing therapeutic genes into target cells. These vectors, derived from genetically modified viruses, encompass a diverse array of systems such as retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAVs). Each viral vector type possesses unique characteristics that make it suitable for specific applications in gene therapy. Retroviruses and lentiviruses, for instance, integrate their genetic material into the host genome, allowing for stable, long-term gene expression. Adenoviral vectors, on the other hand, excel in transient gene expression due to their high transduction efficiency. Adeno-associated viruses offer the advantage of minimal

immunogenicity and the ability to transduce both dividing and non-dividing cells. These viral vectors have been extensively studied and employed in various preclinical and clinical settings, demonstrating their potential for treating genetic disorders, cancers, and infectious diseases (23).

1.2.1 Overview of viral vectors

Retroviruses are RNA viruses that can integrate their genetic material into the host genome, making them useful for stable gene transfer. They have been extensively used in gene therapy and genetic engineering applications. Retroviral vectors are derived from retroviruses by replacing most of their viral genes with therapeutic transgenes (23, 24).

Lentiviruses are a subgroup of retroviruses characterized by their ability to infect both dividing and non-dividing cells and their relatively large cargo capacity. Lentiviral vectors, derived from viruses such as HIV-1, have become popular tools for delivering genes into various cell types, including stem cells and neurons, due to their efficient transduction and stable integration into the host genome (25).

Adenoviruses are DNA viruses that efficiently infect a broad range of dividing and non-dividing cells. Adenoviral vectors are widely used for transient gene expression due to their high transduction efficiency and ability to accommodate large transgenes. However, they induce potent immune responses, limiting their utility for long-term gene therapy (26, 27).

Adeno-associated virus (AAV is a small, non-enveloped virus that is not known to cause any human diseases. AAV vectors are widely used in gene therapy because they efficiently transduce both dividing and non-dividing cells, long-term gene expression, and minimal immunogenicity. However, their cargo capacity is limited (28).

These viral vectors have revolutionized gene therapy and are extensively studied and optimized for various applications in preclinical and clinical settings.

1.2.2 Mechanisms of viral-mediated gene delivery

Viral-mediated gene delivery employs viruses as vehicles to introduce therapeutic genes or nucleic acid materials into target cells. Understanding the mechanisms underlying viral-mediated gene delivery is crucial for optimizing vector design and enhancing efficiency and safety. The first step in viral-mediated gene delivery is the entry of the viral vector into the target cell. Viruses use various mechanisms to interact with cell surface receptors and gain entry, including receptor-mediated endocytosis, membrane fusion, or direct membrane penetration (29, 30). (Figure 2)

Following internalization, viral vectors encounter endosomal compartments within the host cell. To avoid lysosomal degradation and facilitate material release into the cytoplasm, some viruses have evolved mechanisms for endosomal escape, such as membrane disruption or pH-dependent membrane fusion (31, 32).

For viruses delivering genetic material to the nucleus, nuclear import is a critical step. Viral components or complexes often exploit host nuclear import machinery or nuclear localization signals to translocate across the nuclear envelope and deliver their cargo into the nucleus (33, 34).

Retroviruses and lentiviruses integrate their genetic material into the host genome, providing stable and long-term expression of the therapeutic gene. Integration occurs through the action of viral integrase enzymes, which catalyze the covalent attachment of viral DNA to host chromosomal DNA (35, 36).

1.2.3 Advantages and limitations of viral vectors

Viral vectors offer several advantages as gene delivery vehicles, but they also have certain limitations.

Advantages:

Efficient Gene Delivery: Viral vectors can efficiently deliver genetic material into target cells, resulting in high levels of transgene expression. This efficiency is particularly advantageous for applications requiring robust and sustained gene expression, such as gene therapy (25).

Cell Type Specificity: Some viral vectors exhibit tropism for specific cell types or tissues, allowing targeted delivery of therapeutic genes to desired locations within the body. This targeting capability can enhance the efficacy and safety of gene therapy interventions (37).

Long-term Gene Expression: Certain viral vectors, such as adeno-associated virus (AAV), can mediate stable integration of transgenes into the host genome, leading to long-term and persistent gene expression in target cells. This feature is advantageous for treating genetic disorders requiring lifelong therapy (28).

Limitations:

Viral vectors can induce immune responses in the host, leading to the clearance of transduced cells and limiting the duration of transgene expression. Pre-existing immunity to the viral vector or the development of immune responses against vector components can pose challenges for successful gene therapy (38).

The size of the transgene that can be accommodated within viral vectors is limited by the packaging capacity of the viral genome. This constraint restricts the types of genes or genetic elements that can be delivered using viral vectors, particularly for large genes or gene editing tools (39).

Viral vectors may carry the risk of insertional mutagenesis, where integration of the vector genome into the host genome disrupts normal gene function and potentially leads to oncogenesis. Additionally, viral vectors derived from pathogenic viruses may pose safety concerns related to potential reactivation or dissemination (15).

Addressing these limitations through vector engineering, immunomodulation strategies, and careful preclinical and clinical evaluation is crucial for advancing the safety and efficacy of viral vector-based gene therapies.

1.3 Non-viral Vectors in Gene Delivery

Non-viral vectors in gene delivery offer promising alternatives to viral vectors, providing safer and more customizable platforms for nucleic acid delivery into target cells. These vectors encompass a diverse group of delivery systems, including lipid-based vectors such as liposomes and lipid nanoparticles, polymer-based vectors like polyplexes and dendrimers, inorganic nanoparticles such as gold nanoparticles and peptide base vectors such as Cell-penetrating peptides. Non-viral vectors offer advantages such as low immunogenicity, ease of production, and the potential for large-scale manufacturing. Additionally, they can be engineered to possess specific targeting ligands, enhancing their efficiency and specificity in gene delivery applications. While non-viral vectors often exhibit lower transfection efficiency compared to viral vectors, ongoing research aims to optimize their design and formulation to overcome these limitations and unlock their full potential for gene therapy and genetic engineering endeavors (18, 19).

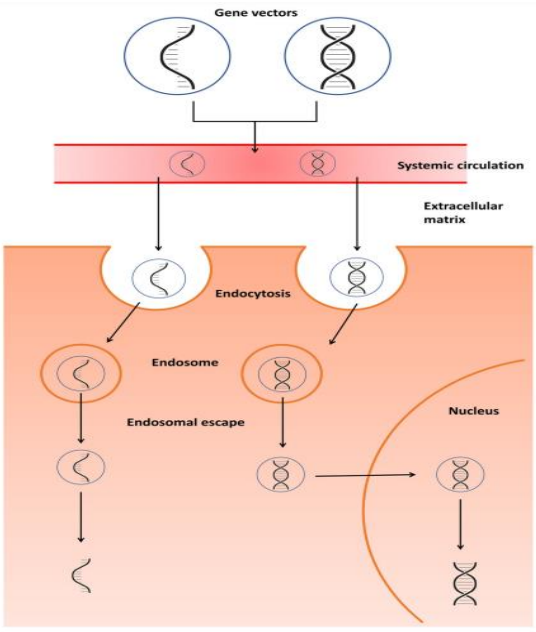

Figure 2. Endocytosis mechanism of gene delivery (14)

1.3.1 Overview of non-viral vectors

Liposomes are spherical vesicles composed of lipid bilayers that can encapsulate and deliver nucleic acids. They are biocompatible, easily modifiable, and can protect nucleic acids from degradation. Liposomes can be surface modified for targeted delivery to specific cell types or tissues, making them versatile gene delivery vehicles (40, 41).

Nanoparticles, including polymeric nanoparticles and inorganic nanoparticles, are particulate carriers with diameters typically ranging from 1 to 100 nanometers. They can efficiently encapsulate or adsorb nucleic acids and protect them from degradation. Nanoparticles offer tunable properties for controlled release and targeted delivery of genetic materials (42).

Synthetic polymers, such as polyethyleneimine (PEI), poly(L-lysine) (PLL), and polyethyleneglycol (PEG), can form complexes with nucleic acids through electrostatic interactions or covalent bonding. These polymer-based vectors protect nucleic acids from degradation and facilitate their uptake by cells (18).

Naked DNA or RNA molecules can be directly delivered into cells without the use of carriers. This approach is straightforward but often less efficient compared to vector-mediated delivery. However, it has been utilized in applications such as DNA vaccination and RNA interference (43).

Cell-Penetrating Peptides (CPPs) are short peptides that can facilitate the intracellular delivery of cargo molecules, including nucleic acids, by traversing cell membranes. These peptides can be chemically conjugated to nucleic acids or formulated into complexes for efficient delivery (44).

These non-viral vectors offer diverse strategies for gene delivery, each with its advantages and limitations. Ongoing research aims to further optimize these vectors and develop innovative delivery strategies for various biomedical applications.

1.3.2 Mechanisms of non-viral-mediated gene delivery

Non-viral-mediated gene delivery relies on a variety of mechanisms to introduce exogenous genetic material into target cells. One common approach involves the use of lipid-based vectors, such as liposomes or lipid nanoparticles, which encapsulate nucleic acids and facilitate their uptake into cells via endocytosis. Once internalized, these vectors can escape from endosomes into the cytoplasm, where the nucleic acids are released and may enter the nucleus for transcription. Another strategy employs polymer-based vectors, such as polyethyleneimine (PEI) or polyethylene glycol (PEG), which can condense nucleic acids into compact particles and promote their cellular uptake through various mechanisms, including electrostatic interactions and membrane disruption. Additionally, inorganic nanoparticles, like gold nanoparticles or quantum dots, can be functionalized with nucleic acids and targeted to specific cell types, where they may enter cells via receptor-mediated endocytosis or membrane penetration. Non-viral vectors offer advantages such as low immunogenicity, ease of production, and versatility in cargo delivery, although their transfection efficiency and stability may vary depending on the formulation and target cell type (45, 46, 17).

1.3.3 Advantages and limitations of non-viral vectors

Non-viral vectors offer several advantages in gene delivery, including their generally safer profile compared to viral vectors, ease of production, and versatility in accommodating various types of materials. They often induce lower immune responses, reducing the risk of adverse reactions in recipients. Additionally, non-viral vectors can be tailored for specific applications through modifications in their composition, such as the addition of targeting ligands or regulatory elements, allowing for precise control over gene expression. However, non-viral vectors typically exhibit lower transfection efficiency and shorter duration of gene expression compared to viral vectors. They may also encounter challenges in delivering genes to certain cell types or crossing cellular barriers (17, 18).

1.4 Comparative Analysis of Viral vs. Non-Viral Vectors

Comparative analysis of viral and non-viral vectors in gene delivery presents an evaluation of their respective advantages and limitations. Viral vectors, such as retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAVs), offer efficient gene transfer and long-term transgene expression due to their ability to integrate into the host genome (23, 25, 26, 28). However, they may induce immune responses, pose risks of insertional mutagenesis, and have limited cargo capacities. In contrast, non-viral vectors, including lipid-based nanoparticles, polymer-based complexes, and inorganic nanoparticles, offer improved safety profiles, scalability, and ease of production (6, 17). They are versatile and can accommodate a wide range of materials but often exhibit lower transduction efficiencies and shorter duration of gene expression compared to viral vectors. Ultimately, the choice between viral and non-viral vectors depends on specific therapeutic goals, target cell types, safety considerations, and scalability requirements (23, 25).

1.5 Recent Developments in Non-Viral Vector Design and Optimization

Recent advancements in non-viral vector design and optimization have focused on enhancing delivery efficiency, targeting specificity, and minimizing cytotoxicity and immunogenicity. One notable approach involves the development of advanced lipid nanoparticles (LNPs), polymer-based delivery systems Cell-penetrating peptides. LNPs, such as ionizable lipid nanoparticles, have been engineered to improve endosomal escape and intracellular delivery of nucleic acids, leading to enhanced gene expression. Additionally, the design of biodegradable and stimuli-responsive polymers has enabled the controlled release of cargo, thereby improving safety and efficacy. Furthermore, the incorporation of targeting ligands and cell-penetrating peptides into non-viral vectors allows for precise delivery to specific cell types or intracellular compartments, minimizing off-target effects. These advancements hold great promise for the development of safer and more efficient non-viral gene delivery systems for a wide range of therapeutic applications (47-49).

Recent developments in cell-penetrating peptides (CPPs) as non-viral vectors have focused on enhancing their efficiency, specificity, and safety for delivering therapeutic materials into target cells. Strategies for optimizing CPP-based vectors include designing novel peptides with improved cell-penetrating properties, conjugating CPPs with targeting ligands to achieve cell-specific delivery and developing CPP-based nanocomplexes for encapsulating and protecting nucleic acids. Additionally, advances in understanding the mechanisms of cellular uptake and intracellular trafficking of CPPs have led to the rational design of optimized delivery systems. These efforts aim to overcome the limitations of CPPs, such as low delivery efficiency and potential cytotoxicity, thereby paving the way for their wider application in gene therapy, drug delivery, and molecular imaging. Recent studies have demonstrated the potential of CPP-based vectors in delivering various cargoes, including nucleic acids, proteins, and nanoparticles, highlighting their versatility and therapeutic promise (50, 51).

1.6 Overcoming Challenges in Gene Delivery

Overcoming challenges in gene delivery is essential for the advancement of gene therapy toward clinical applications. One major challenge is achieving efficient and targeted delivery of therapeutic genes to the desired cells while minimizing off-target effects and immune responses. Strategies to address this challenge include the development of viral and non-viral vectors with improved transduction efficiency, tissue specificity, and safety profiles. Additionally, optimizing delivery routes, such as intravenous, intramuscular, or local administration, can enhance the distribution and uptake of gene therapy vectors. Furthermore, advancements in genome editing technologies, such as CRISPR-Cas9, offer precise targeting of genetic modifications, further improving the specificity and efficacy of gene therapies. However, challenges remain, including the potential for insertional mutagenesis with viral vectors and the need for scalable and cost-effective manufacturing processes. Addressing these challenges requires interdisciplinary collaboration and continued innovation in vector design, delivery methods, and manufacturing techniques (39, 52).

