In depth investigation of Branched Amphipathic Peptide Capsules (BAPCs) for enhanced mRNA transfection efficiency

by

Sungmin Yoon

B.S., Daegu University, 2015
M.S., Texas A&M University-Kingsville, 2017

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biophysics
College of Arts and Sciences

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Abstract

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Approved by:

Major Professor
John M. Tomich

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Your presence has made my journey far from lonely, and I am sincerely thankful for all the members who have wished me well in my Ph.D. journey.

Dedication

I am deeply grateful to my family for their unwavering support and encouragement, which have fueled my determination to pursue education and achieve success.

Preface

Throughout my journey, I have developed a deep appreciation for the nano-scale world and its potential influence on research, technology, and diverse fields. The steadfast support from my lab colleagues and family has been priceless, and I am thankful for their dedication and sacrifices that have propelled me forward.

Chapter 1 - Unlocking the promise of mRNA therapy: advantages, challenges, delivery vectors, and applications

Introduction and background

In 1973, with the culmination of several groundbreaking discoveries related to DNA and gene cloning, including the publication of a seminal paper detailing the successful transfer of genes into human cells, Friedmann and Roblin put forth the visionary concept of gene therapy for the treatment of genetic diseases in humans.[1-4] The concept that a gene can be transported into precise cell types, resulting in its expression that drives therapeutic effectiveness, has the potential to profoundly enhance the quality of life for patients in addressing challenging diseases including infectious conditions, metabolic genetic disorders, cancer, cardiovascular, as well as various others.[5-9] For this application, the therapeutic agent, represented by a gene, is delivered within a vector designed to facilitate its introduction into the cells of the patients.[5] Thus, the development of secure and efficient delivery vectors represents a pivotal engineering challenge in the realm of gene-based therapy. Lentiviral vectors (LVVs)[10, 11], Adenoviral vectors (AdVs)[12, 13], Adeno-associated viral vectors (AAVs)[14, 15] are examples of viral vectors. While viral vectors offer a relatively high efficiency in delivering genetic material to host cells and demonstrate the advantage of sustained gene expression,[16] their major drawbacks include their propensity to elicit immune responses and the potential to cause cellular harm, with the initial reported fatality in a gene therapy clinical trial linked to an inflammatory reaction induced by the specific viral vector, Adenovirus. [17] Consequently, researchers have been actively seeking nonviral vector systems that can ensure enduring gene expression without provoking undesired inflammatory or immune responses, while maintaining low toxicity. Nonviral vectors possess noteworthy qualities, including cost-effectiveness, versatility, immunogenicity avoidance, stability, and a high payload

capacity.[17, 18] These attributes have propelled nonviral vectors into a prominent position in gene delivery research. As non-viral delivery systems, cationic lipids[19-21], polymer-based vectors[21], and peptide-based vectors[22, 23] are typical examples of non-viral vectors. Especially, peptides have been hailed as ideal foundational components for non-viral vectors due to their distinct advantages, including excellent biocompatibility, a wide range of biodegradability, as well as straightforward synthesis and customization.[24, 25] Branched Amphiphilic Peptide Capsules (BAPCs) constitute a novel category of self-assembling peptide nano-capsules.[26-28] Their initial description can be attributed to Dr. Gudlur, et al[29], where they first documented the remarkable self-assembly behavior of these peptides into nanovesicles. Following the initial discovery of BAPCs' intrinsic self-assembling properties, extensive investigations were carried out to assess their capacity for delivering a diverse array of solutes and genetic materials into cellular environments.[30-32] An additional innovative illustration of peptide-based nano-carriers is represented by Aqueous Partitioning Capsules (APC). Dr. Mo, et al.[33] introduced and characterized the peptide Ac-KKKFLIVIKKK-$COHN_2$, known as $K_3h_5nK_3$, and explored its possible application as an adhesive agent. In addition to evaluating the peptide's adhesive qualities, the study also encompassed the assessment of its secondary structure. It revealed that the secondary structures differed depending on the surrounding pH conditions, with nanospheres forming under acidic conditions at pH 2. These cationic properties, owing to the presence of lysine, and the nanospheric shape offer additional potential for drug delivery applications.

As the COVID-19 pandemic swept across the globe, an unprecedented urgency arose in the quest for safe and effective vaccines.[34-37] The demand for these innovative vaccine platforms surged as they presented a versatile approach to addressing the virus's challenges. This urgency was met with a remarkable shift towards the development of messenger RNA (mRNA) vaccines,

driven by their potential to offer rapid and large scale development.[38, 39] Moreover, the flexibility of these platforms to adapt to emerging variants has positioned them as frontrunners in pandemic response.[38, 40] There, However, had been relatively less interest in mRNA compared to DNA for over a decade in therapeutic purposes due to the instability of mRNA and its immunologically active nature.[41-43] This perception changed significantly with two pivotal developments. The first was the groundbreaking work of Weissman and Kariko, who demonstrated that incorporating modified nucleosides into in vitro-transcribed mRNA could substantially reduce its immunogenicity.[41, 44] Building upon this breakthrough, they further revealed that by substituting uridine with pseudouridine in mRNA synthesis, the stability of the molecule was enhanced, consequently increasing its translational capacity.[41, 45] These innovations collectively revolutionized the prospects of mRNA, turning it from an unstable and immune-provoking molecule into a promising and robust platform for various therapeutic applications. The use of mRNA for therapeutic purposes extends beyond the development of SARS-CoV-2 vaccines. Examples of mRNA-based therapeutic applications include monoclonal antibody therapies[43, 46] protein replacement therapies[47-49], and immunotherapies for infectious, genetic & metabolic[50], cardiovascular & cerebrovascular diseases.[43, 51] The field of mRNA therapy has attracted substantial financial investment, leading to the establishment of numerous well-funded biotechnology companies, including Moderna, Argos Therapeutics, RaNA, CureVac, among others.[43]

Nevertheless, despite these developments and advantages, a range of obstacles still lie ahead, requiring resolution before mRNA can be solidified as a comprehensive therapeutic approach applicable to a wide spectrum of both uncommon and prevalent medical conditions. Therefore, it is crucial to attain a more profound comprehension of mRNA delivery system at a

molecular level and its optimal conditions in successful mRNA therapeutics. This chapter will provide an in-depth exploration in four sections: (I) advantages of mRNA over DNA in therapeutic purposes, (II) current challenges facing mRNA therapies for application, followed by (III) the types of nucleic acid delivery vectors, and (IV) current applications of mRNA for therapeutic purposes across various therapeutic areas and an outlook on the future potential of mRNA as a therapeutic modality. Improving the technology for optimizing mRNA structures and developing precisely designed nanoparticles for mRNA-based therapeutics are crucial steps in advancing the potential of mRNA drugs as effective and versatile tools for tackling a range of diseases. Over the past three decades, significant progress has been achieved in the field of mRNA-based therapeutics, leading to impressive enhancements in mRNA stability, functionality, and production.[43, 52] With the significant enthusiasm and promise in this field, we hold a strong optimism for witnessing an accelerated pace of research and progress in mRNA therapy over the next decade. This advancement potentially holds the key to multiple solutions for conditions that are currently difficult to treat, bringing renewed hope to patients with unmet clinical needs that might not be effectively addressed by other available therapeutic methods.[53]

Advantages of mRNA as a therapeutic molecule over DNA

Messenger ribonucleic acid (mRNA) is a single-stranded RNA variety that arises from the transcription of a DNA strand, carrying the genetic coding information for protein synthesis.[54] This intricate process, known as transcription, involves the utilization of four distinct nucleotide bases: adenine (A), thymine (T), guanine (G), and cytosine (C) in DNA. However, during transcription, when mRNA is synthesized from DNA, thymine (T) in DNA is replaced by uracil (U) in mRNA **(Table 1.1)**.

Table 1.1. Comparison of DNA and mRNA molecule

	Strand	Structure	Sugar	Base	Location	Process	Lifetime
Deoxyribonucleic acid (DNA)	Double stranded 	Antiparallel helix	Deoxyribose	Adenine (A), Guanine (G), Cytosine (C), Thymine (T)	In eukaryotes, it's within the nucleus; in prokaryotes, it's in the cytoplasm.	Transcription	Long
Messenger ribonucleic acid (mRNA)	Single stranded 	Single helix	Ribose	Adenine (A), Guanine (G), Cytosine (C), Uracil (U)	In eukaryotes, it's transcribed in the nucleus and then moves to the cytoplasm.	Translation	Relatively short

The process of transcription, which involves synthesizing mRNA from DNA, takes place within the nucleus. As a result of this transcription process, mRNA is produced. This single-stranded mRNA structure is essential for the translation of these genetic instructions into proteins and occurs exclusively within the nucleus. Once the mRNA exits the nucleus and enters the cytoplasm, it can undergo various fates, including translation into proteins, storage for future translation, or degradation.[55] In theory, an mRNA-based strategy has the capacity to generate virtually any protein or peptide by utilizing the cellular protein synthesis machinery, whether this

process occurs within the transfected cell in a laboratory setting or within a living organism.[56] The therapeutic utilization of mRNA has instilled great promise in the battle against a diverse array of incurable diseases. Recently, there have been significant and impressive advancements in biotechnology and molecular medicine that have enabled the generation of practically any functional protein or peptide within the human body by introducing mRNA. This application of mRNA can serve as either a vaccine or a therapeutic agent.[43]

Until recently, DNA-based approaches were considered the most suitable for treating inherited diseases caused by missing or malfunctioning proteins. However, the therapeutic use of DNA poses significant challenges. It necessitates not only the rapid delivery of DNA into the cytoplasm but also efficient entry into the nucleus because DNA must undergo transcription into mRNA before the therapeutic protein can be synthesized **(Figure 1.1A)**.[42, 49] Furthermore, DNA-based therapeutics rely on gaining access to the cell nucleus, where transcription into mRNA takes place. This step is vital for their efficacy, as it necessitates the disruption of the nuclear envelope during cell division. However, achieving successful nuclear delivery presents a considerable hurdle in DNA-based therapy, particularly in the case of non-dividing differentiated cells. The majority of these differentiated cells are post-mitotic, meaning they do not frequently undergo cell division.[58] This obstacle of nuclear delivery represents a significant hurdle in DNA-based therapy. On the other hand, mRNA, upon entering the cytoplasm, can initiate the translation process immediately without the need for nuclear transfer **(Figure 1.1B)**. In contrast to other nucleic acid-based therapies, mRNA transcripts exhibit a relatively high level of

successful delivery to cells and low levels of toxicity.[43, 57] mRNA-based therapeutics offer an additional layer of safety by avoiding integration into the host genome, thereby eliminating the

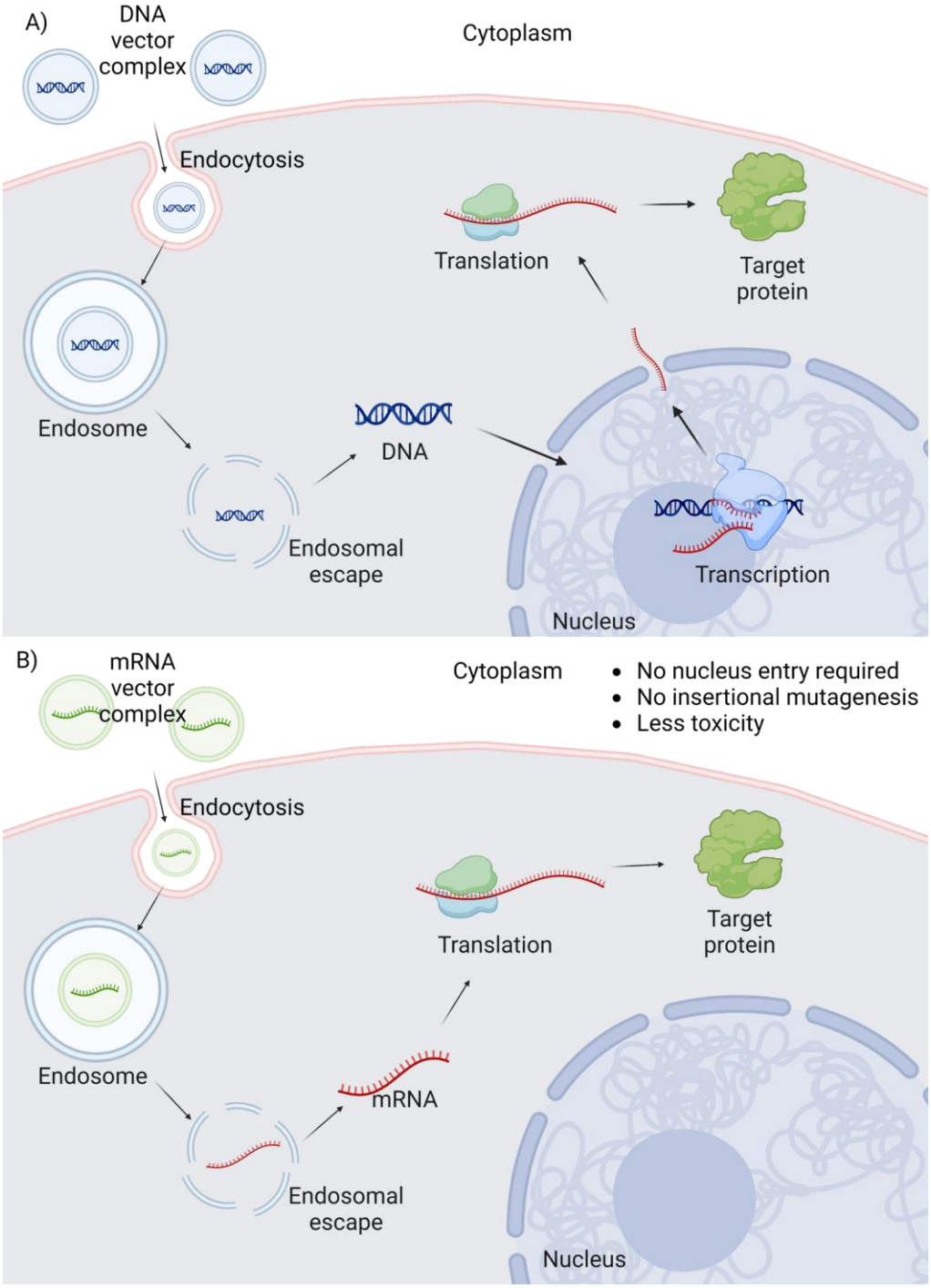

Figure 1.1 Comparing the intracellular translation mechanism

A) DNA's nucleus entry requirement and subsequent cytoplasmic translation. B) mRNA's nucleus-independent translation process. (Schematic created with BioRender.com)

potential risk of inducing insertional mutagenesis.[52] mRNA functions as a transient conduit for genetic information required for protein synthesis, and it does not entail permanent modification of the cellular genetic material. This inherent feature not only enhances the safety profile but also aligns with the preferences of most pharmaceutical applications. Moreover, it is advantageous that mRNA exhibits temporary activity and undergoes complete degradation via natural physiological metabolic pathways.[52, 59] This temporary mRNA activity ensures that the therapeutic or genetic information is only active for a specific period, reducing the risk of long-term side effects or unintended consequences. Additionally, the natural degradation pathways ensure the safe removal of mRNA molecules from the body, minimizing the potential for accumulation or interference with normal cellular processes. This controlled and self-limiting feature enhances the safety profile of mRNA-based therapies and gene expression regulation.

In a comprehensive evaluation, it has been demonstrated that when compared to DNA based therapeutics, mRNA significantly enhances the transfection efficiency of quiescent cells.[60] Additionally, mRNA facilitates accelerated protein synthesis, affords greater precision in regulating expression levels, and lacks supplementary exogenous sequences like antibiotic resistance genes or viral-derived promoters, thus emphasizing the safety and efficacy profile of mRNA.[58, 61, 62] Another notable aspect is the ability to generate mRNA using various approaches, including chemical synthesis or in vitro transcription, albeit acknowledging the limitations inherent to these techniques. These characteristics collectively render mRNA a more appealing molecular candidate for protein supplementation therapy.[42, 48]

Challenges of mRNA development for therapeutic purpose

While the transient characteristic of mRNA offers safety benefits in particular situation, it can also present certain challenges when considering mRNA as a therapeutic agent. In the case of genetic and metabolic disorders demanding extended treatment, the necessity for repeated administration of this biomolecule arises to maintain therapeutic protein levels.[42, 49] The transient nature of this characteristic is a consequence of the ubiquitous presence of RNases, including 5' exonucleases, endonucleases, 3' exonucleases, nonspecific RNases like RNase I and others.[63] Moreover, RNA exhibits greater susceptibility to chemical reactivity compared to DNA, particularly concerning electrophilic additions, alkylation, oxidation, and the like.[64] Additionally, it tends to undergo accelerated hydrolysis in environments with a pH exceeding 6.[63, 64] In light of the aforementioned challenges, significant effort has been directed towards the modification of fundamental mRNA elements and the development of formulations aimed at potentially enhancing its stability and translation efficiency. As a result, researchers have explored various strategies to mitigate the inherent vulnerabilities of mRNA.

The open reading frame (ORF) modifications, specifically codon optimization, are pivotal in enhancing mRNA stability and translation efficiency. Codon optimization significantly influences translation speed, mRNA abundance, and protein folding. High GC-content, despite posing challenges to mRNA secondary structure, translates faster than low GC sequences.[65] The efficiency of translation is controlled by ensuring that the right transfer RNA (tRNA) species are readily available and by adjusting codon usage to match common tRNA sequences. This not only influences translation speed but also has a direct impact on mRNA stability. Replacing rare codons with more common ones speeds up translation by reusing the same tRNA molecules near ribosomes.[66] The adjacency of nucleotides and codons also affects translational rates and

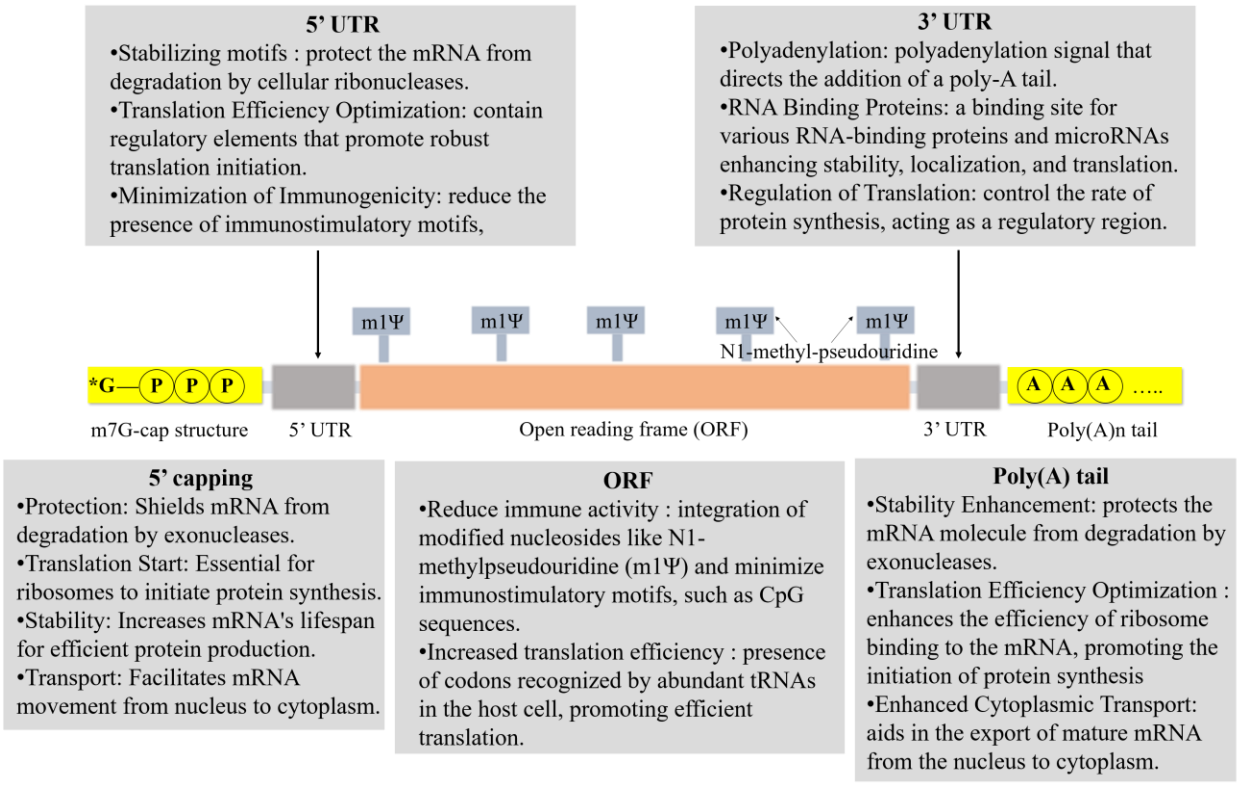

Figure 1.2. The optimization and modification of mRNA's structural components to improve stability, translation efficiency, and reduce immunogenicity

These alterations encompass the open reading frame (ORF), which encodes proteins, along with the flanking 5' and 3' untranslated regions (UTRs). Additionally, structural enhancements such as incorporating a 5' capping structure and modifying the 3' poly(A) tail play pivotal roles in achieving these objectives. Modified and reproduced from 'mRNA therapeutics deliver a hopeful message.' by Zhong, Z, et al.[61], *Nano Today, 23, 16-39*, Figure 1, (https://doi.org/10.1016/j.nantod.2018.10.005).

efficiency.[67] Ensuring the presence of a Kozak sequence in the start codon and modifying the stop codon's sequence are essential steps.[68] Codon-optimized mRNAs have proven effective, especially in vaccine research, where they are used to express non-viral proteins. Some proteins require a delayed translation process, which can be achieved by using uncommon codons. Additionally, substituting nucleotides, like N1-methyl-pseudouridine (1mΨ), enhances base pair stability, improving mRNA translation. Alterations to mRNA secondary structure can increase

stability against endonuclease cleavage and chemical degradation.[69] Furthermore, mRNA transcripts with modified nucleosides have lower immune-stimulating properties, and offer improved safety profiles.[69] These modifications hold great potential for significantly enhancing translation efficiency, establishing ORF modifications as a fundamental aspect of mRNA optimization for a wide range of research and therapeutic applications. The mRNA's stability and translation efficiency are also influenced by regulatory sequence elements found within the 5' and 3' untranslated regions (UTRs). These UTRs play a role in governing protein translation, with the 5' UTR elements and the length of the 3' UTR contributing to this process. They encompass diverse regulatory segments associated with mRNA stability, recognition by ribosomes, interaction with components of the translational machinery, and the formation of mRNA secondary structures. Combining two human β-globin 3′-UTRs in a head-to-tail orientation enhances their stabilizing effect.[70] Those distinct regions in cellular and viral 5′- and 3′-UTRs can also improve mRNA stability and translation efficiency. For specific applications requiring rapid protein synthesis, destabilizing mRNA by inserting AU-rich regions into 3′-UTRs can achieve faster mRNA breakdown and shorter protein expression duration.[71] 5′-capping which is one of post-transcriptional modification play crucial roles in ensuring mRNA stability and efficient translation. In eukaryotic cytoplasm, natural mRNA possesses a 5′ cap structure comprising a 7-methylguanosine (m7G) moiety at its 5′ end, linked to the first nucleotide as m7GpppN.[72] This cap structure serves multiple essential functions, including regulating pre-mRNA splicing, facilitating nuclear export, protecting RNA from exonuclease cleavage, and initiating mRNA translation.[72] The cap 0 structure which is m7GpppNm is particularly vital for effective translation and aids the host in distinguishing self from non-self mRNA molecules.[73] The 5′ cap also screens mRNA against exonuclease activity, promotes pre-mRNA splicing, and

acts as a binding site for the eIF4F heterodimeric translation initiation complex.[74] "Anti-reverse" cap analogs, like Anti-Reverse Cap Analog (ARCA), ensure the correct orientation of the cap structure.[75] ARCA-capped mRNA exhibits enhanced translation efficiency and extended half-life in cultured cells[76], resulting in boosted and prolonged protein expression. These modifications are crucial for optimizing mRNA for therapeutic and research applications. The poly(A) tail varies in length and influences mRNA longevity. Typically, mammalian cell mRNA carries approximately 250 nucleotide-long poly(A) tails, which gradually shorten from 3′ to 5′ during cytoplasmic residence.[74] To optimize mRNA for therapies, tail lengths of around 100 nucleotides are preferred, as this size moderate mRNA decay. Enzymatic polyadenylation, while useful, results in variable tail lengths in RNA preparations. In contrast, in vitro transcription (IVT) from a DNA template yields RNA with a predetermined poly(A) tail length, making it preferable for clinical applications. Overcoming cloning challenges, methods like recurrent restriction digestion, ligation, and propagation have been proposed to extend homopolymeric sequences in circular plasmids, while linear plasmids have been suggested as template alternatives for mRNA synthesis. **Figure 1.2** depicts the various components and modification of mRNA to improve stability, translation efficiency, and reduce immunogenicity.

The effective and secure transportation of mRNA stands out as one of the obstacles in the advancement of mRNA-based therapeutic approaches. The challenge of efficiently delivering mRNA is notably more intricate compared to the delivery of smaller oligonucleotides due to its size, which ranges from 1 to 15 kilobases (kb) or 300 to 5,000 kilodaltons (kDa). This size range is significantly larger than that of smaller RNA molecules like siRNA and miRNA mimics, which typically weigh around 13 to 15 kDa, as well as antisense oligonucleotides and anti-miRs, which fall in the range of 4 to 10 kDa.[42] The cellular uptake efficiency of naked mRNA

molecules stands at a strikingly low frequency. This reduced uptake can also be attributed to the mRNA's negative charge resulting from phosphate groups in the mRNA backbone. This negative electrostatic charge can impede the effective internalization of mRNA by anionic cellular membranes. Moreover, the internalized naked mRNA is subjected to swift degradation rather than being significantly translated within the cells. Due to their huge size, negative charge characteristics, and susceptibility to degradation, naked mRNA molecules face significant barriers in terms of traversing the cell membrane and achieving efficient translocation into the cytoplasm, estimated that only 1/10,000 naked mRNA can uptake by cells.[52]

While addressing the issue of cellular uptake is crucial, another significant barrier in the realm of mRNA therapeutics is the escape from endosomes once they've been internalized by the cell. Once mRNA molecules successfully navigate the hurdles of size, negative charge characteristics, and degradation susceptibility to enter the cell, they often encounter a new challenge: becoming trapped within endosomes.[42] Endosomes, membrane-bound compartments within cells, are critical for the cellular trafficking of various molecules, including mRNA.[38, 43] However, when mRNA molecules are taken up into cells through endocytosis, they tend to become encapsulated within these endosomes.[43] This encapsulation within endosomes can impede the mRNA's ability to fulfill its therapeutic function because the machinery required for translation resides in the cytoplasm, not within the endosomes. To harness the full therapeutic potential of mRNA, it's imperative that these molecules escape from the endosomes and reach the cytoplasm where they can be translated into functional proteins. This process of endosome escape has been a formidable challenge in the development of mRNA-based therapies.

To address the aforementioned challenges, including issues related to instability, cellular uptake, and endosome escape, and to facilitate the widespread application of therapeutic

mRNAs, there is a pressing need for the development of delivery methods that are not only more efficient but also safer. This is a critical requirement for realizing the transformative potential of therapies, including but not limited to vaccination, protein replacement therapy, and genome editing. It necessitates a higher degree of sophistication and precision in the delivery system due to several complex factors. In recent years, a diverse array of materials, encompassing lipids, lipidoids, polymers, peptides, proteins, extracellular vesicles, among others, have been meticulously designed and rigorously investigated for the delivery of mRNA, both in in vitro and in vivo settings.

Types of gene delivery vectors

The emergence of genetic engineering in the early 1970s marked the beginning of biomedical technologies aimed at improving human health by utilizing genetic manipulation methods in a clinical setting. Gene therapy represents a groundbreaking and appealing strategy for tackling human diseases, using carriers or vectors to deliver therapeutic genes into patients' bodies. Various viral and non-viral vectors are employed for the delivery of genes. However, the fundamental requirement in all gene therapy applications is the successful transport of genetic material to host cell through the cell membrane. Each of these delivery systems possesses distinct advantages and disadvantages. In this comprehensive review, we provide an overview of types of viral and non-viral gene delivery systems.