1.7 Clinical Applications of Gene Delivery

Gene delivery has significant clinical applications in treating various genetic disorders, cancers, and other diseases.

For genetic disorders, gene therapy aims to correct or compensate for defective genes responsible for disease development. It involves delivering therapeutic genes to affected tissues or cells. For example, delivery of the Factor VIII or Factor IX gene to liver cells can help produce the necessary clotting factors missing in hemophilia patients (53).

Gene delivery can be used to introduce therapeutic genes that can either kill cancer cells directly or make them more susceptible to other treatments. For example, Oncolytic Viruses are modified viruses that can selectively infect and kill cancer cells (54).

For cardiovascular diseases gene delivery can help regenerate damaged heart tissues and improve heart function. For example, in angiogenesis delivery of genes encoding angiogenic factors can promote the growth of new blood vessels in ischemic tissues (55). In the treatment of cardiovascular diseases, gene delivery can enhance the expression of a protein that improves calcium handling in heart muscle cells (56). Gene delivery is being explored to provide neuroprotective effects and restore function in neurodegenerative diseases. Parkinson's Disease: Delivery of genes encoding for enzymes involved in dopamine synthesis can help alleviate symptoms (57). Gene delivery can enhance the immune response to infections or directly target infectious agents for example delivery of genes that encode for HIV-resistant proteins or RNA molecules that can inhibit viral replication in HIV disease: (58).

1.8 Future Perspectives in Gene Therapy Research Especially by Cell-Penetrating Peptides

Research in gene delivery, especially utilizing cell-penetrating peptides (CPPs), is rapidly evolving, with several future perspectives and directions shaping the field. To enhance targeting and specificity, future research may focus on improving the targeting and specificity of CPP-mediated gene delivery systems to ensure precise delivery to the desired cell types or tissues while minimizing off-target effects (59). To optimize intracellular trafficking, further studies may explore strategies to optimize the intracellular trafficking of CPP-gene complexes, ensuring the efficient release of cargo into the desired cellular compartments for optimal gene expression (60). For combination therapies, future directions may involve exploring the potential of combining CPP-mediated gene delivery with other therapeutic modalities, such as chemotherapy or immunotherapy, to enhance treatment efficacy and overcome resistance mechanisms (61). Future perspectives may involve integrating CPP-mediated gene delivery with advanced gene editing technologies, such as CRISPR-Cas systems, for precise genome modification and correction of genetic disorders (62).

1.9 Cell Penetrating Peptides

In recent years, cell-penetrating peptides (CPPs) such as integrin beta fragments have emerged as promising nonviral vectors for delivering various cargoes into cells, including nucleic acids, plasmid DNA, small interfering RNA (siRNA), and antisense oligonucleotides. This capability makes CPPs valuable in developing new therapeutic strategies due to their ability to integrate and cross biological membranes (63). The cellular internalization processes facilitated by CPPs involve a combination of energy-dependent and energy-independent mechanisms, enabling effective delivery of cargo into the cytoplasm and, in some cases, the cell nucleus (64).

CPPs are short peptides, typically composed of 5–30 amino acids rich in arginine and lysine groups and can be produced at low cost. Their safety has been demonstrated over the last decade (65). Integrin beta fragments exhibit a high affinity for cell

membranes, primarily through interactions with lipid bilayers. The hydrophobic nature of these peptides allows them to readily associate with the hydrophobic core of cell membranes, facilitating efficient translocation across cellular barriers (66).

CPPs possess the unique ability to translocate across cellular membranes and deliver a diverse range of cargoes, including drugs, nucleic acids, and proteins, into cells. This property has positioned CPPs as attractive tools for drug delivery and various biomedical applications. They are believed to penetrate cells through mechanisms such as endocytosis, direct translocation, or a combination of both; however, the exact mechanism of cellular uptake remains not fully understood. Importantly, CPPs represent a category of non-viral gene delivery systems that can overcome some limitations associated with viral vectors (Figure 3). (60)

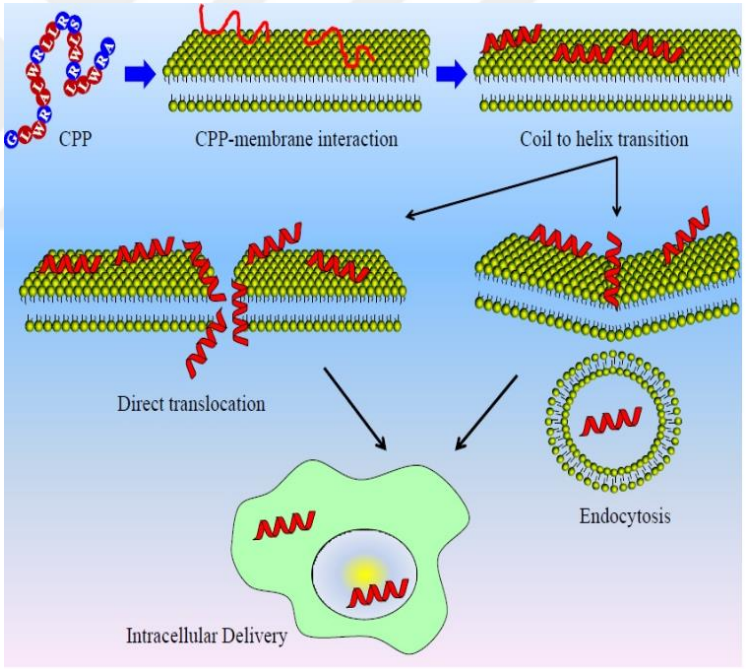

Figure 3. Mechanisms of gene delivery by Cell-Penetrating Peptide (66)

In the field of vaccination, CPPs have shown significant promise, particularly in the context of mRNA vaccines, which hold great potential for cancer management. Defining the sequence of the target antigen accurately is crucial for the formulation and production of effective mRNA vaccines. CPPs facilitate the intracellular delivery

of mRNA by translocating across cellular membranes, enhancing the cellular uptake of mRNA vaccines and leading to improved immunogenicity.

Different CPPs can enter cells either with or without energy, typically transporting cargo via endocytosis for research and therapeutic purposes. However, current applications of drug delivery via CPPs are hampered by a lack of cell selectivity and a limited understanding of the mechanisms underlying their absorption. Despite these challenges, CPPs are extensively studied for gene delivery in transfection protocols. Systemic pharmaceutical distribution is often restricted due to cell and tissue barriers and the poor biomembrane permeability of many therapeutics, limiting their therapeutic utility. CPPs present opportunities to increase medicament concentrations in hard-to-reach locations. Several preclinical studies using CPP-derived therapies have yielded encouraging outcomes in various disease models, prompting clinical trials in certain cases. These studies have opened new avenues for developing novel human therapeutics that are well-tolerated and targeted to intracellular locations.

Certain CPPs, such as TAT (GRKKRRQRRR), penetratin (RQIKIWFQNRRMKWKK), and integrin beta3 fragment (VTVLAGALAGVGVG), which are among the most studied peptides, can also function as nuclear localization signals (NLS). NLS are short sequences rich in positively charged residues that tag a protein for import into the cell nucleus. TAT, penetratin, and integrin beta3 fragment peptides contain these signals, enabling them to direct cargoes inside the nucleus. Several additional CPPs have been structurally and functionally characterized, with the majority currently in preclinical or clinical research. These peptides have been proven to transport a wide variety of biologically active conjugates into cells, including proteins, peptides, DNAs, siRNAs, and small pharmaceuticals. (64)

This comprehensive overview highlights the potential of CPPs in enhancing the delivery of therapeutic agents and their role in advancing biomedical research and clinical applications.

1.9.1 Classification of cell-penetrating peptides

Based on Origin:

Natural CPPs derived from natural proteins and peptides (e.g., HIV-1 Tat peptide). They contain positively charged amino acids (arginine, lysine) and interact with negatively charged cell membrane components (glycosaminoglycans, phospholipids).

Synthetic CPPs are designed through synthetic chemistry (e.g., amphipathic peptides, helical peptides, cyclic peptides). They optimized cargo delivery by modifying size, charge, hydrophobicity, and targeting properties.

Engineered CPPs: developed using protein engineering (e.g., fusion with carrier proteins or antibody fragments). They have Target specific cell surface receptors or ligands to enhance the specificity and efficiency of cargo delivery.

Based on Structure:

Cationic Peptides are Rich in positively charged amino acids (e.g., HIV-1 Tat peptide, penetratin).

Amphipathic Peptides contain both hydrophilic and hydrophobic regions (e.g., Pep-1, MPG).

Hydrophobic Peptides are rich in nonpolar amino acids (Integrin Beta-3 fragment and Grb2(SH2 domain)).

Based on Function:

Direct translocation is the penetration of the cell membrane without endocytosis. Endocytosis utilizes endocytic pathways (e.g., clathrin-mediated). Covalent Conjugation is covalently linked to cargo through chemical linkers. They Ensure CPP and cargo remain associated during uptake. They are peptides for delivering small molecules, proteins, and nucleic acids. (Table 1) (63)

Table 1. Categories of cell-penetrating peptides

Cationic peptides		
1	Penetratin	ROIKIWFONRRMKWKK
2	HIV-TAT	YGRKKRRORRR
3	HIV-1 Rev	KOAIPVAK-amide
4	FHV coat	RRRRRTRRRRRVR-amide
5	Oligoarginines	RRRRRRRRR/ RAAAAARRRRRR
6	CCMV Gag	KLTRAQRRAAARKNKRNTRGC
7	Chimeric dermaseptin S4	ALWKTLLKKVLKAPKKKRKVC
8	VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVE
Amphipathic peptides		
9	Transportan	GWILNSAGYLLGKINLKALAALAKKIL-amide
10	Transportan 10: TP 10	AGYLLGKINLKALAALAKKIL-amide
11	CPP with protease	YTAIAWVKAFIRKLK-amide
12	Pep-1	AC-KETWWETWWTEWSOPKKKRKV-NH-CHa-CH-SH
13	MP GA	AC-GALFLAFLAAALSLMGLWSQPKKKRKV-NH-CH2-CH -SH
14	MPG	Ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-NH-CH -CH2-SH
15	MPGB	RAFLGWLGAWGTMGWSPKKRK-NH-CH.-CH, -SH
16	CADY	Ac-GLWRALWRLLRSLWRLLWKA-NH-CH2-CH,-SH
17	Pepfect6	Stearyl-AGYLLGK(c-TMQ) INLKALAALAKKIL
18	PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL-amide
19	PepFect 15	Stearyl-AGYLLGKLLOOLAAAALOOLL-amide
20	NickFect	NF61: Stearyl-AGYLLGOINLKALAALAKKIL-amide
21	Hel 11-27	KLLKLLLKLWKLLLKLLK
22	InfluencaHA-2 KALA sequence	WEAKLAKALAKALAHLAKALAKALKACEA
23	KLA sequence	Acetyl-KLALKLALKALKAALKLA-amide
24	SC18	GLRKRLRKFRNKIKEK
25	pVEC	LLIILRRRIRKOAHAHSK-amide

Table 1. Categories of cell-penetrating peptides (continued)

Hydrophobic peptides		
26	Kaposis sarcoma fibroblast growth factor Kaposi FGF	AAVALLPAVLLALLAP
27	Signal sequence of lg light chain from Caliman Crocodylus	MGLGLHLLVLAAALQGAMGLGLHLLLAAA LOGA
28	Integrin Beta3 fragment	VIVLAGALAGVGVG
29	Grb2 (SH2, domain)	AAVLLPVLLAAP
30	Fusion sequence HIV-1 gp41	GALFLGFLGAAGSTMGA
31	Hepatitis B virus translocation motif	PLSSIFSRIGDP
32	Sperm-egg fusion protein	SFP23: Ac-KLIATGISSIPPIRALFAAIQIP-amide
33	Human calcitonin partial sequence, hCT-br	LGTYTQDENK(X)FHTFPOTAIGVGAP-amide X: PKKKRKVEDPGVGFA

1.9.2 Comparison of liposomes with CPPs

Nonviral gene delivery systems such as cationic lipid-based methods which have positive charge, are easy to prepare and demonstrate high efficiency. However, they may trigger acute immune responses. Non-lipid cationic polymers exhibit high in vitro efficiency but are toxic to cells and display low efficiency in vivo. They are also shown to have the potential to induce immune responses.

One of the most common transfection methods is Lipofection, utilizing liposomes which are tiny molecules capable of fusing with cell membranes to release their contents (67). Liposomes can be either lipid-based or non-lipid-based. Another transfection method involves cationic polymers, which possess a positive charge that enables them to effectively bind to negatively charged nucleic acids. Liposome transfection techniques are frequently used in the discovery and development of novel drugs, particularly for cancer treatment. Animal cell membranes are composed of a phospholipid bilayer with hydrophilic sides facing both the cytoplasm and the extracellular environment. The lipofection technique leverages microscopic vesicular

structures known as liposomes, which share the same composition as the cell membrane. To create a liposome around the DNA sequence to be transfected, a scientist performs a straightforward process. Depending on the liposome and cell type, the liposome can be endocytosed or directly fused with the cell membrane, allowing the DNA construct to enter the cells.

A widely used proprietary formulation for the transfection of nucleic acids (DNA and RNA) into eukaryotic cells is the Lipofectamine 2000 Transfection Reagent, known for its high expression rate.

When lipids are combined with cationic polymers to create hybrid molecules, they demonstrate notable in vitro success but show reduced activity in vivo. Direct needle injection of nucleic acids, a mechanical method, is simple but exhibits low efficiency, confining gene expression to the needle track. Gene gun technology, relying on pressure, achieves good efficiency but limits gene transfer to targeted areas and requires surgical procedures for internal organs. Electroporation, using electric pulses, displays high efficiency but may cause tissue damage and confines gene transfer to specific regions, necessitating surgical interventions for internal organs. Hydrodynamic delivery, utilizing hydrodynamic pressure, is simple and highly efficient, offering site-specificity; however, it requires catheter insertion in large animals (13). Among nonviral delivery tools, liposomes have become the most used vectors. Liposomes offer high transfection efficiency in vitro, making them ideal for cell-based studies and high-throughput experiments (65). However, they can induce cytotoxicity or trigger immune responses in cells, especially when used at higher concentrations (68, 69). This cytotoxicity can negatively affect cell viability and overall experimental outcomes. In addition, lipids that are used to make liposomes are expensive and challenging to produce (70). Since each method developed so far has its own set of advantages and limitations, further improvements in gene delivery systems are needed.