Viral vector

Gene delivery systems mediated by viruses harness the capacity of viruses to introduce their nucleic acids into host cells, capitalizing on their inherent capability to replicate their genetic material. The effectiveness of viruses as gene delivery vehicles stems from their structural characteristics, which shield the nucleic acids from degradation within the lysosomes.[77-80] Among the effective viral vector platforms currently in use, including lentiviruses, adenoviruses, adeno-associated viruses, retroviruses, pox viruses, herpes viruses, and human foamy viruses (HFV), have demonstrated considerable success.[81, 82]

Lentiviral vectors (LVVs)

In recent times, LVVs have garnered considerable attention as the most extensively researched retroviral vectors for gene delivery.[77] These genetically modified lentiviruses belong to a specialized subclass of retroviruses distinguished by a unique attribute: the presence of an additional three to six accessory viral proteins. These supplementary proteins play a crucial role

in fine-tuning viral gene expression and enhancing infectivity. Notably, LVVs have gained prominence as gene delivery vectors due to their distinctive ability to naturally integrate into nondividing cells—a characteristic that sets them apart from other retroviruses.[83-85] Lentiviral vectors (LVVs) can be pseudotyped to exhibit a precisely tailored tropism, making them a prominent choice for ex vivo gene transfer within the central nervous system.[86] Notably, their application has shown a remarkable absence of significant immune responses and unwanted side effects. LVVs boast several advantages, including their exceptional ability to efficiently infect both dividing and nondividing cells, enable long-term and stable transgene expression, possess low immunogenicity, and accommodate larger transgenes.[87, 88]

Adenoviral vectors (AdVs) and Adeno-associated viral vectors (AAVs)

Adenoviruses (Ads) possess an icosahedral protein capsid that encloses a linear duplex DNA genome consisting of approximately 36,000 base pairs with a dozen capsid proteins without lipid envelop.[12] AdVs have been identified in a diverse array of species, with over 100 distinct serotypes documented. The adenovirus serotypes most frequently employed in gene therapy, specifically types 2 and 5, have likely encountered exposure in the majority of adults.[81] Adenoviruses of types 2 and 5 exhibit the capacity to efficiently transfer genetic material into both dividing and nondividing cells, and their relatively low host specificity enables their utility in gene delivery across a wide spectrum of tissues.[89] Adenoviruses possess the capability to transport substantial DNA fragments, reaching sizes of up to 38 kilobases (kb).[90] However, in contrast to retroviruses, adenoviruses do not integrate into the host genome, resulting in relatively short-term gene expression. While the occurrence of severe illness stemming from natural adenovirus infection is infrequent, it's important to note that gene therapy utilizing adenoviral vectors has, in certain cases, led to significant adverse effects, including fatalities in

some patients. Importantly, adenoviral vectors do not integrate their viral genome into the host genome.[91-93] Adeno-associated vectors (AAV) share certain characteristics with adenoviral vectors. However, due to inherent limitations in their replication and pathogenicity, AAVs are considered a safer alternative compared to adenoviral vectors.[94] AAV vectors exhibit the unique capability to integrate into specific sites within the host genome, thereby yielding sustained transgene expression over an extended period. Furthermore, these stable vectors demonstrate the capacity to infect both dividing and nondividing cells without provoking an immune response. Unlike Adv vectors, which can induce a significant degree of cytopathogenicity and cause substantial harm to target T cells, AAV vectors are notably less detrimental in this regard.[95] However, the successful transduction of AAV vectors necessitates the involvement of helper adenoviruses (Ad) or herpesviruses. This process introduces the potential for integration into the genomes, which carries the risk of inducing mutagenic changes.[96] The relatively lower efficiency of AAV vectors in transduction is attributed to the majority of their DNA residing in an extrachromosomal state, failing to integrate into the host genome. Additionally, AAV vectors are limited in their capacity to accommodate genes larger than 4.5 kilobases (kb) and require vigilant monitoring to detect any potential contamination.[95, 96]

Non-viral vectors

A core engineering challenge in gene-based therapy centers on the development of secure and efficient delivery vectors. Both viral vectors as discussed above and non-viral vectors find application in clinical trials for systemic delivery. Currently, approximately 70% of gene therapy clinical trials conducted to date have harnessed modified viruses as vehicles for gene delivery.[97] While these viral vectors have significantly propelled the field of gene therapy forward, they are accompanied by several limitations, including the potential for restricted packaging capacity[98],

carcinogenesis[99], broad tropism[100], immunogenic responses[101], and challenges associated with vector production.[102] The drawbacks associated with viral vectors in gene delivery could potentially be resolved through the utilization of non-viral vectors. Innovative non-viral vectors have been meticulously designed to tackle these challenges, presenting the opportunity to carry significant genetic payloads and demonstrate lower immunogenicity in contrast to their viral vector counterparts.[103, 104] In this section, we explore categories of non-viral vectors such as cationic lipid vectors, polymer-based vectors, and peptide-based vectors, each offering unique advantages in the realm of gene delivery.

Cationic liposome

Liposomes are spherical vesicles composed of phospholipids.[105] They exhibit a distinctive structure, comprising unilamellar or multilamellar that enclose a central aqueous compartment through a self-assembly process.[106] Liposomes exhibit a size spectrum spanning from 20 nanometer to the micrometer range, and the thickness of the phospholipid bilayer measures approximately 4 to 5 nanometers.[107] Cationic liposomes represent a prominent category of contemporary nonviral polycationic systems. They efficiently condense negatively charged nucleic acids, resulting in the creation of nanoscale complexes. Cationic liposomes possess distinctive attributes, including the ability to encapsulate both hydrophilic and hydrophobic drugs, absence of immune system activation, and precise delivery of bioactive substances to their intended sites of action.[108, 109] However, there exist specific drawbacks linked to this drug delivery system. Notably, liposomes may exhibit notable physicochemical instability. This instability can cause the unintended leakage of the drug, often as a result of liposome fusion leading to the formation of larger particles.[110] Cationic lipids are recognized for their membrane-active properties[111], which could potentially disrupt the normal function and structural integrity

of cellular membranes or subcellular compartments, consequently giving rise to toxicity concerns.[112] Furthermore, the utilization of conventional methods involving organic solvents has adverse environmental implications, poses risks to human health due to toxicity, and frequently causes drug degradation.[113] While liposomes have proven to be highly effective in facilitating the delivery of genetic material for therapeutic purposes, they do present certain limitations. These limitations encompass the capacity to potentially modify the pharmacological behavior of drugs, as well as concerns regarding their stability and the intricacies associated with their preparation processes.

Polymer-based vectors

Polymers can be custom-tailored for a given application by carefully selecting molecular weights, incorporating cell- or tissue-specific targeting components, or making other alterations that grant them distinct physiological or physicochemical attributes.[114] Synthetic polymer nanocarriers have demonstrated potential for clinical use as they can be readily scaled up and their ease of synthesis.[115] Carrier systems that incorporate cationic polymers offer an additional benefit by enabling the creation of smaller, uniformly sized particles, resulting in enhanced transfection efficiency. Cationic polymers possess the capability to condense and compact negatively charged nucleic acids.[116] Polymer-mediated gene and drug delivery offers significant advantages, including the potential to reduce off-target effects and immune responses, and tissue permeability.[117] Polyethylenimine (PEI) stands as the inaugural polycationic polymer, crafted in both linear and branched configurations for applications in gene therapy.[118, 119] While PEI is regarded as a potent non-viral vector for transfection, it can still face issues with limited specificity and effectiveness, and its non-biodegradable nature can lead to cytotoxicity as it accumulates around cells.[18] Considering the frequent need for repeated gene delivery

administrations and the preference for reduced cytotoxicity, non-synthetic biodegradable polymeric vectors, such as chitosan or polyactide, offer a clear advantage compared to non-biodegradable counterparts. However, natural biodegradable polymers, while offering excellent biocompatibility, can be prone to batch-to-batch variations due to differences in their origins.[18]

Peptide based vectors

Peptide-based vectors represent a category of non-viral vehicles composed of less than 50 amino acids that are linked together through covalent peptide bonds.[23] Peptides possess distinctive potential for enhancing nucleic acid delivery owing to their adaptable and modifiable interactions with biomolecules and cellular components.[22] Peptides, with their versatile attributes including the diverse side chain properties of amino acids such as hydrophilicity, hydrophobicity, charge, and polarity[120], offer a compelling foundation for tailoring vectors to cater to specific functions in biological systems, rendering them exceptionally appealing for biomedical applications.[121-123] Among the most widely employed peptide based carriers, cell-penetrating peptides (CPPs) stand out as a preferred choice, owing to their capacity for internalization into cells and their efficient transport of a wide range of payloads across the cell membrane while exhibiting minimal cytotoxicity.[23, 124] Although CPPs have great potential as drug delivery vectors, their inherent chemical instability makes them vulnerable to degradation by both extracellular and intracellular enzymes.[125, 126]

Branched amphiphilic peptide capsules (BAPCs)

Branched Amphiphilic Peptide Capsules (BAPCs) serve as a remarkable example of peptide-based nanocapsules. This concept was originally introduced by Gudlur, et al.[29] BAPCs exhibit amphiphilic properties, featuring one end that includes positively charged lysine residues, making it hydrophilic, and the other end composed of two hydrophobic tails, reminiscent of

diacylglycerol molecules, shown in **Figure 1.3**. These nanocapsules are formed through the self-assembly of two chemically synthesized peptides derived from a natural fragment found within the human dihydropyridine-sensitive L-type calcium channel segment known as CaIVS3 (DPWNVFDFLIVIGSIIDVILSE)[29]. Due to the significant hydrophobic amino acid content in each tail, these peptides are referred to as h_9 for tails with 9 amino acid residues and h_5 for tails with 5 amino acid residues (**Figure 1.3**). The peptides' sequences undergo a reversible transition,

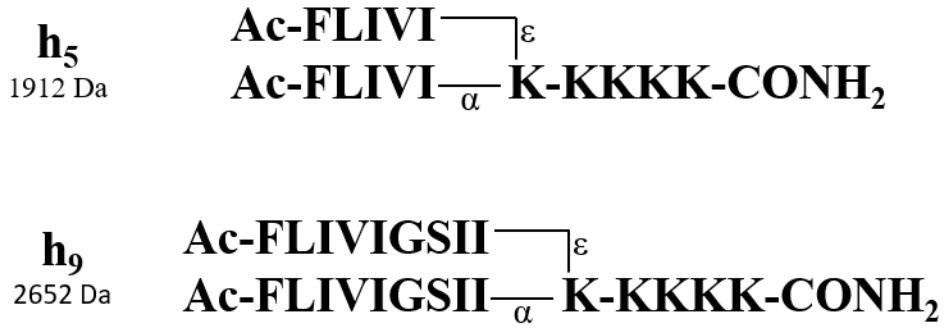

Figure 1.3. Branched amphiphilic peptide sequences of h_5 and h_9

The sequence of the self-assembling peptides bis(h_5)K-K_4 (top) and bis(h_9)K-K_4 (bottom). The fifth lysine residue serves as the branch point, and branch emerge at the N-terminal amine group and the side chain ε amine.

shifting from α-helical monomers in 2,2,2-trifluoroethanol (TFE) to β-like structures upon contact with water, driving their spontaneous self-assembly.[127] When equimolar amounts of branched peptides h_5 and h_9 are mixed and hydrated with water, they rapidly initiate the formation of nanocapsules. After rehydration at both 4 and 37℃, these nanocapsules consistently exhibit a size ranging from 20 to 30 nm. Importantly, BAPCs exhibit notable characteristics such as narrow size range, high thermal stability, resistance to eukaryotic proteolytic degradation, low cytotoxicity, and efficient cellular uptake, surpassing previously discussed delivery systems in these aspects.[30]

Aqueous partitioning capsules (APC)

Another pioneering possible peptide-based nanocarriers is aqueous partitioning capsules (APC). The foundational comprehension of the peptides constituting APC can be credited to a publication authored by Dr. Mo. et. Al.[33] This research paper introduced and extensively characterized the peptide denoted as $K_3h_5nK_3$, which has the sequence Ac-KKKFLIVIKKK-$COHN_2$.[33] The study explored the potential application of $K_3h_5nK_3$ as an adhesive substance,

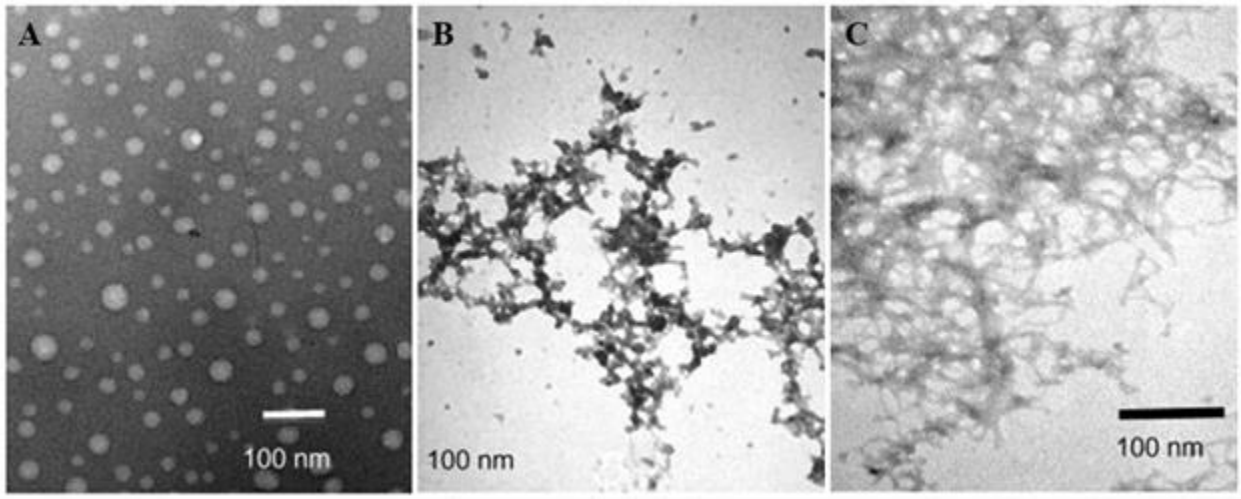

Figure 1.4. TEM images of $K_3h_5K_3$ peptide secondary forms at different pH values

A) $k_3h_5k_3$ peptides suspended at pH 2.0 and imaged at 34,000 x. B) the peptides dissolved at pH 7.0 and imaged at 70,000 x. C) the peptides hydrated at pH 12.0 and imaged at 70,000 x. Images were produced using a Hitachi STEM 4800 EM (Hitachi High Tech Group, Germany). Reproduced from 'A Review of Solute Encapsulating Nanoparticles used as Delivery Systems with Emphasis on Branched Amphipathic Peptide Capsules," by *Barros, et al.*[127], *Archives of Biochemistry and Biophysics, 596,* Figure 11,(https://doi.org/10.1016/j.abb.2016.02.027).

providing a comprehensive examination of its properties and capabilities for adhesive purposes. In addition to evaluating the adhesive properties of the peptide, the research also encompassed an investigation of its secondary structure. This peptide sequence showed that the secondary structures of the peptides exhibited variations depending on the pH conditions of their surroundings, with pH 12 leading to the formation of interwoven nanofibrils potentially contributing to their adhesive properties, pH 7 resulting in a random coil structure, and pH 2 causing a transformation into nanospheres within the acidic environment (**Figure 1.4**).[127] Additionally, we have revealed that the nanospheric shape is sustained even after the surrounding condition returns to neutral condition from an acidic condition, which initially formed the nanospheric structure. These cationic attributes, contributed to the presence of lysine, coupled with the nanospheric configuration, further enhance its potential for applications in drug delivery.