CPPs such as Integrin beta-3 fragments exhibit high affinity for cell membranes, primarily through interactions with lipid bilayers. The hydrophobic nature of this

peptide allows it to readily associate with the hydrophobic core of cell membranes, facilitating efficient translocation across cellular barriers (66). CPPs can overcome some of the limitations of viral vectors and liposome-based delivery systems. The properties of CPP not only allow for effective cellular internalization but also offer potential solutions to challenges encountered with viral vector-based gene delivery systems (71).

CPPs are used successfully to facilitate the delivery of protein-based therapeutics in pathological conditions, such as cancer, inflammatory diseases, oxidative stress-related disorders, diabetes, and brain injury (72). However, protein stability relies on weak non-covalent interactions between secondary, tertiary, and quaternary structures, which must be preserved throughout the delivery process, for their activity and their stability to proteases (73). To improve the efficiency of the delivery of proteins into cells, different types of lipid- and polymer-based vectors including liposomes, microparticles, and nanoparticles have been used, but their efficiency was relatively poor (74). Increasing the efficiency of CPPs, as well as optimizing their gene delivery features, is an important area of research for the advancement of gene therapy. However, CPPs lack nucleic acid-binding amino acid residues and NLS (75).

1.9.3 Using CPPs in mRNA vaccine developing

One of the key challenges in vaccine development is the efficient delivery of genetic material to the target cells, especially antigen-presenting cells (APCs) like dendritic cells. CPPs, due to their cell-penetrating properties, aid in overcoming cellular barriers, allowing the mRNA to enter cells more effectively. This enhanced delivery contributes to an increased expression of the encoded antigen, leading to a stronger immune response. A notable example of CPPs in mRNA vaccine technology is the incorporation of arginine-rich peptides. These peptides contain a high proportion of positively charged amino acids, facilitating interaction with negatively charged cell membranes and promoting cellular uptake. Studies have demonstrated the successful use of arginine-rich antisense oligonucleotides CPPs in improving the delivery and

efficiency of mRNA vaccines, with enhanced antigen expression and subsequent immune responses (76).

By enhancing cellular uptake and bioavailability, CPPs may enable the use of lower vaccine doses while maintaining or even improving efficiency. This can be particularly advantageous in reducing production costs and mitigating potential side effects associated with higher doses (77). Also, CPPs can be modified to include targeting sequences, allowing for the specific delivery of mRNA vaccines to certain cell types or tissues. This targeted approach enhances the precision of vaccine delivery and minimizes off-target effects (78).

Lipid nanoparticles, including liposomes, are also being extensively studied in the development of mRNA vaccines against infectious diseases and cancer (79). Liposome-encapsulated mRNA vaccines have shown efficiency in inducing both humoral and cellular immune responses (80). However, for mRNA vaccines, using CPPs might simplify the formulation process compared to more complex lipid-based systems, potentially reducing production costs (81).

2 BACKGROUND

2.1 Gene Delivery Studies

Gene delivery is a crucial process in gene therapy, a medical field aiming to treat diseases by inserting, altering, or silencing specific genes in a patient's cells. This approach emerged in the late 20th century, offering potential cures for genetic disorders, certain cancers, and other illnesses by fixing faulty genes or introducing new functions into cells. Researchers have been developing effective gene delivery systems for decades to overcome biological barriers. While traditional viral vectors have been highly effective, they come with risks like immune reactions and genetic changes. This has led to the development of safer non-viral vectors, such as lipid-based nanoparticles, Cell penetrating Peptides and polymer complexes. These alternatives offer flexibility, scalability, and lower risk of immune responses, but improving their effectiveness and ability to enter cells remains a major focus of research. Understanding how these delivery systems interact with biological targets is crucial for maximizing their potential in medical treatments. This progress promises personalized and precise gene therapies that could revolutionize modern medicine.

The concept of gene delivery dates back to the 1970s when researchers first attempted to introduce foreign DNA into cells using physical methods such as microinjection (82).

In the 1980s, viral vectors emerged as a promising approach for gene delivery due to their natural ability to infect cells and deliver genetic material (83).

Non-viral methods, such as liposomes, polymeric nanoparticles, and electroporation, gained attention due to their safety and versatility in the 1990s (40). The development of CRISPR/Cas9 technology in the 2010s revolutionized gene editing but also necessitated efficient delivery methods to target specific tissues and cells (84).

Recent advancements in Targeted Delivery and Clinical applications focus on enhancing targeted delivery to specific tissues or cells, as well as translating gene therapy techniques into clinical applications (85).

In the late 20th century, the discovery of cell-penetrating peptides (CPPs) marked a pivotal advancement in gene delivery technology. CPPs, such as the prototypic TAT peptide derived from HIV-1, demonstrated the ability to traverse cell membranes efficiently and deliver various cargoes, including nucleic acids, proteins, and nanoparticles. This discovery sparked a surge of research into CPP-based delivery systems, aiming to harness their remarkable cell-penetrating properties for safe and effective gene therapy applications. Since then, CPPs have been extensively studied and modified to enhance specificity, efficiency, and safety, paving the way for their integration into diverse biomedical applications, including targeted gene delivery strategies.

2.2 Recent Findings and Contributions in Gene Delivery by Cell-Penetrating Peptides

Here are some of the recent findings and contributions in gene delivery studies, especially delivery by cell-penetrating peptides. El-Andaloussi et al. (86) developed a CPP named M918 to deliver proteins and peptides into cells efficiently. The results illustrated that M918 exhibits remarkable cell-penetrating properties, displaying high efficiency and stability in providing a wide range of proteins and peptides into different cell types. The peptide displayed efficient internalization and no significant cytotoxicity, making it a safe and viable option for cellular delivery applications. In another study, Kevin et al. validated CPPs as potential molecules for improving the cytosolic delivery of therapeutic oligonucleotides in the liver, but safety remained a concern for this approach (87). A study by Kato et al. indicated that the design of novel CPPs using unnatural amino acids for plasmid DNA delivery was possible. They found that longer post-incubation led to significantly higher transfection efficiencies (88). A study by Frankel et al. (89) demonstrated the use of CPPs for the transfection of difficult-to-transfect cells, such as primary cells and stem cells. The researchers

developed a CPP-based delivery system that showed high transfection efficiency and low cytotoxicity. This system was particularly effective in delivering plasmid DNA and small interfering RNA (siRNA) into target cells, paving the way for new therapeutic approaches in gene therapy. In a 2023 study, Zhang et al. (90) explored the use of CPP-conjugated nanoparticles for targeted gene therapy in cancer treatment. The study focused on the delivery of therapeutic genes directly to tumor cells using CPPs conjugated with gold nanoparticles. This approach enhanced the specificity and efficacy of gene delivery, resulting in significant tumor reduction in preclinical models. The authors highlighted the potential of CPPs in improving the precision of cancer gene therapy. A recent publication by Wang et al. (91) reported the successful use of CPPs for the delivery of CRISPR/Cas9 components into mammalian cells. The study demonstrated that CPPs could efficiently transport the Cas9 protein and guide RNA into cells, facilitating precise genome editing. This method showed a high editing efficiency and minimal off-target effects, suggesting a promising tool for genetic disease treatment. A 2021 review by Gupta et al. (92) provided a comprehensive overview of non-viral gene delivery systems using CPPs. The authors discussed various CPPs and their modifications to enhance gene delivery efficiency and reduce toxicity. They emphasized the advantages of CPPs over traditional viral vectors, including lower immunogenicity and easier synthesis. The review also highlighted recent advancements in CPP design and their potential applications in clinical gene therapy. A novel study by Kim et al. (93) investigated the use of CPPs for mitochondrial gene therapy. The researchers designed a CPP that specifically targets mitochondria and demonstrated its ability to deliver therapeutic RNA into mitochondrial compartments. This approach holds promise for treating mitochondrial diseases, which are often challenging to address with conventional gene therapy methods.

3 MATERIALS AND METHODS

3.1 Equipment and Materials

The equipment used during the study is given in Table 2.

Table 2. Equipment used in the experiment

Solid-phase peptide synthesis	CEM, Liberty ™ Blue and CEM Discover ™
Razor (Rapid Peptide Cleavage System)	CEM, Liberty ™ Blue and CEM Discover ™
HPLC	Agilent
Vortex	VWR
Pure Water Device	Advantage Milli-Q
CO_2 Incubator	Thermo Scientific Thermo Scientific
Biosafety Cabin	Thermo Scientific Thermo Scientific
pH meter	Thermo Scientific Thermo Scientific
Centrifuge for 15-50mL falcons	Thermo Scientific Thermo Scientific
Centrifuge for Eppendorf	Thermo Scientific Thermo Scientific
Magnetic stirrer	Thermo Scientific Thermo Scientific
Autoclave	Nüve / Witeg
Sonicator	Isolab
Optic microscope	ZeissLab
Fluorescence Microscopy	ZEISS, Germany
Transmission Electron Microscope	Thermo Fisher Scientific TALOS L120 C
-20^{oc} Freezers	Kirsch Froster
-80 Freezers	Kirsch Froster
HPLC	Agilent

The chemicals used during the study are given in Table 3.

Table 3. The chemicals used in the experiment

Fmoc(9-fluorenylmethoxycarbonyl)-D-Amino acids	Sigma-Aldrich
Oxyma	Sigma-Aldrich
Rink Amide Resin	Sigma-Aldrich
Acetonitrile	Sigma-Aldrich
Dichloromethane	Merck
Diethyl ether	Carlo Erba
NN- Dimethry|formamide(DMF)	Carlo Erba
N.N. Dissopropylearbodiimide(DCM)	Merck
Piperidine	Merck
Trisopropylsilane (TIS)	Across
Trifluoroacetic acid (TFA)	Sigma
Dulbecco's Moditied Eagle Medium (DMEM)	Gibco
Fetal Bovine Serum (FBS)	Thermo Sei
penicillin-streptomycin	Gibco
Thypsin EDIA %25	Thermo Sei
Phosphate Buffered Saline (PBS)	GeneMarkBio
DMSO	Merck
Mueller Hinton (MH) agar	Merck
Mueller Hinton (MH) broch	Merck
Tris Acetate EDTA buffer (TAE)	Merck
Ampicillin	GeneMarkBio
OPTI-MEM	Gibco
Lipofectamine™ 2000 Transfection Reagent	Thermo Fisher Scientific
Luria-Bertani (LB) broth	Merck
Luria-Bertani (LB) Agar	Merck

The cells used during the study are given in Table 4.

Table 4. The cells used in the experiment

Escherichia coli DH5-alpha competent cells	Thermo Fisher Scientific
HaLa Cells CCL-2™	ATCC

3.2 Methods

3.2.1 The hypothesized mechanism of peptosome formation

In this study, we developed a novel gene delivery vector. This vector is a hybrid peptide with CPP residues on one side and a segment of human histone H4 on the other side that binds to DNA and investigates the gene delivery efficiency of this peptide into cells. The novelty of our study is related to the presence of Histone 4 protein.
We hypothesized that the histone segment will bind to DNA and these peptides will cover a supercoiled plasmid DNA to form a liposome-like structure. The cell-penetrating part of the peptides that will position outside will start endocytosis when they contact a cell (Figure 4).

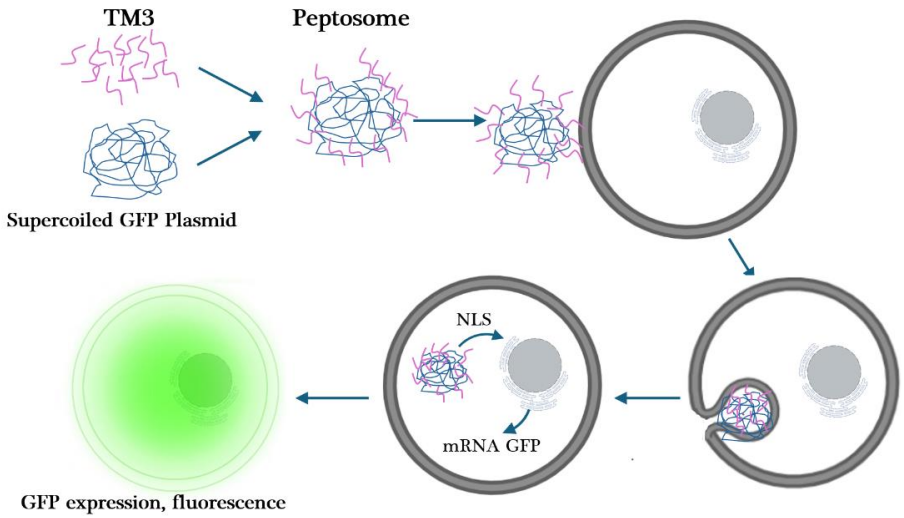

Figure 4. The hypothesized mechanism of peptosome formation, the delivery of DNA into the cell and then to the nucleus.

3.2.2 The Design of TM hybrid peptides

Gene delivery systems must be stable enough in blood vessels to reach the intercellular site of action to achieve a therapeutic effect in target cells. Once there, they need to attach to the cell membrane, enter the cell, escape from endosomes into the cytoplasm, and release the therapeutic gene from the vehicle material. Additionally, if plasmid DNA (pDNA) is delivered, it must diffuse through the cytoplasm and enter the nucleus. Therefore, understanding the properties of gene vehicles and delivery systems is crucial for designing an effective gene delivery system with sufficient transfection efficiency.

3.2.2.1 CPP selection

According to our hypothesis, we selected the cell-penetrating peptides from the category of hydrophobic peptides specified in Table 1. Amino acids found in CPP peptides in this category facilitate binding our hybrid peptide to the cell membrane. For this purpose, we selected the Integrin beta-3 fragment containing the amino acids VTVLAGALAGVGV and the Grb2 (SH2 region) containing the amino acids AAVLLPVLLAAP.