In this investigation, BAPCs and APCs served as carriers for nucleic acid delivery in therapeutic applications. Given the advantages mentioned earlier, the continued exploration and improvement of these delivery vehicles, specifically BAPCs and APCs, are poised to have a pivotal impact on advancing the evolution of inventive drug delivery systems.

Table 1.2. Comparison of viral and non-viral vectors in gene delivery applications

Aspect	Viral Vectors	Non-Viral Vectors
Efficient Gene Delivery	Highly efficient at delivering genes to target cells.[16] Long-lasting gene expression and Suitable for permanent gene therapy.[16]	More suitable for transient gene therapy or certain applications.[16]
Target Cell Specificity	Can target for cell-type specificity such as dividing or non-dividing cells.[89]	Can be modified for cell-specific targeting.[16]
Immunogenicity and cytotoxicity	Potential to induce immune responses.[87, 88, 101] Limited repeated use.[6]	Less immunogenic.[103, 104] Repeated administration may be possible.
Integration Risk	Viral vectors can integrate into the host genome, potentially causing insertional mutagenesis.[6] Safety concerns exist.[6, 87, 88]	Non-viral vectors do not integrate reducing the risk of mutagenesis.[17] Safer in terms of genetic alterations.[17]
Limited Cargo Capacity	Limited cargo capacity for therapeutic genes due to vector size constraints.[8, 90, 95, 96]	Can accommodate larger genetic payloads.[18] Suitable for delivering larger genes.[18]
Manufacturing Complexity	More complex and expensive to produce and purify viral vectors. May require specialized facilities.	Simpler and cost-effective manufacturing.[115] Easier scalability.[115]
Risk of Pre-existing Immunity	Some individuals may have pre-existing immunity to the viral vectors, reducing effectiveness.[8]	Lower risk of pre-existing immunity. More suitable for broad patient populations.

Current applications of mRNA for therapeutic purposes

mRNA-based therapeutics are anticipated to emerge as a potent treatment option for various challenging medical conditions, including infectious diseases, inherited metabolic disorders, cancer, and other health issues. Numerous studies have highlighted the advantages of mRNA over DNA and traditional protein drugs, showcasing its ability to achieve improved transfection efficiency and prolonged protein expression. mRNA can be readily synthesized through in vitro transcription (IVT) processes, making it relatively straightforward to

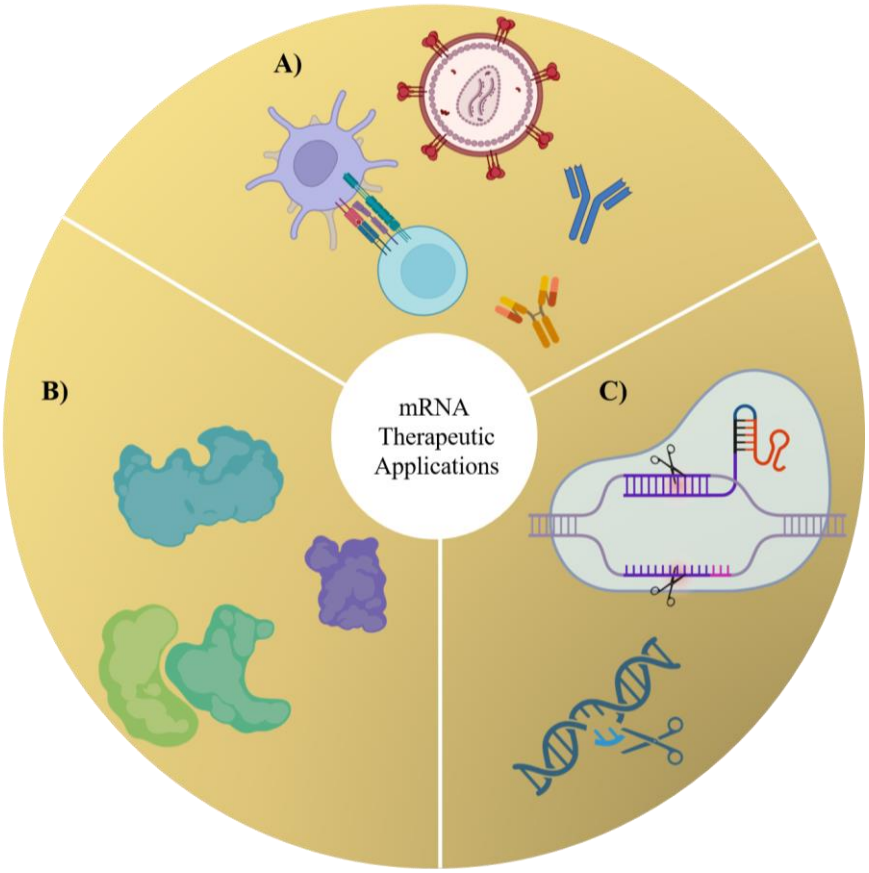

Figure 1.5. mRNA therapeutic applications

mRNA drugs have opened the door to a multitude of promising treatments for diseases that were once refractory or considered incurable. These treatments encompass A) immunotherapy, B) protein replacement therapy, and C) cell reprogramming and gene editing therapy.
(Schematic created with BioRender.com)

manufacture and swiftly apply to diverse therapeutic contexts. The field of mRNA therapy has attracted substantial financial investments, leading to the establishment of well-funded biotechnology companies like Moderna, CureVac, BioNTech, Argos Therapeutics, RaNA, Translate Bio, Ethris, Arcturus, and Acuitas. Evidently, mRNA has emerged as one of the most promising domains in drug development, offering extensive possibilities for exploration in the long term. In this section, we provide a comprehensive overview of the latest advancements in mRNA-based drug technologies and their diverse applications.

mRNA-based immunotherapy

Cancer immunotherapy has a rich history of utilizing mRNA-based technologies for research and clinical applications. In 1995, researchers demonstrated that injecting naked RNA encoding a specific cancer antigen into muscles could trigger antigen-specific antibody responses.[129] Subsequently, they found that exposing dendritic cells (DCs) to mRNA encoding tumor antigens, or total mRNA extracted from tumor cells and administering them to tumor-bearing mice, induced T-cell immune responses and inhibited tumor growth.[130] These discoveries, coupled with the growing availability of vaccine targets from the cloning of novel tumor antigens, accelerated the development and clinical translation of mRNA-transfected DC vaccines.[131, 132] Over the years, clinical trials refined the treatment protocols for DCs transfected with IVT mRNA, enhancing their antitumoral activity by optimizing DC maturation and co-delivering mRNA encoding immune response modulators. However, researchers have revisited the direct in vivo administration of IVT mRNA encoding tumor antigens due to the complexity and cost of cell therapies.

Intradermal delivery of IVT mRNA, whether in its naked form or as a protamine-complexed, sequence-optimized variant, was the subject of clinical trials. These trials revealed

the expression of the encoded antigen within skin cells, culminating in the initiation of T cell and antibody responses.[133] Notably, when administered intradermally, naked mRNA alone primarily induced a T helper 2 (TH2)-type antigen-specific immune response. Conversely, the introduction of adjuvants such as granulocyte-macrophage colony-stimulating factor (GM-CSF)[134] or the complexation of IVT mRNA with protamine provoked a pronounced shift towards a TH1-type response.[135] Early clinical trials that involved protamine-complexed IVT mRNA or combined mRNA with GM-CSF confirmed the safety and feasibility of intradermal vaccination, demonstrating its efficacy in stimulating antigen-specific antibody and T cell immune responses.[136] Subsequent optimizations yielded a pharmaceutical-grade two-component vaccine, uniting a naked mRNA encoding the antigen with a protamine-complexed mRNA adjuvant developed by CureVac. This vaccine exhibited formidable potency in preclinical investigations and is currently under evaluation in ongoing clinical trials, targeting patients with prostate cancer (NCT0083146) and non-small-cell lung cancer (NCT00923312).[137] These findings underscore the promising potential of intradermal mRNA-based vaccines for robust immune response elicitation, offering renewed optimism for enhanced therapeutic interventions.

In an alternative approach, IVT mRNA underwent optimization for *in situ* transfection of DCs. The primary goals were to enhance the stability and translation efficiency of the IVT mRNA and to improve the presentation of the mRNA-encoded antigen on both murine and human DCs, particularly via MHC class I and II molecules.[138-140] Intriguingly, direct injection of naked IVT mRNA into lymph nodes emerged as the most effective means of eliciting robust T cell responses. This method resulted in the selective and efficient uptake of IVT mRNA by resident DCs in the lymph nodes, achieved through macropinocytosis, subsequently facilitating DC maturation via TLR7 signaling. The presentation of the antigen encoded by IVT mRNA,

combined with the immunostimulatory environment within the lymph nodes, led to the generation of potent antigen-specific T cell responses of the proinflammatory T_H1 type and significant antitumor immunity in animal models.[141, 142] Further enhancement of DC immune responses following IVT mRNA delivery was achieved by co-administering the DC-activating ligand FLT3 or co-transfecting DCs with the TriMix, comprising IVT mRNAs encoding immunomodulators CD40L, CD70, and truncated, constitutively active TLR4.[143] Recent efforts have initiated the first-in-human trials involving intranodal injection of naked IVT mRNA encoding cancer antigens, developed by BioNTech, in patients with melanoma (NCT01684241).[144]

Personalizing cancer immunotherapy has been made more accessible through the utilization of in vivo administered mRNA technology due to its adaptability, resilience, and cost-effectiveness.[145] Recently, the first clinical trial (NCT02035956) for an individualized cancer vaccine has commenced.[52] This groundbreaking approach involves analyzing tumor specimens from each patient using next-generation sequencing to identify individual immunogenic mutations. Subsequently, a personalized IVT mRNA vaccine is developed, encoding a polypeptide composed of epitopes that align with these unique mutations. This innovative application of IVT mRNA potentially marks the inception of drug engineering based on an individual's genomic information. Beyond its role in active immunization and immunomodulation, IVT mRNA is under investigation as a versatile tool for transiently modulating immune cells. For example, researchers have successfully transfected IVT mRNA encoding tumor antigen-specific T cell receptors (TCRs) or chimeric antigen receptors (CARs) into T cells or natural killer cells ex vivo through electroporation.[52] These modified cells, with mRNA-encoded receptors, possess the capacity to selectively recognize and eliminate tumor

cells expressing the target antigen, with the transient nature of mRNA delivery reducing the risk of unintended side effects caused by uncontrolled immune cell expansion. This versatile technology holds tremendous promise for revolutionizing cancer immunotherapy.

In recent decades, mRNA-based vaccines have emerged as a transformative approach to combating infectious diseases. In 1993, researchers demonstrated that liposome-encapsulated IVT mRNA, encoding an influenza nucleoprotein, could induce a robust virus-specific T cell response in mice.[146] This early breakthrough laid the foundation for the exploration of mRNA-based vaccines for infectious diseases. More recently, self-amplifying IVT RNA, combined with synthetic lipid nanoparticles (LNPs) and administered intramuscularly, has demonstrated the capacity to induce protective antibody responses against respiratory syncytial virus (RSV) and influenza virus in murine models.[147] Three distinct IVT mRNA-based vaccine strategies for infectious diseases are undergoing pharmaceutical development. For HIV infections, individuals receiving highly active antiretroviral therapy have been subjected to immunization with DCs transfected with IVT mRNA encoding various HIV proteins. Phase I/II clinical trials employing IVT mRNA encoding different viral proteins have confirmed the safety of these vaccines and the induction of antigen-specific CD8+ and CD4+ T cell responses.[148, 149] While one of these trials documented increased HIV inhibition by antigen-specific CD8+ T cells in ex vivo analysis, no observable antiviral effects were observed during the clinical trial itself.[149] Ongoing preclinical investigations are exploring two distinct approaches employing IVT mRNA as prophylactic influenza vaccines. The first approach involves intradermal administration of a two-component vaccine, comprising an mRNA adjuvant and naked IVT mRNA encoding influenza hemagglutinin antigen, either individually or in conjunction with neuraminidase-encoding IVT mRNA.[52] These regimens have demonstrated their ability to elicit protective immune responses

against the corresponding influenza strains in aged and newborn mice, along with conferring long-lasting protective immunity in ferrets and swine.[150] In parallel, a second strategy harnesses self-amplifying IVT mRNA containing sequences derived from positive-stranded RNA viruses. Initially conceived for a flavivirus model, this approach demonstrated that intradermal delivery of less than 1 ng of IVT genomic mRNA could confer protective immunity against flavivirus infection, akin to that achieved with attenuated virus strains. Ongoing research efforts in the field of RNA-based vaccines for infectious diseases have concentrated on utilizing recombinant RNA replicon systems originating from the alphavirus family.[151, 152] Furthermore, within the realm of infectious diseases, a groundbreaking achievement has occurred with the swift creation and distribution of COVID-19 vaccines, firmly grounded in mRNA technology. These vaccines, such as the Pfizer-BioNTech (BNT162b2) and Moderna (mRNA-1273) vaccines, represent a paradigm shift in vaccine development. The SARS-CoV-2 virus responsible for COVID-19 features spike, nucleocapsid, envelop, and membrane protein abbreviated to S, N, E, and M, respectively.[43] These vaccines utilize mRNA to induce an immune response against the SARS-CoV-2 virus. Specifically, mRNA vaccines focus on the S glycoprotein or receptor binding domain (RBD) subunit of the virus.[153] By introducing mRNA encoding the S glycoprotein into muscle or dendritic cells, these vaccines trigger a cascade of immune responses. This process leads to the presentation of the S glycoprotein by major histocompatibility complexes (MHC) class I and II, stimulating humoral immunity.[154] Consequently, B lymphocytes produce neutralizing antibodies that effectively block viral binding and cell entry.[155] Furthermore, mRNA vaccines activate cellular immunity by generating specific cytotoxic T cells (CD8+).[156] These T cells play a crucial role in identifying and eliminating cells infected with SARS-CoV-2 **(Figure 1.6A)**.