3.2.2.2 Histone protein selection

Histones are core proteins that help in the packaging of DNA into chromatin, and they include several types such as H2A, H2B, H3, and H4 (Figure 5). Each type has specific variants that contribute to the regulation of gene expression (Table 5). The H4 protein is the smallest Histon protein, and it also has special positively charged amino acids. These two features facilitate the binding of negatively charged DNA to the histone portion of the hybrid peptide which is richer in arginine and lysine from the N-terminal 12^{th} to 20^{th} amino acid containing GKGGAKRHR (Figure 6).

Table 5. The types and properties of Histone proteins. The table provides details on the molecular weight, the number of amino acid residues, and the content of basic amino acids (lysine and arginine) for different histones

Histone	Molecular Weight	Number of Amino Acid Residues	Content of Basic Amino Acids (% of total)
H1*	21,130	223	Lys:29.5, Arg: 1.3
H2A*	13,960	129	Lys:10.9, Arg: 9.3
H2B*	13,774	125	Lys:16.0, Arg: 6.4
H3	15,273	135	Lys: 9.6, Arg: 13.3
H4	11,236	102	Lys:10.8, Arg: 13.7

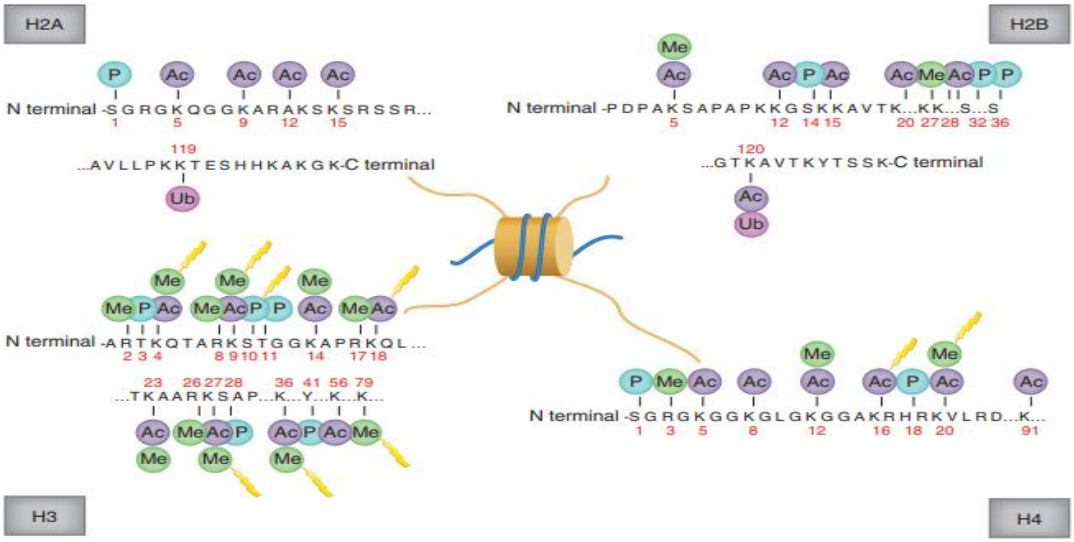

Figure 5. The sequences of various Histone proteins

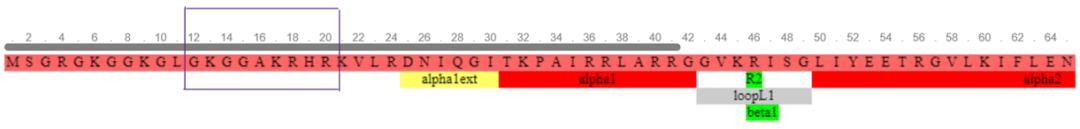

Figure 6. Histon H4 protein sequence

Eight peptides named TM1 to TM8 were designed by combining distinct amino acid residues from various CPPs with part of the histone H4 protein, which is richer in positively charged amino acids that bind to DNA (Table 6).

The sequences written in bold were taken from histone H4 protein. TM1 contains a fragment of integrin beta-3 with 13 amino acids bound to H4. TM2 and TM3 contain different shorter parts of beta-3 integrin. TM4 consists only of a part of histone H4 without a leading CPP. TM5 consists of a fragment of integrin beta-3 without a histone H4 component. TM6 uses the growth factor receptor-bound protein SH2 domain as a leading CPP. TM7 and TM8 are different versions of TM3 featuring changes in the CPP leading region.

Table 6. Properties of synthesized peptides

TM peptides	Sequences	Molecular Weights (g/mol)
TM1	VTVLAGALAGVGV**GKGGAKRHR**	2074.46
TM2	VTVLAG**GKGGAKRHR**	1506.77
TM3	VGV**GKGGAKRHR**	221.43
TM4	**GKGGAKRHR**	966.11
TM5	VTVLAGALAGVGV	1126.36
TM6	AAVLLPVLLAAP**GKGGAKRHR**	2095.57
TM7	LGL**GKGGAKRHR**	1249.48
TM8	LGV**GKGGAKRHR**	1234.74

3.2.3 Synthesis and purification of TM hybrid peptides

Peptide synthesis was performed using a peptide synthesizer with the standard Fmoc protocol at Acibadem University. The Liberty™ Blue device can accommodate 20 natural amino acids and 5 unnatural amino acids. During the synthesis, the temperature in the reaction vessel was maintained at 50 °C, controlled via microwave energy.

Generally, in Solid Phase Peptide Synthesis (SPPS), peptides were constructed on resin from the C-terminal to the N-terminal. The resins utilized in this study included Rink Amide resin (0.70 mmol/g loading capacity) for synthesizing peptides with an amide end and Cl-TCP (Cl) ProTide resin (0.42 mmol/g) for synthesizing peptides with a carboxyl end. To avoid undesired reactions, the amino acids employed in this process had protective groups.

Before synthesis, the resins were swelled in DMF for 1 hour. In the initial step of the synthesis cycle, the first amino acid (0.2 M in DMF) was attached to the resin through its C-terminal (Figure 7). To add the second amino acid, piperidine (20% in DMF) was used to deprotect the Fmoc group on the N-terminal of the first amino acid. The carboxyl group of the second amino acid was activated using Oxyma® (1.0 M in DMF) and DIC (0.5 M in DMF). In the final step of each cycle, the N-terminal protecting group of the most recently added amino acid was removed as previously described.

Cleavage

Upon completion of the synthesis, the resin was transferred to the Razor (Rapid Peptide Cleavage System) device and washed three times with DCM before cleavage. The peptides were cleaved from the resin using a solution of TFA / H2O / TIS (95 / 2.5 / 2.5, v/v/v) at 37°C. During this process, the side chain protecting groups of the amino acids were also removed under the same conditions. The cleavage time varied between 30 and 45 minutes, depending on the number of arginine residues in the peptide. For peptides containing three or fewer arginine residues, the cleavage time was 30 minutes. For peptides with more than three arginine residues, an additional 5 minutes was added for each arginine, up to a maximum of 45 minutes.

The cleaved peptides were collected using cold diethyl ether (-20°C) and centrifuged for 3 minutes at 3500 rpm, which precipitated the crude peptide, free from chemicals. This precipitation step was repeated three times. Finally, the precipitated peptides were dried under nitrogen for further purification (Figure 7).

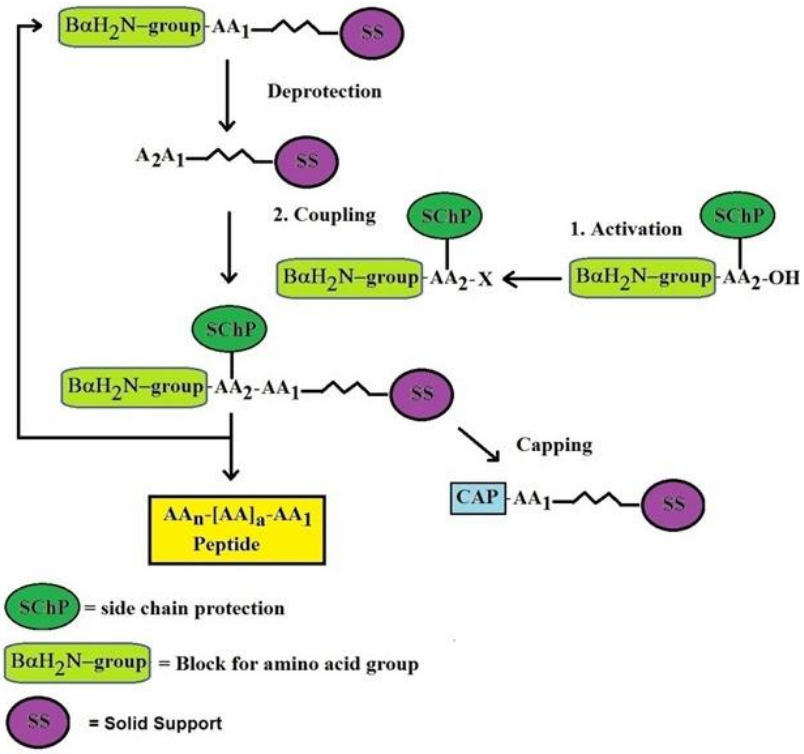

Figure 7. General Procedure for Solid Phase Peptide Synthesis

Purification:

The peptides were purified using an Agilent Technologies 1260 Infinity HPLC system. UV absorption was detected at 214 nm and 280 nm. Analytical HPLC was conducted at a flow rate of 0.2 ml/min, while semi-preparative HPLC was performed at a 1 ml/min flow rate. Two mobile phases were used: Eluent A was 0.05% TFA in H2O, and Eluent B was 0.25% TFA in acetonitrile. The synthesized peptides were analyzed over 30 minutes using a linear gradient ranging from 5% to 80% B. The gradient of the solvent was adjusted appropriately for each compound. Peptides were separated by their peaks on the HPLC, frozen at -80°C.

3.2.4 Nucleic acid preparation

pcDNA3-EGFP, which expresses green fluorescent protein (Enhanced GFP) when introduced into cells, was used to investigate the efficiency of TM peptides in the cellular delivery of DNA molecules.

pcDNA-EGFP is a mammalian expression vector commonly used in molecular biology for the expression of the Enhanced Green Fluorescent Protein (EGFP). The vector contains a strong promoter, typically the cytomegalovirus (CMV) promoter, which drives high levels of gene expression in a wide variety of mammalian cells. The EGFP gene, a mutant of the original green fluorescent protein (GFP) from the jellyfish "Aequorea victoria", exhibits enhanced fluorescence and stability, making it an excellent reporter gene. This plasmid also includes features such as a high-copy-number origin of replication for amplification in *E. coli*, and a selectable marker, usually an antibiotic resistance gene, for the selection of transfected cells. The pcDNA-EGFP vector is widely utilized for studying gene expression, protein localization, and cellular processes due to the visibility and quantifiable nature of the EGFP fluorescence (Figure 8).

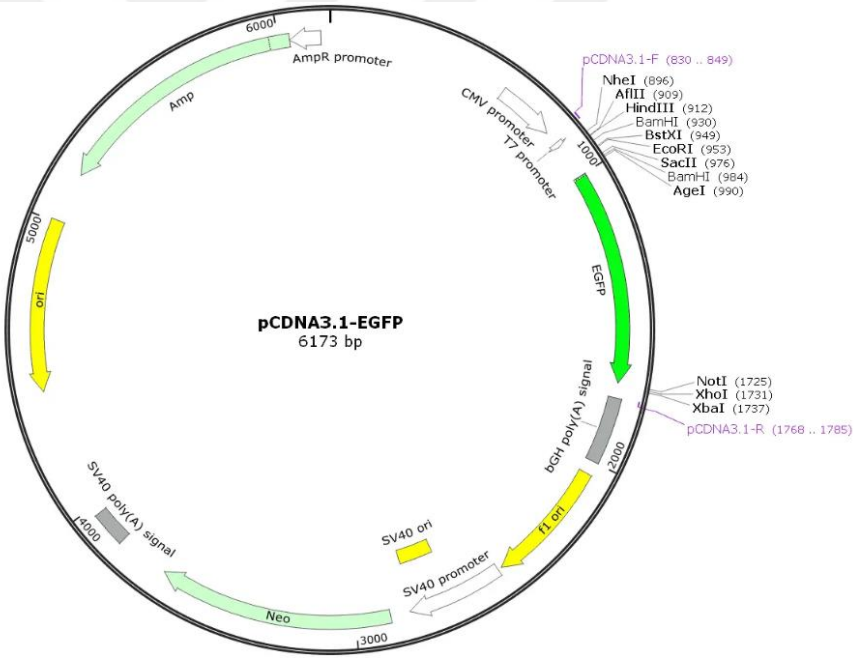

Figure 8. The plasmid map of the pcDNA-EGFP vector. Key features include the ampicillin resistance gene for bacterial selection, the neomycin resistance gene for selecting transfected mammalian cells, and the EGFP gene for expressing green fluorescent protein

3.2.4.1 Transformation of competent *E. Coli* DH5α by pcDNA-EGFP

To transform competent DH5α *E. Coli* with the pcDNA-EGFP plasmid, 1-5 µl plasmid was mixed with 200 µl competent cells (1-5 µl usually 500 pg-100 ng) in a microcentrifuge tube. The pUC19 control DNA was used as a control. The mixtures were incubated on ice for 30 min, followed by heat shocking for 90 s in a 45°C water bath. The tubes were returned to the ice for 2 min, then added to antibiotic-free LB broth medium and incubated at 37°C for 45 min with shaking. The cells were centrifuged briefly, and the pellet was resuspended in 100 µl of medium. The suspension was inoculated onto LB agar plates with appropriate antibiotics. The plates were incubated at 37°C for 16-18 hours (Figure 9).

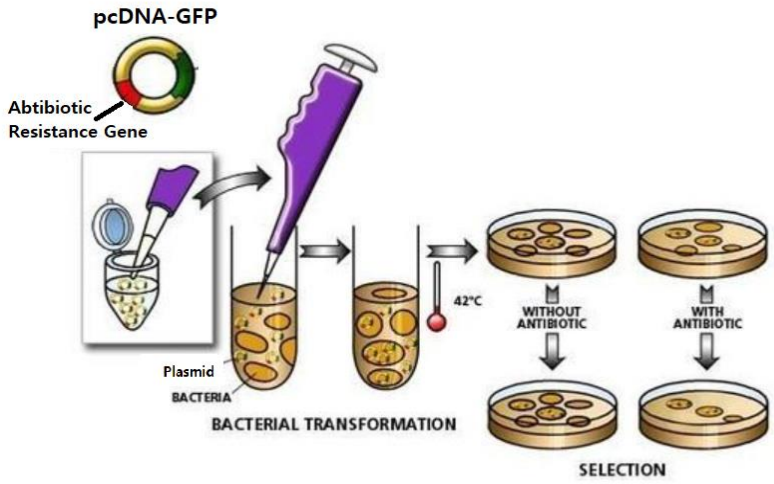

Figure 9. Transformation of Competent *E. coli* cells

3.2.4.2 Isolation of pcDNA3-EGFP plasmid by alkaline lysis method

For this purpose, the pcDNA3-EGFP plasmid was isolated by Alkaline lysis method. 2-3 µL of stock containing the transformed *E. coli DH5α strain* with an EGFP plasmid, previously stored at -80°C, cultured at the LB Agar culture medium with 100 mg/ml Ampicillin and was incubated at 37°C for 16-20 hours. One single colony of Overnight culture was added into an LB broth culture medium containing 100 mg/ml Ampicillin. The culture was incubated overnight at 37°C.

Alkaline lysis protocol was used to isolate bacterial plasmid DNA. Three different solutions are used as follows: solution I (resuspension buffer), solution II (lysis buffer), and solution III (neutralizing buffer). Solution I: For 200 mL, 1.212g Tris base and 0.75g EDTA were dissolved in 160 mL of distilled water and the pH was adjusted to 8.0 with HCl. The final volume was adjusted to 200 ml. Solution II was prepared fresh, by dissolving 0.8g of NaOH in 95 mL of distilled water and 5 mL of 20% SDS with a total volume of 100 ml. For solution III: For 100 mL, 29.45g of potassium acetate was dissolved in 50 mL of distilled water. The pH was adjusted to 5.5 with glacial acid and the final volume was increased to 100 mL with ddH_20. 100 µg/mL of RNAse A was added inside the resuspension buffer. The overnight grown cultures were centrifuged for 1 min at 14,000 rpm. After centrifuge, the supernatant was discarded, and the bacterial pellet was dissolved in 200 µL of Solution I. The samples were vortexed until the pellet was completely dissolved. After resuspending the pellet, 200 µL of solution II was added to the tubes and the samples were mixed by carefully inverting the tubes a few times. Then 200 µL of solution III was added to each tube and then the samples were centrifuged for 10 min at 14,000 rpm. After the centrifuge, it was seen that the samples still contained some white pellets in the mixture, therefore the samples were centrifuged again for 10 minutes at 14,00 rpm. When the centrifuge was complete, 600 µL of the supernatants were transferred to fresh tubes, and the white pellets were discarded. 900 µL of cold 100% EtOH was added to the new tubes containing the supernatants and after this, the samples were mixed by inverting the tube a few times and then centrifuged for 20 min at 14,000 rpm. After the centrifuge, the supernatants in each tube were discarded and then the tubes were left for air drying until all the EtOH evaporated. Finally, the samples were resuspended in 45 µL sterile ddH_20 and stored at -20 $^{\circ C}$. The average plasmid DNA Concentration (ng/µL) was measured.

3.2.5 Preparation of TM peptide/plasmid DNA complexes (peptosome)

Each TM peptide was dissolved separately in deionized water, and several 10-fold dilutions were prepared for plasmid and TM peptides. Different examinations were performed to obtain final efficient concentrations.

After 5 mg of all peptides were taken separately and dissolved in 1 ml of pure water, the transfection efficiency of different dilutions was up to 10^{-6}. The transfection efficiency for the best dilution of each peptide was tested by using different volumes of 10, 20, 50, 100, and 200µl of each dilution in the mixture. Each peptide was tested three times to ensure reliability and reproducibility.

3.2.6 Imaging of peptosomes by transmission electron microscopy (TEM)

The formation of peptosomes when plasmid DNA was mixed with hybrid peptides was analyzed by TEM.

Each sample (20 µL) was deposited on parafilm, a grid was placed on, and waited for 2-3 hours until drying. Then to create contrast, one drop of tannic acid solution (Uranyless), was dropped on the sample. After waiting for 5 minutes, the samples were washed by dipping 20 times in distilled water in 3 separate beakers and left to dry for one night. Peptosomes, along with TM3 and pcDNA3-EGF were imaged by Transmission Electron Microscopy.

3.2.7 Transfection

HeLa cells (CCL-2™, ATCC) were seeded in 24-well culture plates ($40\text{-}45\times10^4$ cells/well) and incubated overnight in 1 mL of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin stock solution at 37°C. The following day, the medium was aspirated, and the cells were carefully washed two times with PBS 1X at 24-hour intervals. The next day, the cells were used when they reached 85%–90% confluency.

Several distinct transfection processes were conducted for optimization, and the details of each transfection (including the average cell count, number of cells plated, number of wells used, and the total and used volumes of cell suspension and cDMEM) are documented.

The experimental groups contained 10^{-1} to 10^{-6} dilutions of both GFP plasmids stock with 300 ng/µL concentration, and TM peptides, were prepared in 10, 20, 50, 100, and 200 µL. 5 mg of TM peptides were dissolved in 1 ml demonized water before making dilutions. To optimize transfection efficiency by TM peptides we have prepared mixtures of TM peptides and pcDNA3-EGFP with different ratios and concentrations and they were tested individually in separate reactions with one group kept constant and the other diluted to find the optimal concentration. Different transfection parameters such as DMEM, Opti-MEM amount, and Pptosome incubation time were optimized for each study.

An example is the transformation planning of GFP plasmid with TM3 peptide and Lipofectamine in a 24-well plate (Figure 10). GFP Plasmid was used at a concentration of 300 ng/µL and diluted to 10^{-4}. TM3 Peptide used was diluted to 10^{-5}.
For optimization, various dilutions of plasmid and peptide were tested in previous experiments. According to Table 7, different amounts of plasmid and TM3 peptide were mixed and incubated for 15 minutes at room temperature. Finally, 100 µL of the total amount of the mixture was added to 900 µL of OptiMEM to reach a total of 1 ml for each well plate containing 40-45×10^4 cells/well Hela cells.

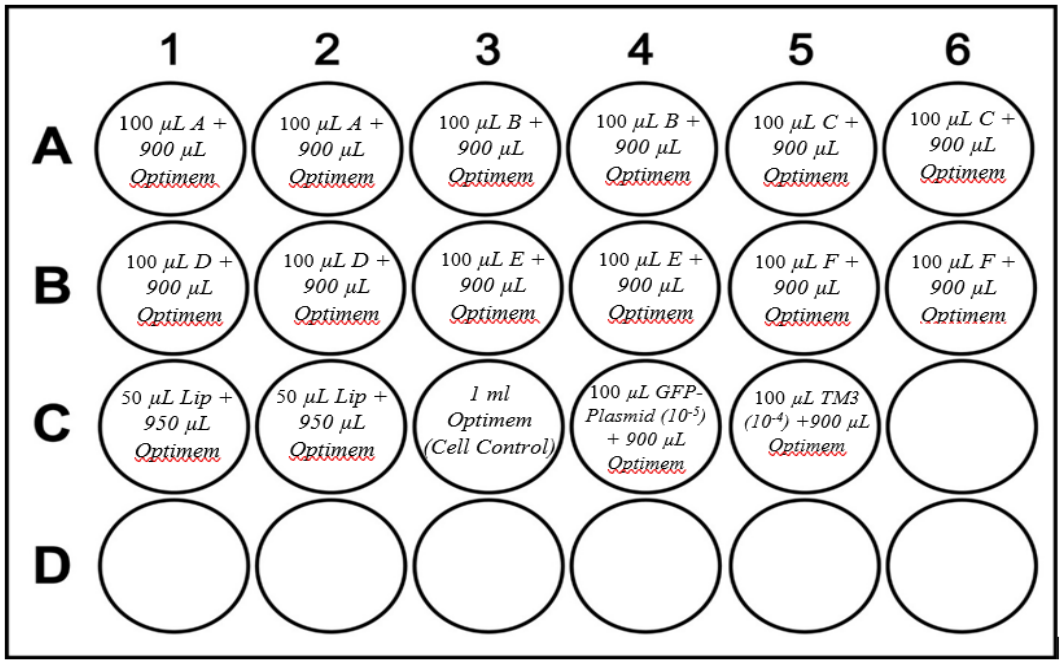

Figure 10. Planning of transfection by TM3 peptide

Table 7. Optimization of transfection of TM3 peptide

Sample	A (µL)	B (µL)	C (µL)	D (µL)	E (µL)	F (µL)
pcDNA3-EGFP Plasmid	100	100	100	100	100	100
TM3 Peptide	10	20	50	100	150	200

The best transfection efficiency was obtained by mixing 100 µL of each sample. So, the final concentration is 1.3 pM for pcDNA3-EGFP and 200 pM for TM3 peptide. As a result, it made a ratio of 154 TM3 molecules per plasmid.

Also, control wells were filled with 1 mL of Opti-MEM, and Lipofectamine control wells were prepared according to the protocol in Table 8 The preparation of Lipofectamine involved two initial mixtures as given in the protocol of "Invitrogen™ Lipofectamine™ 2000 Transfection Reagent, Catalog number: 11668019- Thermo fisher scientific": Mixture 1, containing 4 µL of Lipofectamine 2000 and 50 µL of Opti-MEM, and Mixture 2, containing 250 µL of Opti-MEM and 80 µL of GFP plasmid (300 ng/µL) which diluted to 10^{-1}. These two mixtures ' equal volumes (50 µL) were combined in a microcentrifuge tube and thoroughly mixed by pipetting. Then, 50 µL of this combined mixture was added to the Lipofectamine control wells, along with 950 µL of Opti-MEM.

Table 8. Lipofectamine™ 2000 Transfection Reagent preparation

Materials	**Mixture 1 (µL)**	**Mixture 2 (µL)**
Lipofectamine 2000	4	-
Opti-MEM	50	250
GFP-Plasmid (10^{-1})	-	80
Total Amount Taken for Transfection:	50	50

3.2.8 Image processing and analysis of cells

The cells were observed under a fluorescence microscope with a GFP filter at 24 and 48 h with excitation at 470 nm and emission at 525 nm to investigate the

transformation of cells and expression of GFP. Transfection efficiency was evaluated by comparing the fluorescence image of the cells and their brightfield counterpart. Transfection efficiency was calculated by the ratio of the number of cells yielding green fluorescence signals expressing GFP to the total number of cells from the corresponding brightfield image.

4 RESULTS

4.1 Design of Synthesized TM Peptides

Eight peptides designated TM1 through TM8, were synthesized by combining specific amino acid residues from various CPPs with segments of the histone H4 protein, which is known for its high content of positively charged amino acids that bind to DNA (Table 5).

To predict the 3D structure, computational programs such as the RPBS web portal-PEPFOLD3 are used. All the structures start with red, indicating the histone H4 part. The transition from red to orange, then yellow, green, and finally blue, indicates the progression from the histone H4 part to the CPP part.

TM1 (VTVLAGALAGVGVGKGGAKRHR, 2074.46 g/mol) forms an alpha-helix to the presence of hydrophobic residues (V, L, A) and has the potential for hydrogen bonding. The positively charged K and R residues may form interactions with negatively charged DNA (Figure 11. A).

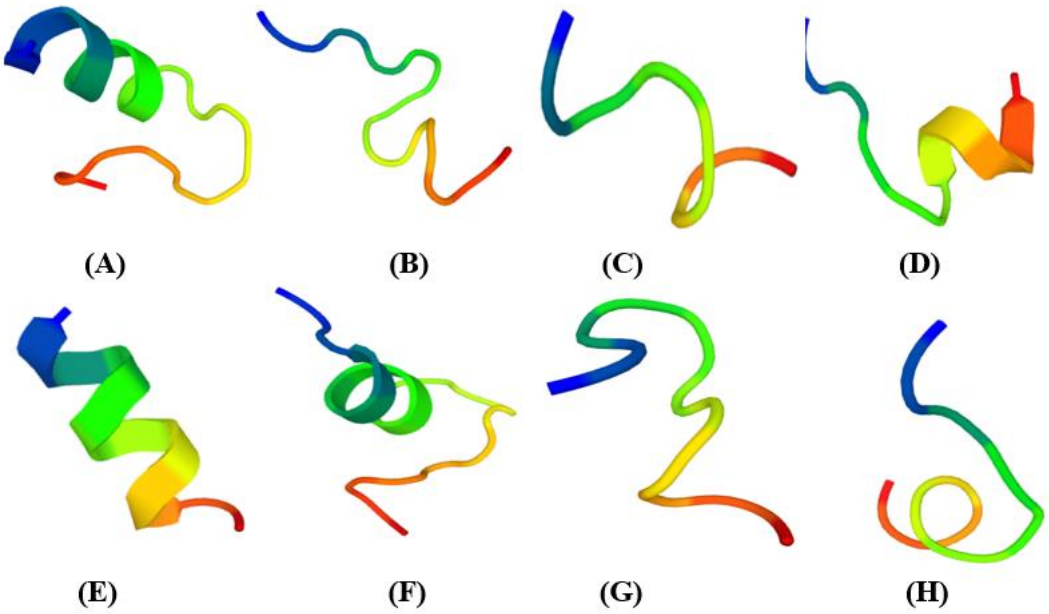

Figure 11. Three-dimensional structure of TMs. (A) TM1. (B) TM2. (C) TM3. (D) TM4. (E) TM5. (F) TM6. (G) TM7. (H) TM8.

TM2 (VTVLAGGKGGAKRHR, 1506.77 g/mol) is Similar to TM1 but shorter and potentially forms a smaller alpha-helix. The presence of G and K residues suggests flexibility and potential interactions with DNA (Figure 11. B).