The speed at which mRNA technology enabled the development and authorization of COVID-19 vaccines underscores its agility and adaptability in confronting new and swiftly evolving infectious threats. These vaccines have not only exhibited exceptional efficacy but also proven their capacity for large-scale production and global distribution, resulting in millions of doses administered worldwide. This remarkable achievement offers hope for managing the current pandemic and establishes a model for addressing future infectious challenges. mRNA vaccine platforms are positioned to swiftly respond to emerging viral threats, providing effective strategies against known pathogens and potential future global health concerns.

Utilizing IVT mRNA for Protein Replacement

The application of IVT mRNA has emerged as a promising avenue for protein replacement therapy (**Figure 1.6B**). This approach involves supplementing proteins that are either deficient or dysfunctional within the body, as well as substituting foreign proteins that can modulate cellular pathways, including therapeutic antibodies. The inception of IVT mRNA-based protein replacement can be traced back to 1992 when it was first employed to address protein deficiencies.[157] Since the first inception, nucleoside modifications mitigated the immune-stimulatory properties of RNA and significantly improved mRNA's translational efficiency, thus opening the door to therapeutic applications.[44] This newfound approach introduced modified nucleosides, such as 2-thiouridine and 5-methylcytidine, into IVT mRNA. In one notable study, IVT mRNA encoding surfactant protein B (SPB) was aerosolized and administered to mice suffering from a congenital lung disease caused by SPB deficiency. The results were promising, as the treatment protected the mice from respiratory failure and extended their lifespans.[158] Similar success was observed in experiments involving pseudouridine-modified IVT mRNA encoding erythropoietin, with therapeutically relevant levels of erythropoietin detected in mice

and macaques.[159] Furthermore, this approach extended its reach to address asthma by delivering nucleoside-modified mRNA encoding the regulatory T cell transcription factor FOXP3. This intervention effectively rebalanced pulmonary T-helper cell responses, safeguarding mice against allergen-induced tissue inflammation and airway hyperresponsiveness.[160] Furthermore, the direct injection of IVT mRNA, modified to include pseudouridine and 5-methylcytidine, and encoding vascular endothelial growth factor A (VEGFA), demonstrated notable advantages in enhancing cardiac function and prolonging the survival of mice in a myocardial infarction model.[161] In a separate context, another promising application of mRNA technology involves addressing cystic

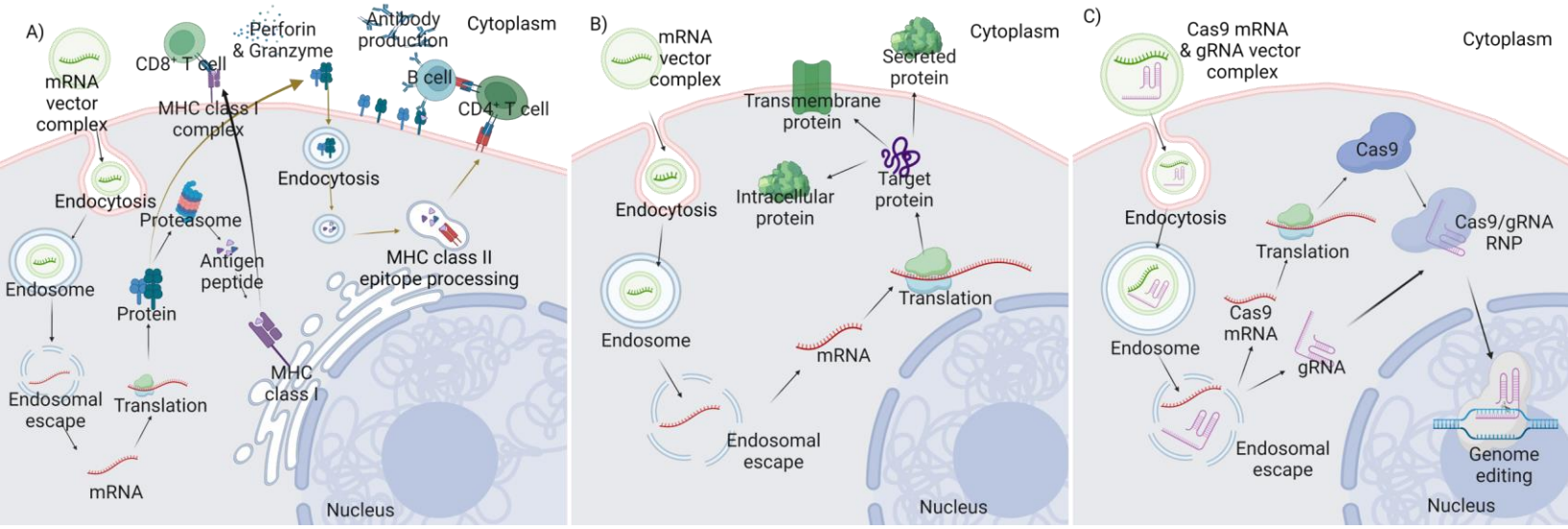

Figure 1.6. Comprehensive mRNA mechanisms and their transformative potential in therapeutic applications

Cellular uptake of mRNA vector complex begins with endocytosis followed by endosomal escape, and mRNA release into the cytosol. mRNA is then translated into protein for therapeutic applications, including A) immunotherapy, B) protein replacement therapy, and C) gene editing.
(Schematic created with BioRender.com)

fibrosis (CF), a life-threatening autosomal recessive condition affecting approximately 100,000 individuals globally.[162] This severe ailment results from mutations in the CF transmembrane conductance regulator (CFTR) gene, responsible for encoding an ATP-binding cassette-class C

protein.[162] The intranasal application of LNPs carrying CFTR mRNA has demonstrated substantial promise, with outcomes indicating the restoration of as much as 55% of the typical chloride efflux observed in healthy mice.[163, 164] Additionally, the mRNA therapy known as MRT5005, designed to augment CFTR protein expression, has progressed into the phase I/II stage of clinical investigation.[165] This marks a significant advancement in the pursuit of therapeutic solutions for cystic fibrosis, a condition characterized by impaired chloride transport due to mutations in the CFTR gene. Nasal administration of LNPs loaded with CFTR mRNA showcases a potentially transformative approach in addressing this challenging genetic disorder, and the ongoing clinical trials underscore the growing importance of mRNA-based therapies in the field of cystic fibrosis research.[164]

IVT mRNA-based protein replacement therapy faces technical challenges, including cell-specific modifications, proteolytic processing, and signal peptides. These hurdles require careful cell selection, and in some cases, signal peptide modifications. Despite these challenges, IVT mRNA-based protein replacement therapy holds promise for a wide range of applications, particularly for proteins with broad therapeutic potential, low-dose requirements, and established profiles in humans.[52]

Cell reprograming and gene editing application

Reprogramming cellular states through nucleoside-modified IVT mRNA applications has shown promise in modulating cell phenotype and function, representing a compelling avenue of research. Notably, in 2010, it was demonstrated that IVT mRNA containing pseudouridine and 5-methylcytidine, encoding the Yamanaka stem cell factors, offered a safe and efficient method for reprogramming cells to pluripotency, leaving no residual traces of transgenes.[166] Moreover, this IVT mRNA technology was not limited to pluripotency induction; it also facilitated the

direct differentiation of induced pluripotent stem cells (iPSCs) into myocyte-like cells. Subsequent developments have introduced several variants of the original approach, aiming for more effective induction of pluripotent stem cells or cell fate conversion.[167, 168] These advancements leverage the IVT mRNA's high transfection efficiency in vitro, transient expression with no genomic integration, and its capability to transfer complex mixtures. Importantly, the absence of residual transgene expression in IVT mRNA-induced iPSCs has simplified their utilization in disease modeling, toxicology assessments, and holds great promise for regenerative medicine.[169-171] Furthermore, IVT mRNA transfer could potentially be harnessed to generate clinically valuable differentiated cells.

Over the past decade, genome editing with IVT mRNA-encoded engineered nucleases has emerged as a viable alternative to conventional gene therapy **(Figure 1.6C)**. Tools like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 offer powerful mechanisms for site-specific genome modification. However, these methods carry the risk of nonspecific editing due to prolonged presence of editing enzymes derived from DNA-based vectors.[172] To address this concern, IVT mRNAs encoding ZFNs, TALENs, and Cas9 have demonstrated successful genome editing both ex vivo in embryonic cells from various species and in vivo in rodents.[173-176] Recent breakthroughs have even enabled site-specific gene modifications in cynomolgus monkeys, opening new avenues for creating primate models of human diseases.[177] IVT mRNA has also found utility in transposon-mediated stable gene transfer, with applications both in vitro and in vivo. For instance, the expression of transposases from systems like Sleeping Beauty, piggyBac, or Tol2 via IVT mRNA injection resulted in stable genomic transposition in mammalian cells[178] and rodents.[179]

Table 1.3. Clinical trials for mRNA-based therapies (protein replacement, immunotherapy, and gene editing)

Therapeutic approach	Protein target	Disease	ClinicalTrials.gov Identifier	Phase	Reference
Immunotherapy	3 TAAs selected from a warehouse and p53 RNA; Neo-Ag based on NGS screening	TNBC	NCT02316457	I	Kowalski, P et al. [180]
Immunotherapy	Zika virus antigen	Zika virus	NCT03014089	I	Kowalski, P et al. [180]
Immunotherapy	H10N8 antigen	Influenza	NCT03076385	I	Robert A, F et al. [181]
Immunotherapy	CMV glycoprotein H pentamer complex	CMV infection	NCT03382405	I	Kowalski, P et al. [180]
Immunotherapy	Neo-Ag	Melanoma; Colon cancer; GI cancer; Genitourinary cancer; HCC	NCT03480152	I/II	Arthur, E et al. [182]
Immunotherapy	Anti - HER2	Breast Cancer	-	Preclinical	Yulia, R et al. [183]
Immunotherapy	TAAs	NSCLC	NCT00923312	I/II	Zhuoya, D et al. [184]
Immunotherapy	OX40L, IL-23, and IL-36γ	Solid tumor or lymphoma	NCT03739931	I	Yasmin G, M et al. [185]
Immunotherapy	TAA for melanoma	Melanoma	NCT00204516	I/II	Zhong, Z et al. [61]
Immunotherapy		prostate cancer	NCT0083146	I/II	Yichen, Z et al. [137]
Immunotherapy	TAA-transfected DC	Prostate cancer	NCT01278914	I/II	Kowalski, P et al. [180]

Protein Replacement	CFTR	Cystic fibrosis	NCT03375047	I/II	Eric W F W Alton et al.[186]
Enzyme replacement (endocrine)	Factor VIII	Hemophilia A	-	Preclinical	Chen, C.-Y. et al. [187]
Enzyme replacement (endocrine)	Factor IX	Hemophilia B	-	Preclinical	DeRosa, F. et al. [188]
Enzyme replacement (endocrine)	MMUT	Methylmalonic acidemia	-	Preclinical	An, D. et al. [189]
Enzyme replacement (intracrine)	CFTR	Cystic fibrosis	-	Preclinical	Haque, A. K. M. A. et al. [190]
Genetic Editing	CRISPR-Cas9/TTR	Transthyretin Amyloidosis	-	Preclinical	Julian D. G et al. [191] Jonathan D, F et al. [192]
Genetic Editing	CRISPRCas9/BCL11A	ß-thalassemia	NCT03655678	I/II	Kowalski, P et al. [180]
Genetic Editing	CRISPRCas9/E6 and E7	Cervical intraepithelial neoplasia	NCT03057912	I	Zheng, H et al. [193]
Genetic Editing	CRISPRCas9/CD19	ß cell leukemia and lymphoma	NCT03166878	I/II	Waseem, Q et al. [194]
Genetic Editing	ZFN mRNA/CCR5	HIV	NCT02500849	I	Shu, S et al. [195]
Regenerative	VEGF - A	Ischemic Heart Disease	-	Preclinical	Lior, Z et al. [161] Leif, C et al. [196]

Conclusions

The journey of gene therapy and the remarkable rise of mRNA-based therapeutics have unveiled a new era of possibilities in medicine. From its inception in 1973, the concept of gene therapy has evolved significantly, offering the potential to transform the lives of patients grappling with a wide spectrum of medical conditions, ranging from genetic diseases to infectious and cardiovascular disorders. While viral vectors initially held the spotlight for gene delivery, concerns over immune responses and safety issues have propelled non-viral vectors, particularly peptide-based nano-carriers like BAPCs and APCs, into the forefront of research.