TM3 (VGVGKGGAKRHR, 221.43 g/mol) is a shorter peptide, likely to be more flexible and the G and K residues suggest potential turns or loops in the structure, with R and K interacting with DNA (Figure 11. C).

TM4 (GKGGAKRHR, 966.11 g/mol) is a short peptide, that forms turns and loops, and it is rich in positively charged residues (K, R), suggesting strong DNA binding potential (Figure 11. D).

TM5 (VTVLAGALAGVGV, 1126.36 g/mol) lacks charged residues, likely forming hydrophobic interactions. It Could form alpha-helix due to its hydrophobic nature (Figure 11. E).

TM6 (AAVLLPVLLAAPGKGGAKRHR, 2095.57 g/mol) makes the combination of hydrophobic and hydrophilic regions and it can form alpha-helix or beta-sheet structures with potential loops, flexible regions due to P, and DNA interactions via K and R residues (Figure 11. F).

TM7 (LGLGKGGAKRHR, 1249.48 g/mol) forms flexible loops or turns. The presence of L and G suggests some hydrophobic and flexible regions, with DNA interactions via K and R residues (Figure 11. G).

Finally, TM8 (LGVGKGGAKRHR, 1234.74 g/mol) which is like TM7, makes loops and turns. It has hydrophobic and flexible regions (L, G) and it can facilitate DNA interactions via K and R residues (Figure 11. H).

4.2 Purification of Synthesized Peptides

The initial analysis of the TM peptides, synthesized via the SPPS method, was conducted using HPLC. The chromatogram analyses of these synthesized peptides are presented below. The x-axis represents the retention time in minutes, and the y-axis represents the absorbance units (AU) at a specific wavelength. Retention times and the corresponding percentages of solution B were determined using a linear gradient. Subsequently, the peptides were purified with a focus gradient by narrowing the scanned area of solution B%.

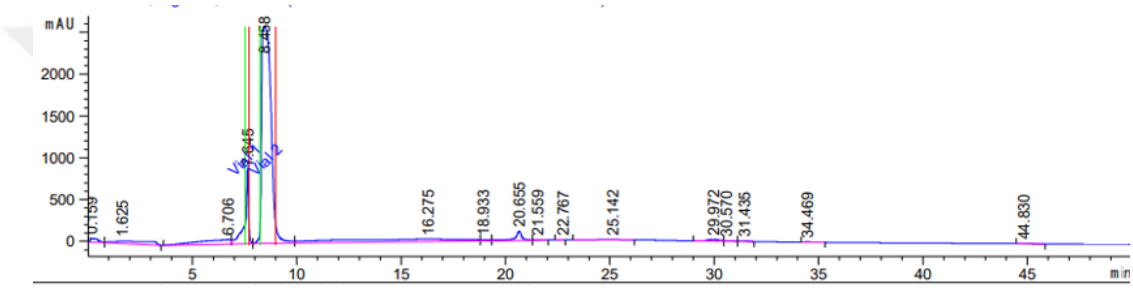

Figure 12. HPLC chromatogram of TM1 peptide

The HPLC chromatograms represent different samples, showing distinct peaks at various retention times. In Figure 12, the most prominent peak appears at 8 min with the highest intensity around 2200 mAU, alongside several smaller peaks.

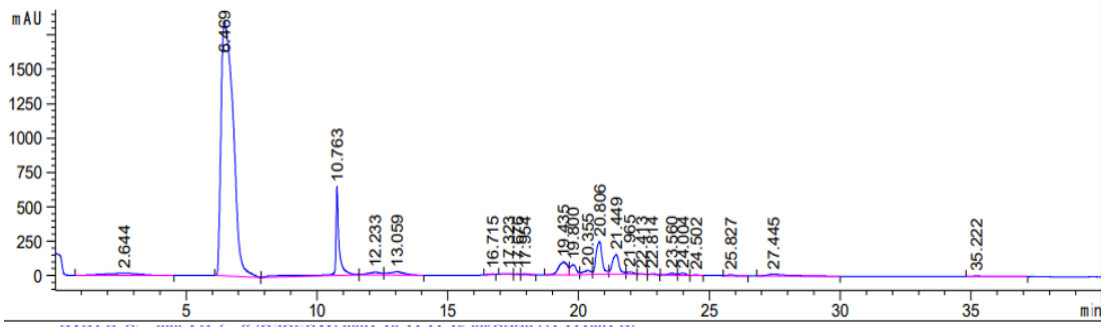

Figure 13. HPLC chromatogram of TM2 peptide

Features a significant peak between 6 to 8 min with an intensity of about 1700 mAU and additional smaller peaks are shown in Figure 13.

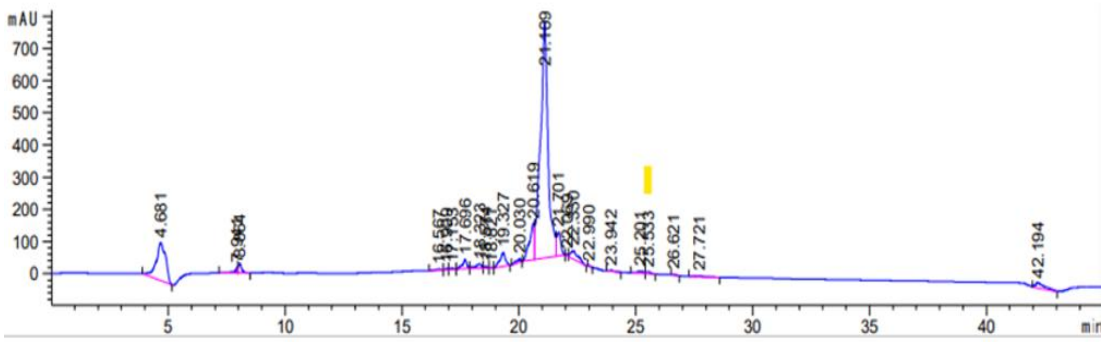

Figure 14. HPLC chromatogram of TM3 peptide

TM3 peptide was obtained by collecting the eluent from the peak between 21 and 22 min. A prominent peak is observed at this period, indicating the primary component of the TM3 peptide eluting from the column. Several smaller peaks appear before and after the main peak, suggesting the presence of impurities or other elements in the sample. The chromatogram indicates that the main peak is well-resolved, implying successful separation of the TM3 peptide from other impurities under the given HPLC conditions (Figure 14).

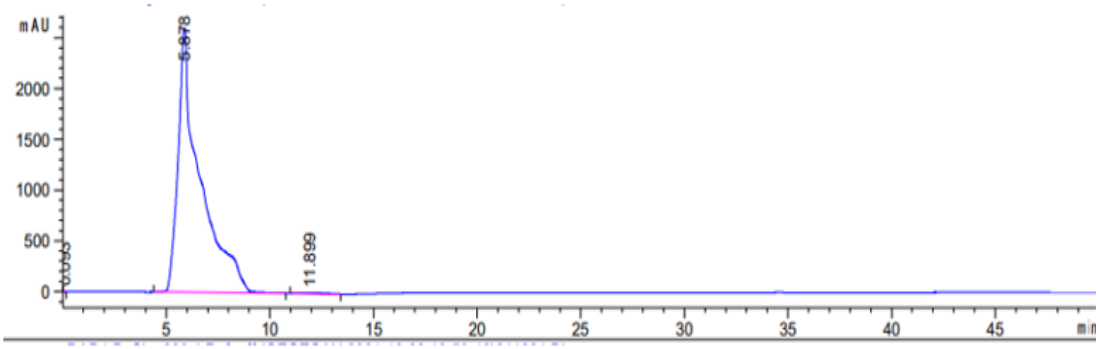

Figure 15. HPLC chromatogram of TM4 peptide

This chromatogram has a prominent peak at approximately 5.878 minutes, indicating a major component eluting. The peak has a high intensity, suggesting a significant concentration of the corresponding compound. There is also a smaller peak at about 12 minutes, indicating the presence of another compound in a lower concentration. The baseline is relatively flat beyond these peaks, indicating the absence of significant other components in the sample (Figure 15).

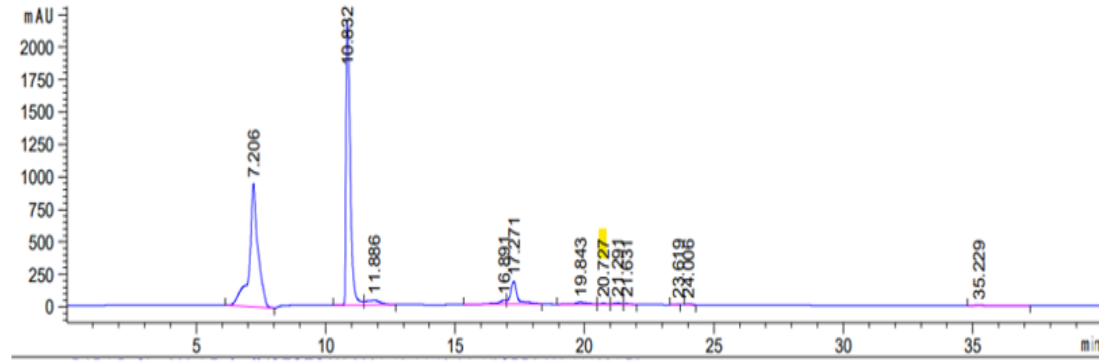

Figure 16. HPLC chromatogram of TM5 peptide

TM5 peptide chromatogram displays multiple peaks, with the most prominent at 10.892 minutes. There are additional peaks at 7.206 and 11.886 minutes. This suggests a more complex mixture with several compounds present in varying concentrations. The peak at 10.892 minutes is the highest, indicating the predominant component in the sample, while the other peaks represent minor components (Figure 16).

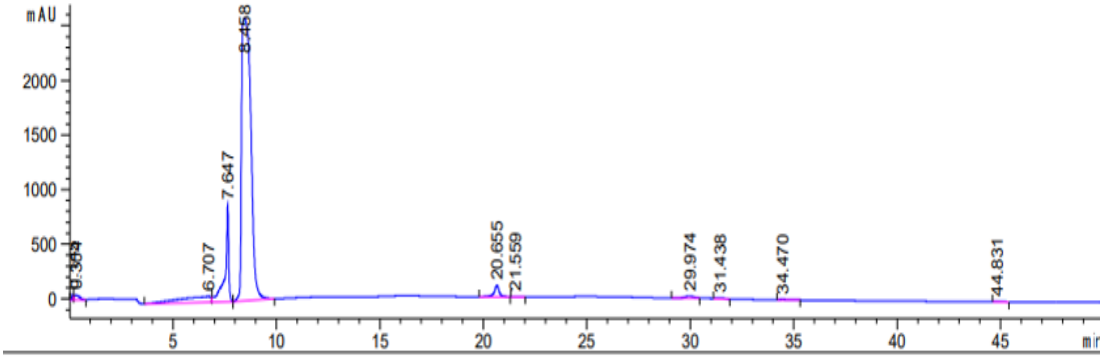

Figure 17. HPLC chromatogram of TM6 peptide

The TM6 peptide chromatogram also shows multiple peaks, with the highest peak occurring at 8 to 9 minutes. Additional peaks are observed at about 6, 7, and other times. This indicates a complex sample with multiple components, with the compound eluting at 8.458 minutes being the most concentrated. The other peaks represent various minor components in the mixture. The baseline remains relatively flat between peaks, indicating a well-separated chromatographic profile (Figure 17).

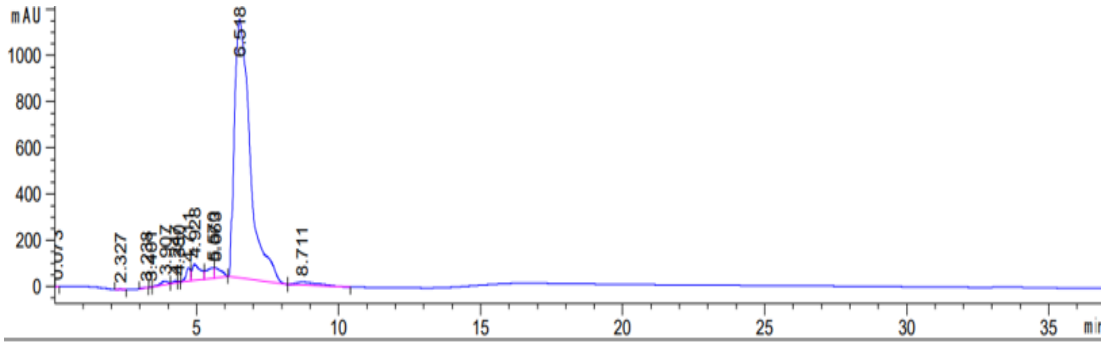

Figure 18. HPLC chromatogram of TM7 peptide

The TM7 peptide chromatogram displays multiple peaks at various retention times, with the most prominent peak appearing at 6.518 minutes, reaching approximately 1000 mAU in height. This significant peak indicates the presence of the primary compound in the sample, likely constituting the highest concentration among the detected substances. Several smaller peaks appear at different retention times. The overall baseline is stable, indicating good resolution and separation of the detected compounds (Figure 18).

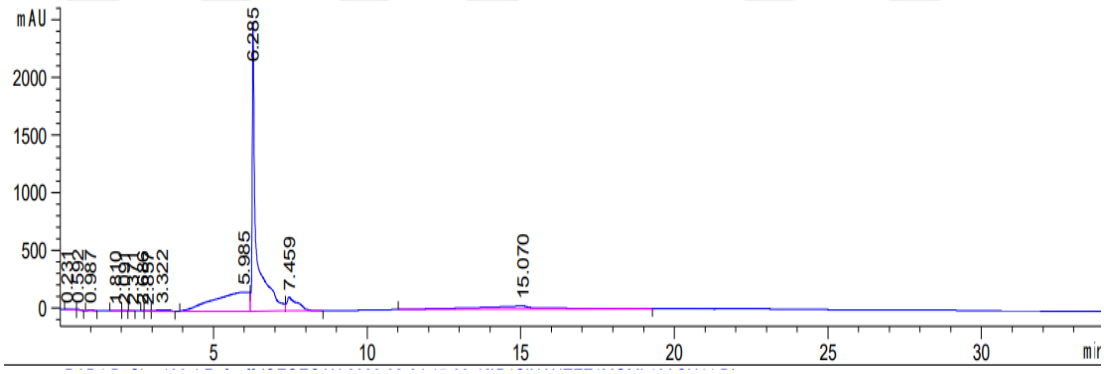

Figure 19. HPLC chromatogram of TM8 peptides

The TM8 peptide chromatogram also presents multiple peaks with the most substantial peak occurring at 6.285 minutes, reaching around 2000 mAU in height, suggesting this is the major compound present in the sample at a relatively high (Figure 19).

4.3 Peptosome Formation

The formation of peptosomes when plasmid DNA was mixed with hybrid peptides was analyzed by Transmission Electron Microscopy (TEM).

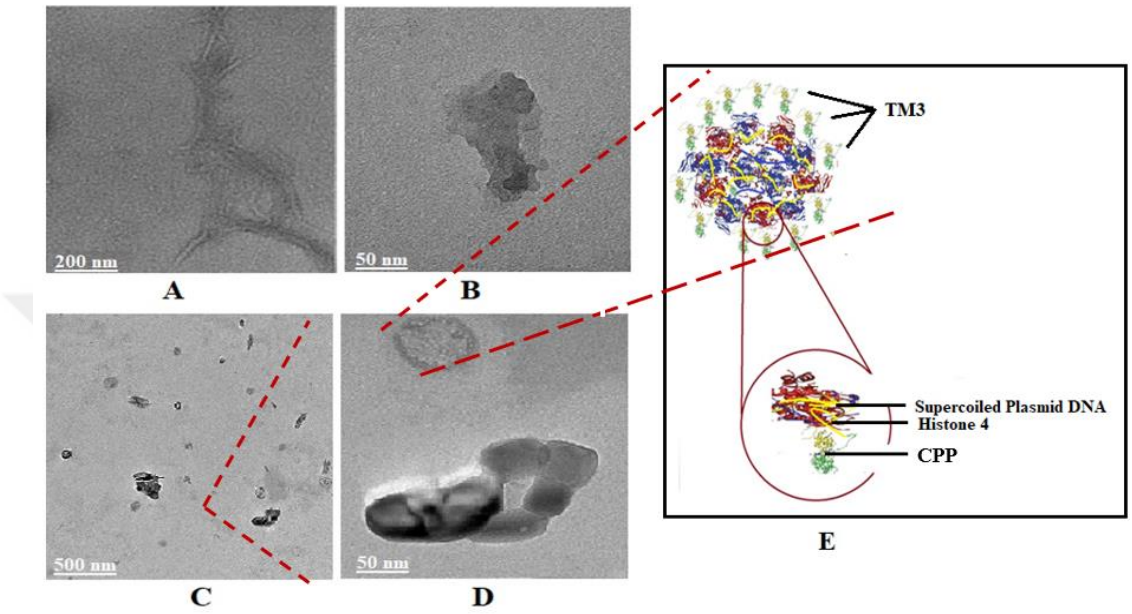

Figure 20. TEM (Transmission Electron Microscopy) image and predicted structure of peptosomes. A: Probable bundles of DNA strands; B: Probable aggregates of TM3 peptide; C: Probable supercoiled Plasmid DNA and TM3 peptide complexes (peptosomes) which is about 50 to 100nm in size; D: A group of peptosomes in higher magnification, E: Predicted structure of peptosome as supercoiled plasmid DNA surrounded by TM3 peptides.

TEM images showed the formation of peptosomes as predicted, by mixing plasmid DNA with TM3 peptide. This unique structure, consisting of an integrin beta-3 segment and a nine-amino acid region of histone H4, forms the basis of this gene delivery vector. The histone H4 domain of TM3 facilitates DNA binding, ensuring that the CPP component remains available for effective binding and penetration into cells. The binding of the histone component of TM3 to DNA positions their free CPP component outside the "peptosome," making them available for cell binding and penetration. TEM images of peptosomes indicated spherical formations with two different densities, a thinner layer covering a more massive region in the middle as can

be expected by the free CPP part of TM3 outside and the histone part bound to the surface of supercoiled plasmid DNA inside. (Figure 20).

4.4 Transfection

Transfection of cells was possible when we used peptides featuring a combination of an integrin beta-3 fragment and a nine-amino acid segment of histone H4. The expression of the GFP plasmid was observed under Fluorescence Microscopy.

Although it was possible to transfect the plasmid with TM1 and TM2 into cells, the best transfection efficiency was achieved with TM3. The transfection efficiency was better for TM3 than for Lipofectamine at both 24 and 48 h in terms of the percentage of cells transformed (Figure 22).

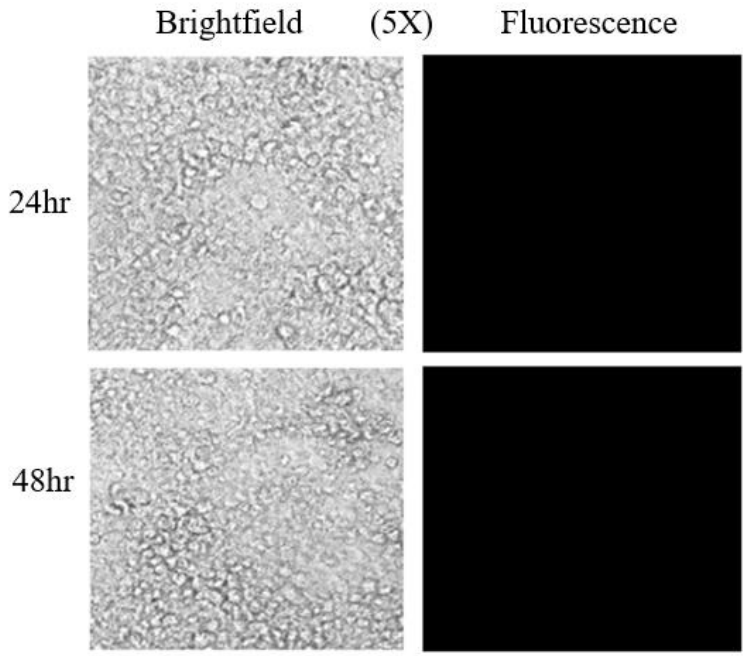

Figure 21. GFP expression in HeLa cells: Non-transfected cells

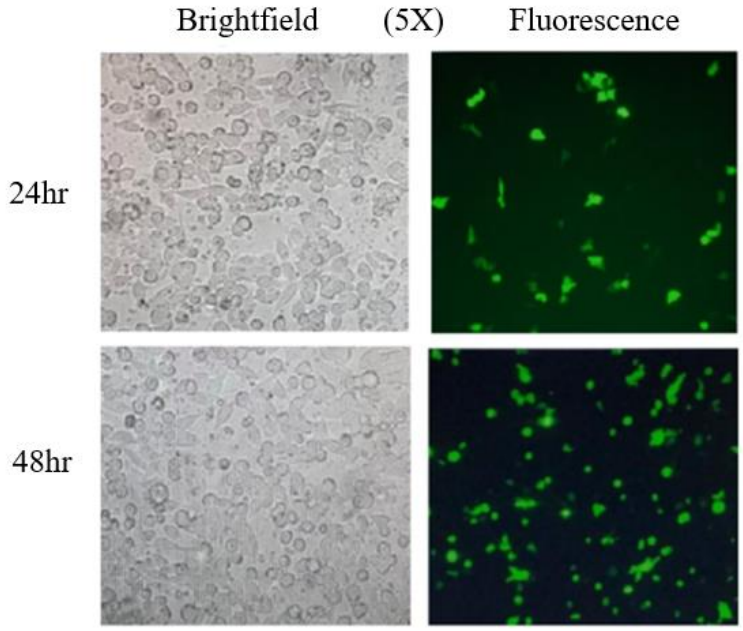

Figure 22. GFP expression (seen as green fluorescence) in HeLa cells: transfected by Lipofectamine 2000

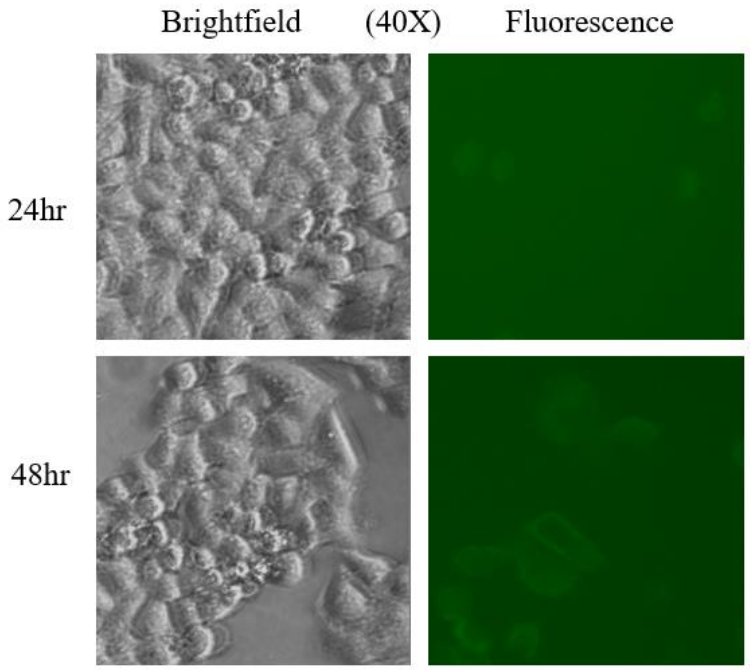

Figure 23. GFP expression (seen as green fluorescence) in HeLa cells: transfected by TM1

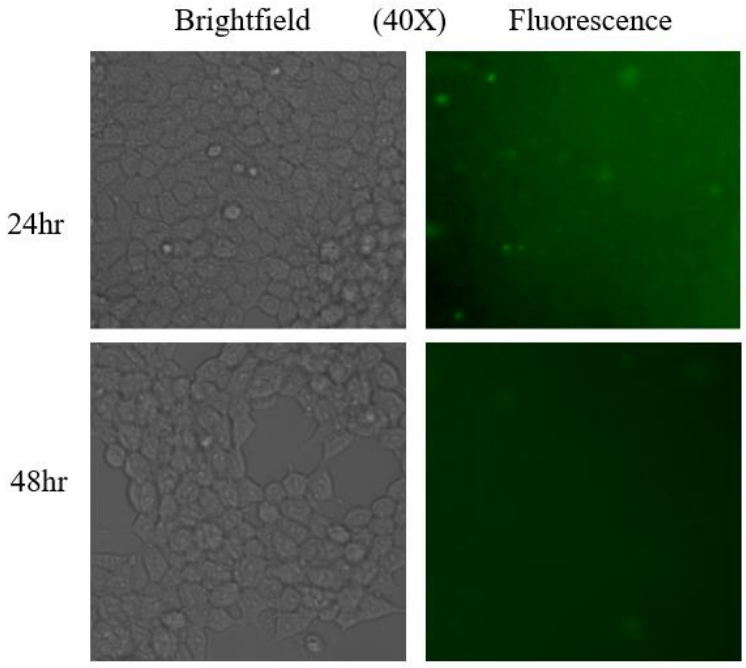

Figure 24. GFP expression (seen as green fluorescence) in HeLa cells: transfected by TM2

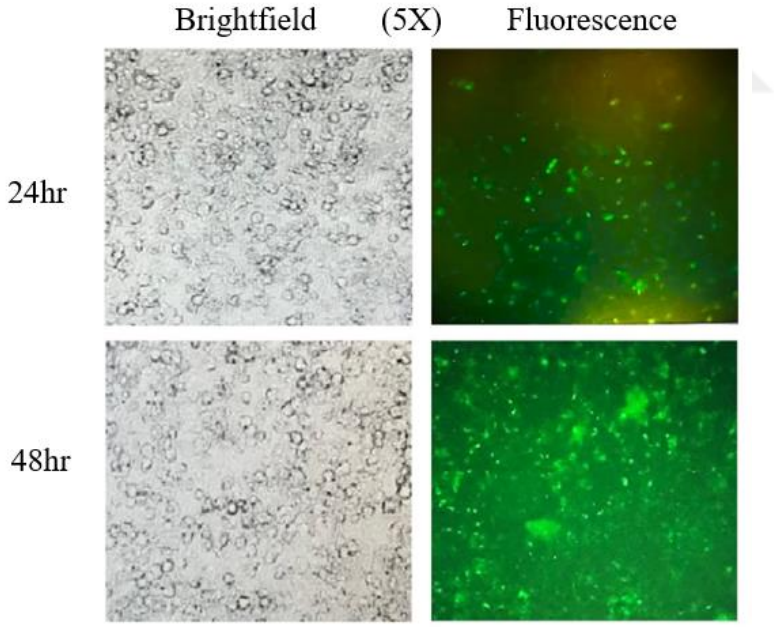

Figure 25. GFP expression (seen as green fluorescence) in HeLa cells: transfected by TM3. 5X magnification.