The COVID-19 pandemic ushered in a seismic shift in vaccine development, with mRNA vaccines emerging as frontrunners due to their rapid adaptability and scalability. This shift was underpinned by groundbreaking work that enhanced the stability and reduced the immunogenicity of mRNA, unlocking its therapeutic potential beyond vaccines. mRNA-based therapies now extend to monoclonal antibodies, protein replacement, and immunotherapies, with substantial investment in biotechnology companies catalyzing their development. These peptide-based nano-carriers, including BAPCs and APCs, have played a pivotal role in optimizing mRNA delivery, enhancing its safety and efficacy. They offer a promising avenue for ensuring the enduring success of mRNA-based therapeutics.

Nonetheless, the journey ahead is not devoid of challenges. The need to delve deeper into the molecular aspects of mRNA delivery systems and optimize their conditions for therapeutic success remains paramount. The advancement of mRNA drugs hinges on refining their structures and designing precise nanoparticles. Over the past three decades, considerable progress has been made in enhancing mRNA stability, functionality, and production. Looking forward, our optimism in the field of mRNA therapy, bolstered by the contributions of nano-carriers like

BAPCs and APCs, is unwavering. The capacity to meet unmet clinical demands and provide answers to presently formidable medical conditions offers renewed optimism for patients. With expectations of a rapid acceleration in research and development over the next decade, mRNA therapy, bolstered by these inventive delivery systems, is positioned to emerge as a versatile and efficacious approach that supplements and strengthens our array of therapeutic strategies.

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Chapter 2 - Improving mRNA delivery through Branched Amphiphilic Peptide Capsule (BAPC) surface charge modification and efficacy check in in vitro and in vivo

Introduction

In the dynamic field of biomaterials and nanotechnology, innovative drug delivery systems have been at the forefront of scientific endeavors.[1-4] Among these cutting-edge advancements, Branched Amphiphilic Peptide Capsules (BAPCs) have emerged as a promising class of nanocarriers with the potential to revolutionize the targeted delivery of therapeutic cargo for various biomedical applications. BAPCs are positively charged self-assembling nanostructures with an amphiphilic nature and an intricate branched architecture.[5-8] Their distinctive composition provide cargo such as mRNA the capability of being encapsulated or surface-bound while remaining cationic. Possessing a positive charge promotes efficient intracellular uptake and endosomal release of biomolecules.[9-11] The distinctive feature of BAPCs is their capacity to form stable bilayers, making them ideal candidates for encapsulating and protecting sensitive bioactive cargo from degradation and premature release.[8] Our research aims to unravel the intricacies of BAPCs in their capacity as a nucleic acid delivery vector. This study extends beyond characterizing the physical and chemical properties of BAPCs; it delves into their functional aspects, encompassing the binding kinetics, mRNA delivery capabilities, and their performance in both in vitro and in vivo settings.

One of the central themes in our research revolves around the strategic modification of BAPCs to increase their mRNA delivery capabilities, a crucial aspect in the field of nucleic acid therapy where effective mRNA delivery to target cells is paramount. To unravel the intricacies of

this process, we meticulously investigated the formation of bilayers within BAPCs. It's important to note that while BAPCs tend to form bilayers simultaneously, tracking and comprehending the intricacies of this process can be challenging. To overcome this challenge and gain a precise understanding of bilayer formation, we employed a step-by-step approach using maleimide-modified iron oxide Magnetic Nano Beads (MNBs). The maleimide groups present on the surface of MNBs exhibit a selective reactivity with sulfhydryl groups, allowing for the creation of a stable thioether linkage.[12-14] By utilizing solid MNBs in our approach, in contrast to relying solely on peptides, we effectively monitored the transition from monolayer to bilayer formation. This sequential approach enabled us to gain a comprehensive understanding of the structural evolution of BAPCs as they progressed into self-assembling capsule.

This methodological decision significantly enhanced the precision and clarity of our observations, ensuring a comprehensive exploration of the subtle nuances associated with BAPCs as gene delivery vector. Furthermore, our exploration of bilayer formation harnessed biophysical techniques, including the assessment of intermolecular π–π interactions and fluorescence quenching.[15-17] These analytical methods provided vivid insights into the primary driving forces behind bilayer formation in BAPCs, particularly the intermolecular π–π interactions between the aromatic rings of peptides involved in bilayer formation, specifically the phenylalanine residues within these peptides. As fluorescence decreases in response to the formation of bilayers, it serves as an invaluable indicator of the π–π interactions and, by extension, the successful formation of bilayers, an achievement that underscores the reliability of BAPCs as a gene delivery platform.

To complement the demonstration of bilayer formation in the MNB experiment, we utilized Nano Tracking Analysis (NTA) to evaluate the ability of BAPCs to form capsules and

determine their size distribution in a liquid suspension. NTA, a robust technique combining light scattering and Brownian motion,[18-20] allowed us to obtain insights into the size distribution of BAPCs. This approach unveiled distinctive capsule shapes and yielded precise measurements of mean and mode sizes with remarkably low standard deviations. These findings contribute significantly to the characterization of BAPCs, laying the foundation for their effective utilization in biomedical applications.

The surface charge of gene delivery vectors is a critical determinant of their effectiveness, particularly for facilitating interactions with cell membranes and enhancing cellular uptake.[21-23] In this context, the positive surface charge profile plays a central role in optimizing the therapeutic potential of gene delivery vectors. However, our research revealed that the BAPCs-mRNA-Heparin complex exhibited a negative surface charge due to the presence of negatively charged heparin and mRNA at the cationic BAPCs are fully saturated by negatively charged molecules, mRNA and heparin. To address this challenge, we explored various strategies to impart a positive surface charge to the outermost layer of the BAPCs complex. Our investigations led to an advancement, highlighting the salient role of lysine in surface modification. We discovered that the introduction of lysine molecules, either in the form of commercial poly-L-lysine (PLL) or lab made oligo-L-lysine with ten lysine residues (OLL_{10}), enabled a dose-dependent increase in the positive charge of the complexes. This finding provides a promising avenue for customizing the surface charge of BAPCs-mRNA complexes, essential for enhancing gene delivery efficiency. To comprehensively evaluate the potential of positively charged BAPCs coated with lysine for mRNA delivery, we conducted both in vitro and in vivo experiments. In the in vitro assessments, we employed mRNA encoding the Green Fluorescent Protein (GFP) as a model system to monitor gene expression within mammalian cells. Our

results demonstrated that the positively charged BAPCs coated with lysine substantially enhanced GFP expression, underscoring the effectiveness of this modification in an in vitro setting.

Moving beyond in vitro studies, we transitioned to in vivo investigations, a critical step in translating nucleic acid delivery systems to clinical applications. In the in vivo context, we assessed the performance of BAPCs-mRNA complexes, specifically investigating luciferase mRNA expression as a marker of gene delivery efficiency. Our research explored the impact of spray drying as a method for enhancing the stability and efficacy of gene delivery systems in an in vivo environment. We discovered that the choice of carrier agents, such as mannitol and lactose, significantly influenced the duration and magnitude of luciferase expression.

Furthermore, it's worth noting that the BAPCs complex exhibited in vivo luciferase expression without the application of the spray drying method. However, when it came to the positively charged BAPCs complex coated with lysine, we encountered a negative outcome. This particular formulation failed to elicit luciferase expression in the in vivo setting. These findings underscore the complexity of in vivo experiments, where a multitude of factors including the immune system, tissue barriers, genetic diversity, and the diverse physiological microenvironments within living organisms all come into play, influencing the response to gene delivery systems.

This contrast between our in vitro and in vivo gene expression outcomes serves as a reminder of the intricate challenges and nuances that underpin the optimization of gene delivery systems. It highlights the critical importance of comprehending the multifaceted nature of in vivo responses, guiding us towards the development of more effective gene delivery strategies.

Materials and methods

Solid phase peptide synthesis

All linear peptides were assembled on clear amide resin (Peptides International, Louisville, KY) by solid-phase peptide synthesis strategy using Fmoc-protected amino acids. This method was employed on the PS3 peptide synthesizer (Gyros Protein Technologies, Uppsala, Sweden) at room temperature. Each cartridge was loaded with 0.5 mmol of the required amino acid, 0.068 grams of 1-Hydroxy-7-azabenzotriazole (HOAt), and 0.190 grams of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU). For the synthesis of peptides on the PS3 peptide synthesizer, the reagents utilized consisted of dimethylformamide (DMF), 20% piperidine, and 0.4 M N-methylmorpholine (NMP) (Thermo Fisher Scientific, Waltham, MA). Peptide synthesis was completed while under N_2. Following the completion of the reaction, the resin was subjected to dichloromethane (DCM) washes followed by vacuum drying. The weight of the resin was determined, and the peptide was readied for cleavage. The peptides were then cleaved from the resin at room temperature, employing a mixture consisting of 98% trifluoroacetic acid (TFA) and 2% distilled, deionized water. The solution was filtered and transferred into ice-cold diethyl ether and underwent three extra washes using diethyl ether. The peptides were subjected to lyophilization by being suspended in deionized water. Following this, the peptides were dried under vacuum conditions and subsequently stored as solids at room temperature.

Synthesis of Branched Amphiphilic Peptide-Magnetic Nanobeads (BAPCs-MNBs)

A cysteine residue was incorporated at the C-terminus of the oligo-lysine segment within the bis-(Ac-FLIVIGSII)-KKKKK-C-$CONH_2$ peptide. This cysteine residue was covalently

bonded to maleimide groups situated on the surface of magnetic nanobeads (Ocean NanoTech, San Diego, CA) using a solvent mixture consisting of 75% Ethanol and HEPES. As per the method described by Natarajan et al.[24], we employed 600 nanomoles of peptide for every 0.5 milligrams of MNBs, which marked the point of peptide saturation on the MNBs' surface. This led to the hydrophobic segments of the peptides aligning themselves towards the organic solvent, prompting the peptides to adopt a helical secondary structure, thus preserving their mono-dispersion. The peptides formed capsules when they assumed a beta structure in an aqueous solution. We synthesized peptide monolayer-coated magnetic nanobeads using the bis-(Ac-FLIVIGSII)-KKKKK-C-$CONH_2$ peptide in its chloride salt form, following the same procedure as previously detailed. Afterward, the particles were re-dispersed in pure TFE (trifluoroethanol). Another peptide, bis-(Ac-FLIVIGSII)-KKKKK-$CONH_2$, was introduced at double the concentration used to create the initial bilayer, which equated to 1200 nanomoles per 0.5 milligrams of MNBs. To encourage the formation of a bilayer on the magnetic nanobeads, water was gradually added to dilute the TFE, resulting in a final water-to-TFE ratio of 9:1. This promoted the creation of a beta structure, enabling the branched sequences to interact and construct a bilayer on the magnetic nanobeads. Following a resting period of 20–30 min on a magnetic rack, the BAPc-MNBs were attentively collected and then concentrated by means of a rotavapor featuring a water bath set at 40℃. This meticulous procedure guaranteed the thorough removal of TFE, ultimately yielding the final product, BAPc-MNBs, which were subsequently re-suspended in pure water.

Zeta potential measurement

Zeta potentials in each sample were assessed utilizing a Zetasizer Nano ZS (Malvern Instruments Ltd, Westborough, MA). Zeta potential measurements were carried out with the power adjusted to a maximum of 200 V, involving a total of 340 measurements per sample, and the resulting mean Zeta potential was reported.

Nanoparticle Tracking Analysis (NTA)

NTA assessments were conducted utilizing a NanoSight LM14 instrument (Malvern Panalytical, UK). This instrument was equipped with a sample chamber featuring a 405 nm laser, and digital imagery was captured through a CMOS camera (Hamamatsu Photonics K. K., Shizuoka, Japan). The introduction of samples into the sample chamber was achieved using sterile syringes (BD Discardit II, New Jersey, USA) until the liquid reached the tip of the nozzle. The NTA 3.3 Dev Build 3.3 301 software was employed to capture and analyze the data. The samples were measured for a duration of 60 sec at a frame rate of 25.0 frames per sec, utilizing a slide shutter setting of 1200 and manual gain adjustment. All measurements were carried out under room temperature conditions. The mean size and standard deviation values yielded by the NTA software align with the arithmetic calculations based on the sizes of all particles analyzed by the software.

Trypan Blue Exclusion assay

Cellular viability was assessed using the Trypan Blue exclusion assay. Following trypsinization, cultured cells were resuspended in growth medium. A 0.4% Trypan Blue solution was gently mixed with the cell suspensions. The determination of viable and non-viable cells

was conducted using the Countess™ 3 Automated Cell Counter (Invitrogen, Waltham, MA). The Countess™ 3 Automated Cell Counter employs specialized software to automatically identify and differentiate between viable and non-viable cells based on Trypan Blue staining. The percentage of viable cells was calculated by comparing the number of unstained (viable) cells to the total cell count obtained through the Countess™ 3 software. All experiments were conducted in triplicate to ensure reliability, and the results were reported as the mean ± standard deviation.

Gel retardation assay

Confirmation of the interaction between BAPCs and nucleic acids was achieved through a gel retardation assay. The resultant BAPCs-mRNA complex was adjusted to a final volume of 10 µL, then combined with 2 µL of 6× loading buffer. This mixture was loaded onto a 1.2% agarose gel supplemented with 3 µL of ethidium bromide (EtBr). Electrophoresis was carried out in a running buffer of 1× TAE at a voltage of 130 V for a duration of 15 min. Subsequently, the nucleic acid bands were visualized using UV light with a 300 nm wavelength.

In vitro GFP mRNA delivery

For GFP transfection experiments, cells were seeded at 80,000 cells/well in 0.5 mL of complete medium in an 8-well chamber and incubated. Following an overnight incubation, the culture medium was substituted with Opti-MEM Reduced Serum (Thermo Fisher Scientific, Waltham, MA) containing BAPC-GFP mRNA complexes featuring diverse weight by weight (w/w) ratios. Subsequently, the cells were incubated at 37℃ for 4 h in the presence of these complexes, after which they were replenished with fresh DMEM media. After 48 h, cells were washed with phosphate buffered saline (PBS) and fixed with 200 µL of 4% paraformaldehyde

(PFA) in PBS for 10 minutes at room temperature. Following the completion of transfection procedures and subsequent cell fixation, cell membranes were permeabilized with 0.1% Triton X for 1 hour (Thermo Fisher Scientific, Waltham, MA). After permeabilization of the cell membranes, the cells were washed with PBS and nuclei was stained with 4', 6'-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA). Cell transfected with Lipofectamine 3000™ were used as a positive control. GFP transfection efficiency of cells treated with BAPC-nucleic acid complexes was monitored by a LSM 880 laser-scanning microscope (Carl Ziess, Gottingen, Germany) and images were analyzed by using ZEN 3.5.