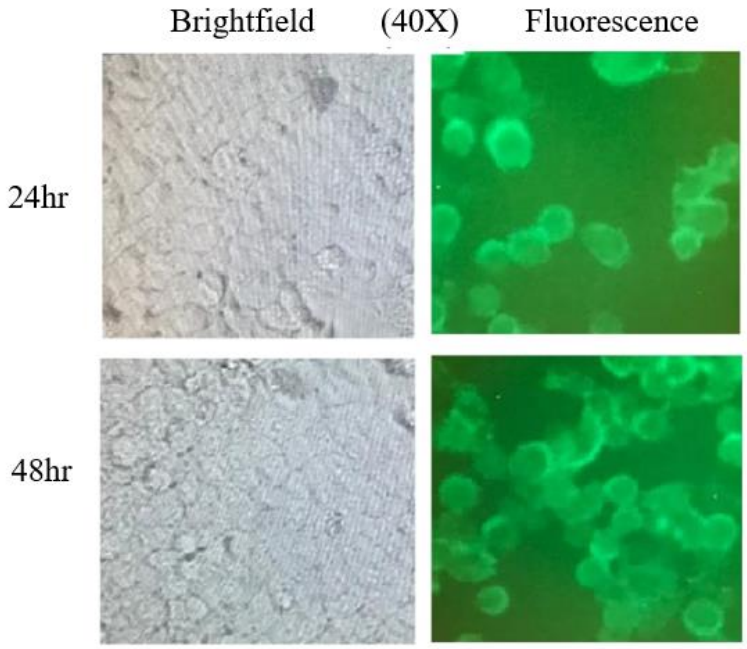

Figure 26. GFP expression (seen as green fluorescence) in HeLa cells: transfected by TM3. 40X magnification

TM4, which contained only part of histone H4, and TM5, which only contained a fragment of integrin beta 3, did not permit transfection (Figure 21). When we used the growth factor receptor-bound protein SH2 domain in place of integrin beta-3 (TM6), the transfection activity of the peptide was completely lost (Figure 21). In TM7 and TM8, the CPP component of TM3 was modified by changing one or two valine residues to leucine to improve transfection efficiency. However, these changes resulted in the complete loss of transfection activity (Figure 21). Thus, TM3 was identified as the peptide with the best transfection activity (Figure 25, 26). The resulting peptosomes, particularly those formed with the TM3 peptide, exhibited promising transfection abilities. The success of TM3 in transfection can be attributed to the strategic combination of CPP and the DNA-binding domain of histone H4. The histone domain of TM3 binds to DNA molecules, leaving the CPP part free and available for binding and penetration into cells. This unique structure forms the basis of the peptosomes, ensuring efficient gene delivery. The formation of peptosomes was confirmed through Transmission Electron Microscopy (TEM), providing visual evidence of the complexes formed between TM3 and plasmid DNA.

4.5 Transfection Efficiency

Upon repeated experimentation, it was observed that TM3 consistently demonstrated superior transfection efficiency compared to other peptides tested.

The transfection efficiency of Lipofectamine 2000 and TM3 at 24 and 48 h in HeLa cells is presented in Table 9.

Table 9. Lipofectamine 2000 and TM peptides transfection efficiency in 24hr and 48hr in HeLa cells. TM3 showed the highest transfection efficiency at 76% compared to 52% of Lipofectamine 2000.

Reagent/Time	24hr (%)	48hr (%)
Lipofectamine 2000	27	52
TM1	12	16
TM2	15	18
TM3	**52**	**76**
TM4	0	0
TM5	0	0
TM6	0	0
TM7	0	0
TM8	0	0

The percentage of transfected cells expressing GFP increased at 48 hours compared to 24 hours evaluation.

The evaluation of hybrid peptides revealed that TM3, featuring a combination of an integrin beta-3 fragment and a nine-amino acid segment of histone H4, exhibited the highest transfection efficiency. TM3 achieved transfection efficiency of 52% in 24 hours and 76% in 48 hours. These values were 27% and 52% with Lipofectamine2000 respectively.

5 DISCUSSION AND CONCLUSION

In recent years, proteomics has significantly advanced our understanding of cancer biology and provided new avenues for cancer treatment. Proteomics, the large-scale study of proteins, is essential for understanding the complexities of cancer-causing mechanisms at the molecular level as well as to produce new therapeutic tools. As a result of these studies, anticancer monoclonal antibodies became important biological drugs for cancer treatment (94, 95). Small synthetic peptides that are much easier to produce than monoclonal antibodies are also being used increasingly to develop new therapeutic regimens. Besides their direct use for treatment, they may also be used as anti-cancer drug carriers (96). In this study, we evaluated their potential for delivery of genetic material into the cells. The special design of these peptides may enable targeting cancer cells.

Gene therapy has emerged as a promising method for treating genetic diseases, cancer, autoimmune, and infectious diseases that are otherwise incurable (70, 97). The most important problem for gene therapy is the lack of an effective and safe gene delivery system that can be used both in vitro and in vivo. DNA and RNA vaccines, which require gene delivery, have recently become important tools for protecting against infections and treating cancer (98, 99).

Currently, researchers use a variety of methods to deliver genes, including both viral vectors and nonviral transfection agents. The use of viral vectors and liposomes has been a common strategy, but limitations and safety concerns have led researchers to explore alternative delivery methods. Viral vectors such as retroviruses, adenoviruses, and adeno-associated viruses that are extensively utilized in gene therapy, have gained popularity because of their effectiveness in gene transfer.

However, the use of viral vectors poses potential safety issues, including the risk of immune reactions, in addition to limitations associated with these vectors. Immune responses triggered by viral vectors may limit their therapeutic applications. For example, Retroviruses, for instance, utilize an integrase enzyme to integrate the desired

gene into the human cell genome, enabling long-term expression. However, concerns arise regarding insertional mutagenesis, wherein the site of integration cannot be controlled, potentially leading to adverse effects, such as the inactivation of tumor suppressor genes or the creation of oncogenes that could transform cells into cancer cells (6). Therefore, alternative transfection tools such as liposomes, nanoparticles, peptides, and techniques using naked DNA have emerged as safer and more economical techniques. Some of these tools have less toxicity and lower potential to trigger immune responses, making them advantageous in terms of scalability (70).

Liposomes, as nonviral vectors, are widely used in delivery systems. They can encapsulate nucleic acids, offering flexibility in cargo selection, but they have some disadvantages such as difficult production, high costs, and limited penetration. Whereas liposomes can exhibit cargo leakage during circulation, CPPs offer improved cargo protection, minimizing premature release and enhancing drug stability (100).

Even so, the application of CPPs is constrained by several notable limitations. Firstly, CPPs often exhibit non-specific uptake, leading to off-target effects and potential toxicity in non-target cells. Additionally, the efficiency of CPP-mediated delivery can be highly variable depending on the type of cargo, the cell type, and the specific CPP used. Another significant limitation is the potential for rapid degradation by extracellular proteases, reducing the effective concentration of CPPs available for cell entry. Furthermore, the endosomal entrapment of CPP-cargo complexes can hinder the release of the therapeutic agents into the cytosol, thereby diminishing their intended biological activity. These challenges underscore the need for further optimization and development of CPPs to enhance their specificity, stability, and delivery efficiency for therapeutic applications (63). After considering all this, among all gene delivery agents, CPPs have gained attention in recent years as nonviral vectors that can efficiently deliver various cargoes into cells. In most of the research involving CPPs, peptides such as penetratin, Tat, and polyarginine peptides were initially used for translocation purposes (101-103). Because of their advantages, such as stability in extreme temperatures and easy production, CPPs emerged as promising tools for gene delivery (104). CPPs generally exhibit low immunogenicity and cytotoxicity

compared to some liposomal formulations (105). These characteristics are crucial for the development of safe and effective drug delivery systems. CPPs can also be easily modified via chemical conjugation with different molecules to optimize their properties, including stability and specificity. Thereby, CPPs can be tailored to meet the requirements of specific drug and gene delivery applications (106, 107).

El-Andaloussi et al. (86) developed a CPP named M918 for the efficient delivery of proteins and peptides into cells. The results illustrated that M918 exhibits remarkable cell-penetrating properties, displaying high efficiency and stability in delivering a wide range of proteins and peptides into different cell types. The peptide displayed efficient internalization and no significant cytotoxicity, making it a safe and viable option for cellular delivery applications. In another study, Kevin et al. validated CPPs as potential molecules for improving the cytosolic delivery of therapeutic oligonucleotides in the liver, but safety remained a concern for this approach (87). A study by Kato et al. indicated that the design of novel CPPs using unnatural amino acids for plasmid DNA delivery was possible. They found that longer post-incubation led to significantly higher transfection efficiencies (88).

This research presents a novel, easy-to-produce peptide with low cell toxicity as a highly efficient gene delivery tool. The novelty of our gene delivery agent is that it is a hybrid peptide with CPP residues on one side and a human histone H4 segment that binds to DNA on the other side. We designed several peptides by combining different peptide sequences derived from different CPPs with the DNA-binding component of histone H4 protein. The H4 segment cannot deliver genes when used without a leading CPP sequence. Among our hybrid peptides, the most efficient gene delivery vector was TM3 (Table 2). The binding of the histone component of TM3 to DNA positions their free CPP component outside the “peptosome,” making them available for cell binding and penetration (Figure 3). TEM images of peptosomes indicated spherical formations with two different densities, a thinner layer covering a more massive region in the middle as can be expected by the free CPP part of TM3 outside and the histone part bound to the surface of supercoiled plasmid DNA inside.

TM3 was identified as the peptide with the best transfection activity. The resulting peptosomes, particularly those formed with the TM3 peptide, exhibited promising transfection abilities. The success of TM3 in transfection can be attributed to the strategic combination of CPP and the DNA-binding domain of histone H4. The histone domain of TM3 binds to DNA molecules, leaving the CPP part free and available for binding and penetration into cells. This unique structure forms the basis of the peptosomes, ensuring efficient gene delivery.

The CPP sequence was an important factor in the transfection efficiency of peptosomes. In the transfection we didn't include any CPP part as in TM4, which contained only part of histone H4, the transfection feature of the peptide was completely lost. A small portion including just three amino acids of integrin beta fragment used in TM3 enabled the highest transfection efficiency. Although transfection was possible when the whole integrin beta is used in TM1 and a larger part in TM2 compared to TM3, their transfection efficiency was much lower than in TM3. TM5, which only contained a fragment of integrin beta 3, may permit transfection, however, it didn't enable the expression of GFP. This may be due to the lack of histone sequences which function as NLS directing DNA into the nucleus enabling the transcription and expression of the transfected gene. When the Growth factor receptor-bound protein SH2 domain is used in place of integrin beta-3, in TM6, the transfection activity of the peptide is completely lost. In TM7 and TM8, the CPP component of TM3 was modified by changing one or two valine residues to leucine to improve transfection efficiency. However, these changes resulted in the complete loss of transfection activity. All these results showed that the CPP sequence used in peptides to form peptosomes is very critical.
TM3's effectiveness in transfection, coupled with its cost-effective synthesis together with demonstrated safety of CPPs over the last decade, positions it as a promising candidate for further development.

Exploring the variety of TM3 in delivering different types of genes and cargoes could enhance its applicability across a broader spectrum of gene therapy applications.

Our findings shown in Table 2 demonstrate a notable increase in the percentage of transfected cells expressing GFP at 48 hours compared to the 24-hour evaluation for both reagents, indicating a time-dependent enhancement of transfection. This observation suggests that longer incubation times might be advantageous for achieving higher transfection rates using these reagents. Moreover, our analysis of hybrid peptides revealed that TM3 exhibited superior transfection efficiency compared to Lipofectamine2000. Specifically, TM3 achieved transfection efficiencies of 52% and 76% at 24 and 48 hours, respectively, whereas Lipofectamine2000 yielded lower efficiencies of 27% and 52% for the same time points.

These results indicate the potential of TM3 as a promising transfection agent (Figure 6), surpassing the performance of the widely used Lipofectamine2000. The enhanced transfection efficiency of TM3 could be attributed to its unique composition, combining elements that facilitate cellular uptake and nuclear localization. Further investigation is warranted to elucidate the underlying mechanisms driving the superior performance of TM3 and to optimize its delivery system for various applications in molecular biology and biotechnology. Overall, our study underscores the importance of exploring novel transfection agents and highlights TM3 as a promising candidate for future research and applications in gene delivery. The application of peptosomes in cancer treatment, particularly for delivering therapeutic genes or CRISPR/Cas9 components, may offer targeted and effective strategies for combating cancer at the genetic level. Considering the relevance of gene therapy in vaccine development, peptosomes could play a role in enhancing the gene delivery of DNA or RNA vaccines. This study's findings contribute to addressing the challenges associated with current gene delivery methods and provide a novel approach that could revolutionize the field of gene therapy. Further trials, including animal studies, are needed to evaluate the transfection efficiency and toxicity in vitro and in vivo and develop TM3, which has several advantages over current gene delivery agents, as a gene delivery tool. One of the limitations of this study was the lack of comparison of TM3 transfection efficiency with previously reported CPPs as transfection agents. This should be included in further studies of the evaluation of TM3. The cost of small peptide production by a peptide synthesizer is much lower compared to the production of lipids used to produce

liposomes. Extension of CPPs by histone sequences H4 is not expected to increase considerably. The production of small peptides is much easier and faster compared to liposome lipids, increasing their feasibility. The development of TM3 and the concept of peptosomes may open new avenues for advancing gene delivery systems and may bring us one step closer to overcoming the challenges in treating genetic diseases and cancer through gene therapy.

The implications of these findings are profound for the field of cancer proteomics. Proteomic analysis can provide insights into the molecular mechanisms by which TM3 enhances transfection efficiency, thereby facilitating the optimization of peptide-based delivery systems for cancer therapy. Moreover, understanding the interactions between TM3 and cellular proteins could lead to identifying new therapeutic targets and developing more effective cancer treatments.

Solved Problems in Established Methods

Traditional gene delivery methods, including viral vectors and non-viral systems like liposomes, face several limitations. Viral vectors are highly efficient but carry risks of immune responses and insertional mutagenesis, which can lead to adverse effects such as the creation of oncogenes. Non-viral systems like liposomes, while safer, often result in cytotoxicity and are expensive to produce. The new peptosome system addresses these issues by offering high transfection efficiency with lower toxicity and production costs.

Limitations

Despite the promising results, this study has some limitations. The long-term stability and potential immunogenicity of the peptosomes in vivo have not been thoroughly investigated. Additionally, the study primarily focuses on in vitro transfection efficiency, and further research is needed to evaluate the performance and safety of peptosomes in animal models and clinical settings.

Perspectives

Future research should aim to explore the in vivo applications of peptosomes, assessing their stability, immunogenicity, and overall safety in animal models. Additionally, optimizing the peptide design could further enhance transfection efficiency and specificity. The peptosome technology holds potential for various therapeutic applications, including gene therapy and vaccine technology, making it a promising alternative to current gene delivery systems.

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