In vivo luciferase activity assay

The spray drying procedure, conducted by 'CritiTech', involved 4 mg of dry powder formulations. These formulations were divided into three groups: the mRNA-only treated group, the mannitol carrier group, and the lactose carrier group. Each group consisted of three mice. The 4 mg of dry powder included sugar carriers (mannitol or lactose) along with BAPCs/Fluc mRNA, where the mRNA amount within the 4 mg of powder was 10 μg. An in vivo murine model was employed, with three mice per group. Each mouse received a 50 μL injection of rehydrated powder from the 4 mg of dry powder formulations, which contained the specified 10 μg of Fluc mRNA. Luciferase activity was monitored at 24, 48, 72, 96, and 120 h post-injection to assess mRNA delivery and gene expression kinetics in vivo. The in vivo testing was performed at Princeton University in the laboratory of our collaborator Alex Ploss, ensuring a thorough evaluation of the experimental groups and their respective treatments.

For in vivo bioluminescence imaging assay, the study involved four distinct groups: BAPCs+mRNA, BAPCs+mRNA+poly I:C, BAPCs+mRNA+Heparin+OLL_{10}, and

BAPCs+mRNA+Heparin+OLL_{10}+poly I:C. Each group consisted of three mice, and all formulations were prepared in liquid form. A consistent injection volume of 50 μL was administered to each mouse, with 10 μg of Fluc mRNA delivered per mouse. A comprehensive monitoring of luciferase activity was conducted at various time intervals, including 4, 24, 48, 72, 96, and 120 h following the injection. This rigorous in vivo evaluation was carried out by researchers at Princeton University to assess mRNA delivery and the kinetics of gene expression within the living organisms.

Results and discussion

Biophysical analysis to characterize the properties of Branched Amphiphilic Peptide Capsules (BAPCs)

To study the creation of bilayers within BAPCs, we utilized iron oxide Magnetic Nano Beads (MNBs) that had undergone modification with maleimide functional groups. The peptide employed to form a monolayer on these magnetic beads was custom-designed with an additional cysteine residue at its C-terminus, specifically known as h_9Cys (bis (Ac-FLIVIGSII)-K-K_4-C-$CONH_2$) (**Figure 2.1A**). This particular adjustment allowed for the maleimide group to selectively react with sulfhydryl groups under conditions maintaining the pH within the physiological range.[13] As a result, a stable thioether linkage was formed, which is known to be non-reversible.[14] We utilized 600 nanomoles of , h_9Cys peptides/0.5 mg of MNBs to generate these thioether conjugates.[24] Before the formation of branched amphiphilic peptides (BAPs), indicating the formation of a monolayer, the MNBs exhibited notable negative surface charges,[25] as depicted in **Figure 2.1B**. Following the application of BAPs, there was an observed elevation in the surface charge of the MNBs from -12.07 ± 1.46 to +28.13 ± 5.30. This increase is believed to be attributed to the introduction of surface lysine residues by the BAPs onto the MNBs. Following the BAPs formation on MNBs, a second peptide devoid of cysteine, called h_9, (bis (Ac-FLIVIGSII)-K-K_4-$CONH_2$) was introduced into the system (**figure 2.1A**). The confirmation of bilayer formation on MNBs in an aqueous environment was achieved through the assessment of intermolecular π–π interactions and fluorescence quenching. It is established that intermolecular π-π interactions can induce fluorescence quenching.[15] Given that one of the primary forces driving bilayer formation in BAPCs is the intermolecular π–π interactions between the aromatic rings in phenylalanine of each peptide (**Figure 2.1C**), the reduction in

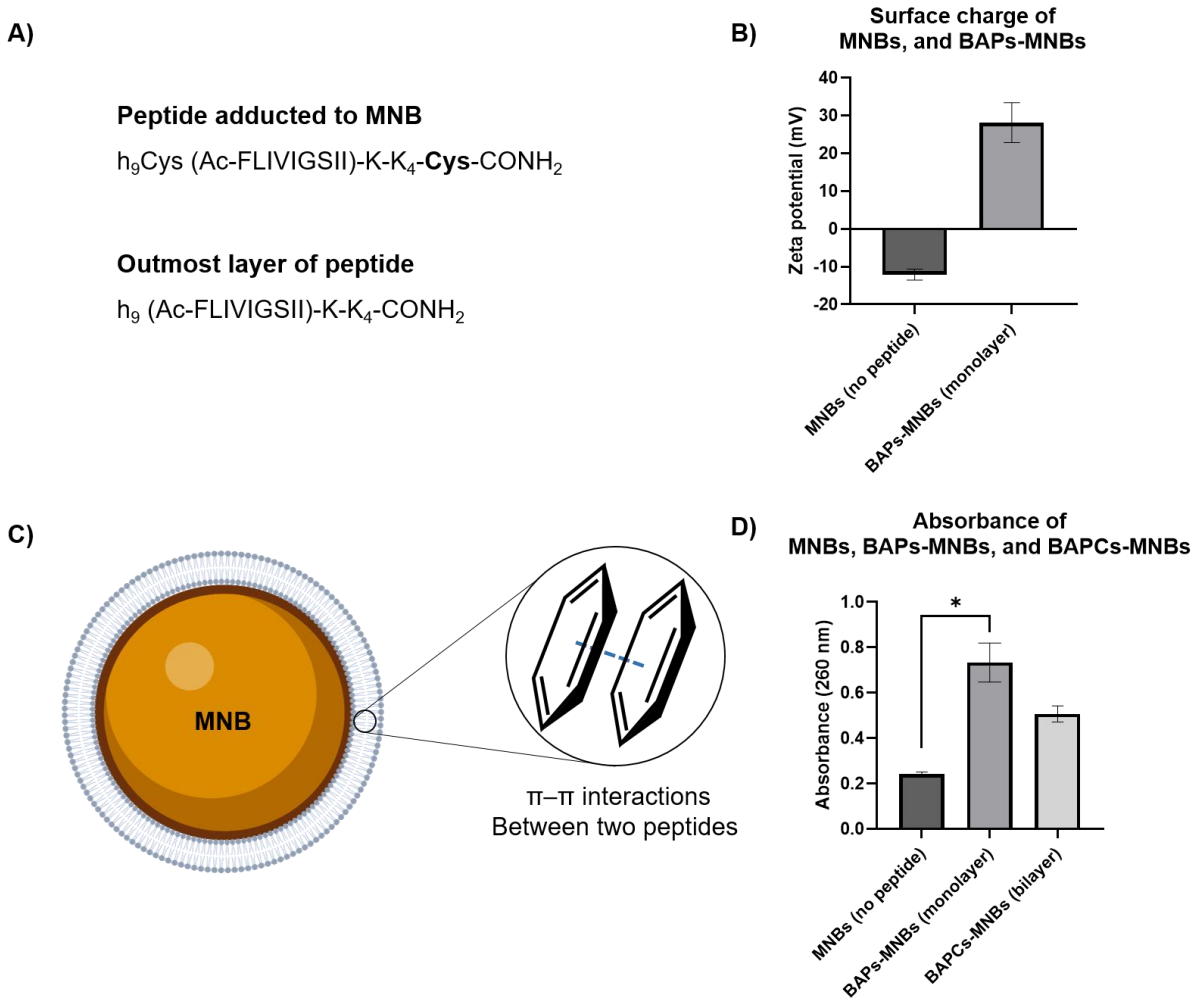

Figure 2.1. Biophysical characterization of BAPCs utilizing Magnetic Nano Beads (MNBs) to verify bilayer formation

A) Peptide sequences for monolayer and bilayer formation on MNB. The sequence of the cysteine added version of h_9, called h_9Cys, for the monolayer formation on MNB (top) and original h_9 sequence for bilayer formation (bottom). B) Zeta potential data demonstrating surface charge changes. zeta potential data indicating a shift from a negative charge on bare MNBs to a positive surface charge after monolayer formation, referred to as "BAPs-MNBs. C) Schematic image of BAPC-MNB and π–π interactions between two peptide strands. D) Absorbance changes from bare MNBs to BAPs-MNBs to BAPCs-MNBs at 257 nm. Absorbance decrease shown between monolayer and bilayers assumably by π–π interaction quenching effect. Kruskal–Wallis test followed by a Dunn's multiple comparison test relative to the MNBs (no peptide) was performed using GraphPad Prism version 8.0.2, n = 3; significance determined at * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (Schematic created with BioRender.com)

fluorescence at a wavelength of 257 nm, which corresponds to the maximum fluorescence absorption of phenylalanine, serves as an indicative measure of the π–π interactions between the peptides and the formation of bilayers. As shown in **Figure 2.1D**, fluorescence decrease was shown after bilayer formation. This is due to the interactions of bilayer formation of BAPCs on MNBs.

In conjunction with the use of MNB experiment, Nano Tracking Analysis (NTA) was employed to characterize the capsule-forming capacity and size of the BAPCs particles in a liquid suspension. NTA is a powerful technique that combines light scattering and Brownian motion to determine the size distribution of particles in a liquid medium.[19] In this process, a

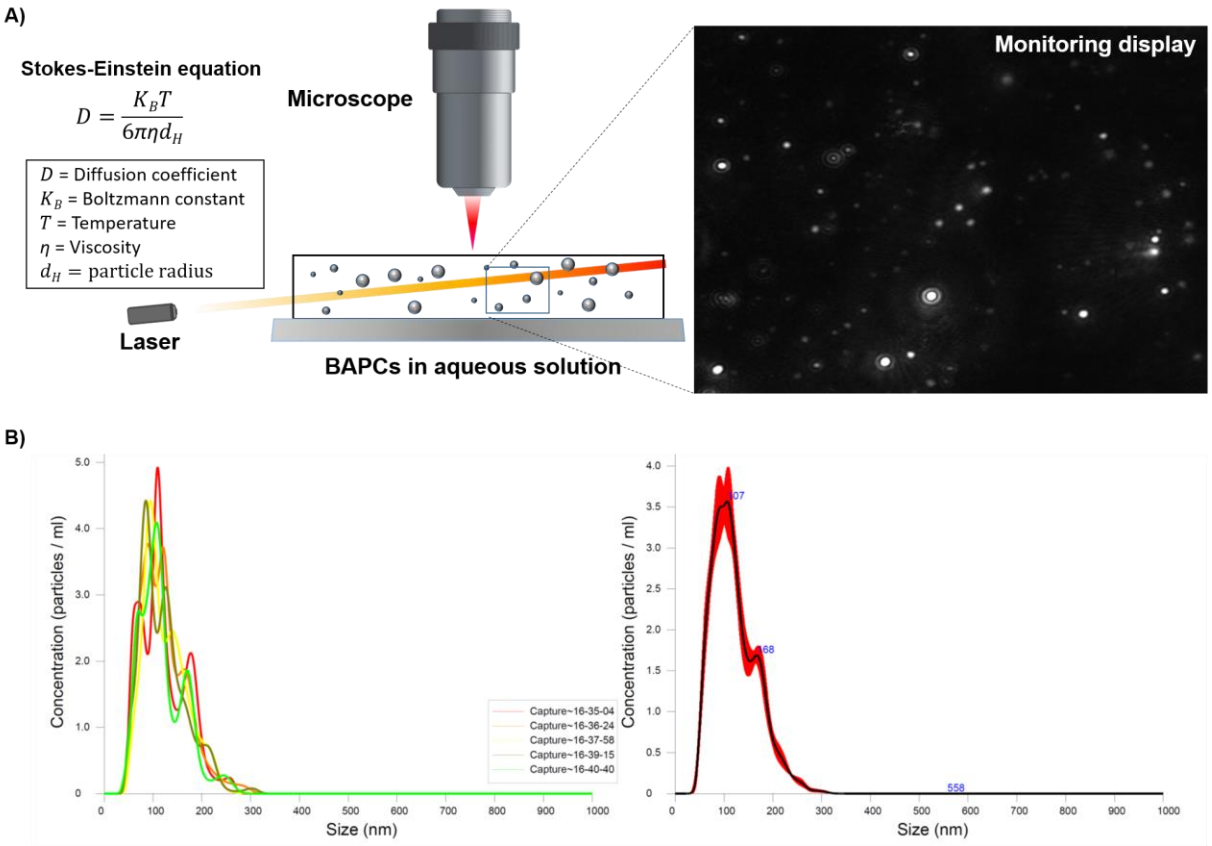

Figure 2.2. NTA data of BAPCs unveiling distinctive capsule shape and size properties

A) Schematic image of NTA operation and monitoring display showing 5 individual data runs. B) NTA graph data indicating BAPCs averaged size distribution and particle concentration.
(Schematic created with BioRender.com)

sample chamber is illuminated by a laser beam, and the scattered light is meticulously collected by a high-quality 20x microscope objective, which is then recorded by a camera.[20] The NTA software analyzes individual particles, and their hydrodynamic diameters are calculated using the Stokes-Einstein equation[20] **(Figure 2.2A)**. The NTA analysis of BAPCs, generated in deionized H_2O, provided fascinating insights. It unveiled distinctive capsule shapes, as depicted in **Figure 2.2A**, and yielded a mean size of 121.2 nm, with a remarkably low standard deviation of 1.1 nm. Additionally, the analysis revealed a mode size of 97.2 nm, accompanied by a standard deviation of 4.8 nm, as shown in **Figure 2.2B**. This robust analytical approach offers valuable information for the characterization of these unique particles.

Binding kinetics and saturation point of BAPCs with mRNA

In order to gain comprehensive insights into the interaction between BAPCs and mRNA, we employed gel retardation assay to investigate the time-dependent dynamics of this interaction. This method, operating under UV wavelength, enables visual differentiation of unbound and bound mRNA on BAPCs, facilitating the analysis of the kinetics and dynamics of this interaction. As illustrated in **Figures 2.3A**, the mRNA binding capacity steadily intensified over time, ultimately reaching nearly complete mRNA binding within 10 minutes.

After checking the complete binding time, BAPCs and mRNA binding saturation point was investigated. To prevent the overconsumption of materials and mitigate adverse effects resulting from an excessive use of nanoparticles or an abundance of mRNA, it is imperative to ascertain the most suitable mRNA binding capacity for BAPC-mRNA complexes. These determinations contribute to enhancing treatment efficacy while curbing therapeutic expenses as well. We prepared BAPC-mRNA complexes by mixing varying amount of BAPCs (200 ng, 600 ng, 1000 ng, 1400 ng, 1800 ng, 2200 ng and 2600 ng) with fixed mRNA amount (200 ng) to achieve BAPC-mRNA complexes of weight per wight (w/w) ratio of 1:1, 3:1, 5:1, 7:1, 9:1, 11:1 and 13:1, respectively. BAPC-mRNA complexes with over 9:1 (w/w ratio) did not show visible mRNA bands, suggesting that at that ratio there is a complete saturation of mRNA binding sites (**figure 2.3B**). It is known that water-soluble molecules with a high negative linear charge density, such as dextran sulfate or heparin, have been reported to facilitate the release of oligonucleotides from cationic carriers.[26, 27] In our pursuit of enhancing mRNA release post-cellular uptake, we explored the use of heparin. However, excessive heparin coating on the BAPC's surface can lead to reduced binding capacity for mRNA and premature mRNA release before uptake by the target cells. Therefore, it is crucial to use a minimally optimal amount of

heparin that ensures mRNA binding without causing premature release. **Figure 2.3C** illustrates that a BAPC:mRNA:Heparin w/w ratio of 9:1:0.24 achieved this delicate balance by minimizing heparin usage while ensuring a consistent BAPCs to mRNA ratio of 9 : 1 (w/w ratio) (**Figure 2.3B**).

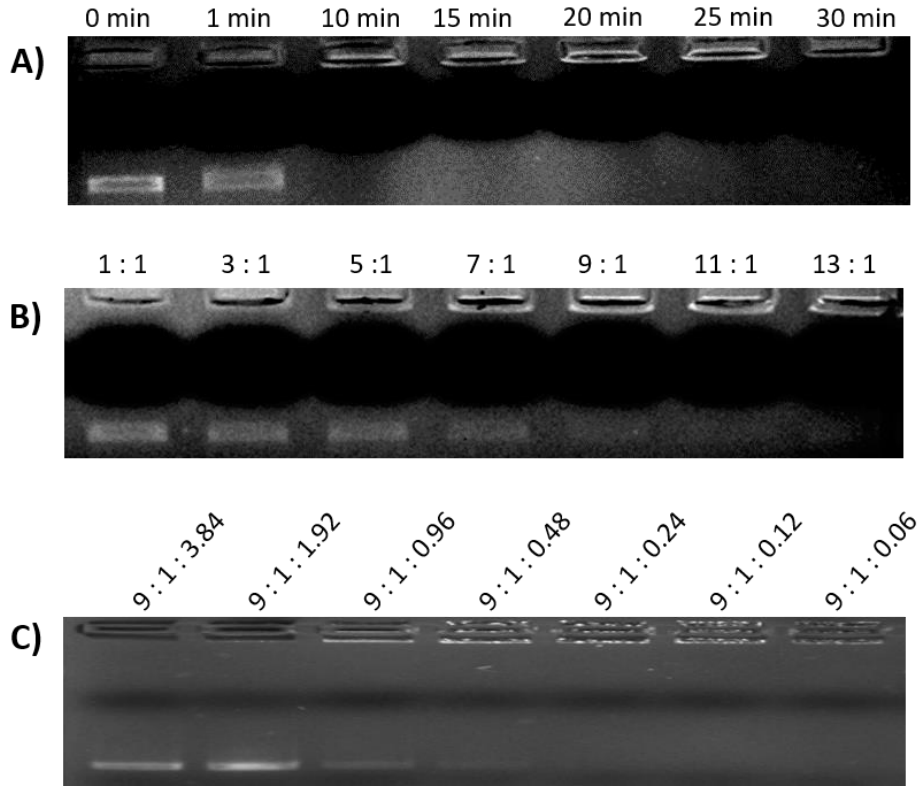

Figure 2.3. Dynamics and optimization of BAPC-mRNA interaction by gel retardation assay

A) Time-dependent mRNA binding capacity on BAPCs. Time-dependent dynamics of the interaction between BAPCs and mRNA using a gel retardation assay, revealing a gradual increase in mRNA binding capacity over time, achieving nearly complete binding within 10 minutes. **B)** Optimizing BAPC-mRNA complexes for saturation. BAPC-mRNA complexes prepared with different BAPC-to-mRNA weight-to-weight (w/w) ratios. Complexes with a w/w ratio exceeding 9:1 exhibit no visible mRNA bands, indicating complete saturation of mRNA binding sites. **C)** Balancing heparin usage in BAPC-mRNA-Heparin complexes. Optimization of a BAPC:mRNA:Heparin complex with a w/w ratio of 9:1:0.24,

achieving a delicate balance between heparin usage and maintaining a consistent BAPC to mRNA ratio. This minimizes premature mRNA release while ensuring efficient binding.

Surface charge modification of BAPCs-mRNA complex with cationic molecules for positive surface charge

The surface charge of vectors designed for gene delivery stands as a pivotal determinant of their effectiveness. Specifically, the positively charged nature of these vectors holds paramount significance in fostering interactions with negatively charged cell membranes, facilitating endocytosis, and subsequent intracellular delivery.[21-23] This inherent attribute is particularly advantageous for successful gene transfer. Moreover, the surface charge profile exerts a pronounced influence on critical aspects such as biodistribution, circulation time, and immune responses.[28, 29] These factors collectively impact the overall success of gene therapy endeavors. Hence, it becomes evident that the surface charge, especially one with a positive charge, assumes a central role in shaping the therapeutic potential and efficacy of gene delivery vectors. Nevertheless, it's important to note that in the case of the BAPCs-mRNA-Heparin complex, a negative surface charge prevails due to the presence of negatively charged heparin and mRNA on the surface of BAPCs. Addressing this challenge is imperative, and efforts must be made to devise strategies that can impart a positive surface charge to the outermost layer of the BAPCs complex. In order to measure the surface charge of BAPC complex, we used zeta potential measuring method. The measurement of zeta potential involves introducing a solution into a cell with two gold electrodes. Applying voltage to these electrodes causes particles to migrate towards the electrode with an opposing charge.[30] Particle velocity is assessed using a Doppler technique, as a function of voltage, within the cell where a laser beam is transmitted, and fluctuations in scattered light intensity occur at a frequency that correlates with the speed of the particles; multiple voltage settings are employed to gauge particle speed, and this information

is used to determine the zeta potential.[30] The modification of gene delivery vectors to achieve a positive surface charge is a crucial endeavor in optimizing their efficiency. In this context, the utilization of PEG-diamine and divalent cations, such as Mg^{2+}, Ca^{2+}, and Zn^{2+}, presents a promising strategy. However, **Figure 2.4A** shows a challenging and inconsistent charge distribution on the BAPCs complexes despite these interventions. The introduction of PEG-diamine, known for its positive charge in solution, was expected to play a vital role in achieving the desired surface charge alteration. Yet, the outcome indicates a failure to consistently induce a positive charge on the BAPCs complexes (**Figure 2.4B**). This outcome necessitates a more comprehensive exploration into the underlying mechanisms responsible for this unexpected result. In parallel, the incorporation of divalent cations, such as Mg^{2+}, Ca^{2+}, and Zn^{2+}, was anticipated to interact with the negatively charged constituents of the BAPCs complex, potentially neutralizing the overall charge or even turning it positive. However, the data suggest that this approach too did not yield a consistent positive surface charge. Lysine, an essential amino acid, possesses a distinctive attribute with profound implications in the context of gene delivery vectors. Under physiological conditions, its intrinsic pKa values confer a positive charge to the molecule. The intrinsic positivity of lysine under physiological pH conditions makes it an attractive option for surface modification in gene delivery applications. In our experimental work, we employed commercial poly-L-lysine (PLL) and explored lysine's potential as the outermost coating molecule for BAPCs complexes, which led to an exciting breakthrough. The results revealed a dose-dependent increase in the positive charge of the complexes, as depicted in **Figure 2.4C**. Considering the substantial size of commercial PLL, we postulated that using lab made oligo-L-lysine with 10 lysine residues (OLL_{10}) might achieve a positive surface charge

with a reduced amount of OLL_{10} and w/w ratio. As illustrated in **Figure 2.4D**, when we used a combination of BAPCs: mRNA: Heparin: OLL_{10} in a w/w ratio of 9:1:0.24, The zeta potential

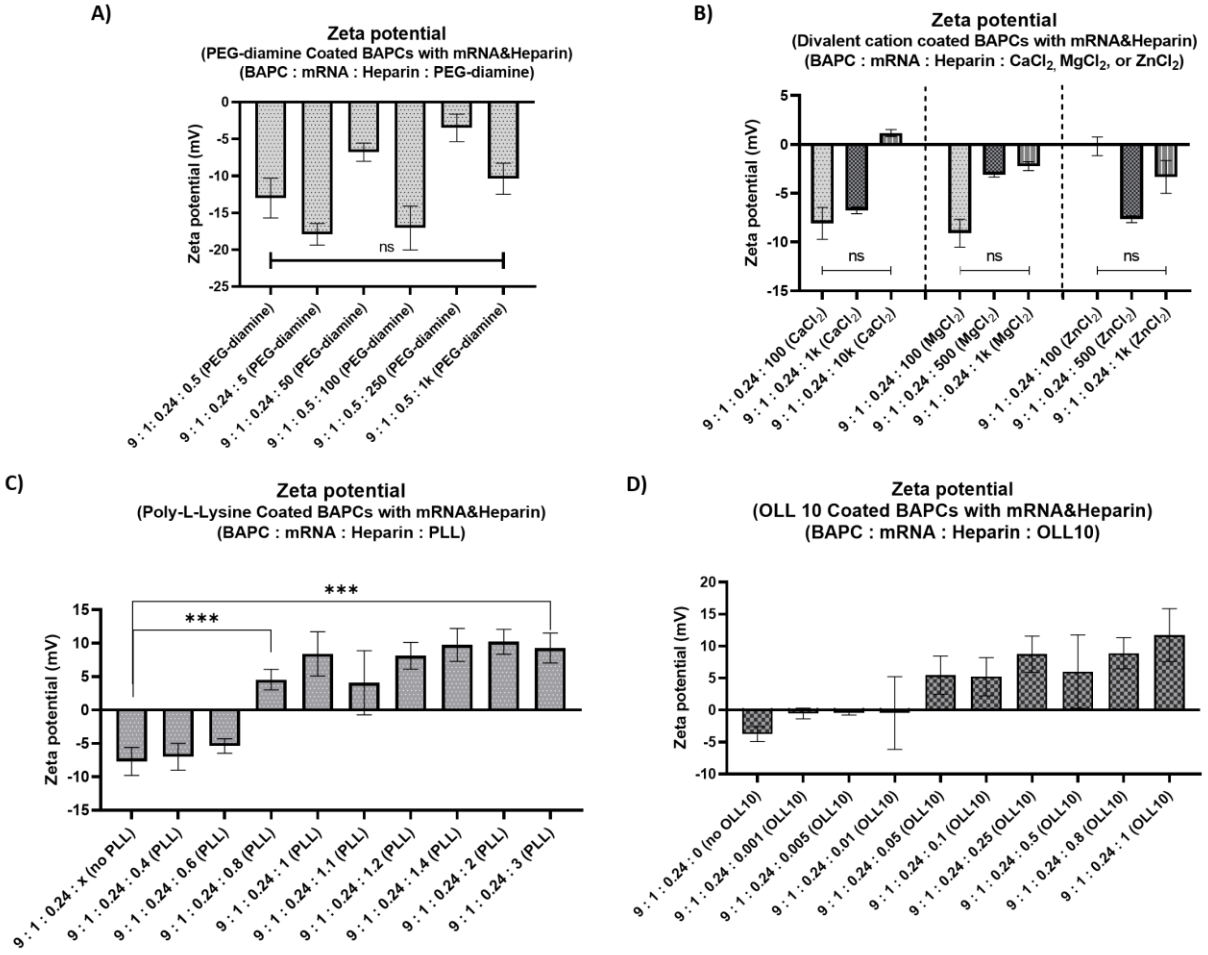

Figure 2.4. Surface charge modification of BAPCs-mRNA complex with cationic molecules

Zeta potential of BAPCs complex with A) PEG diamine, B) Divalent cations, Ca^{2+}, Mg^{2+}, and Zn^{2+}. Dose dependent increase of zeta potential with C) PLL and D) OLL_{10}. Error bars represent standard deviation. The Kruskal-Wallis test, followed by Dunn's multiple comparison, was conducted using GraphPad Prism version 8.0.2, (n = 3); significance determined at * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

data revealed a positive surface charge that reached saturation at a specific ratio, with a significantly lower w/w ratio of OLL_{10} in comparison to PLL. This discovery marks a significant advancement in our pursuit of attaining the desired surface charge, a crucial factor for efficient gene delivery. The ability to fine-tune the surface charge in a dose-dependent manner presents a

promising avenue for customizing the characteristics of BAPC as a gene delivery vector. This adaptability holds great potential for tailoring the vectors to specific applications and optimizing their effectiveness, thereby propelling the field of gene therapy forward.

In vitro GFP expression with positively charged BAPCs-mRNA complex

In order to validate the capability of positively charged BAPCs coated with lysine to improve the internalization of mRNA into mammalian cells, an experiment was performed using mRNA that encodes the Green Fluorescent Protein (GFP). GFP is a commonly utilized fluorescent marker in the field of molecular and cell biology, chosen for its capacity to mark and trace cellular elements and functions. Leveraging its distinctive fluorescent characteristics, GFP serves as a valuable instrument for tracking gene expression and pinpointing protein distribution within cells, thus playing a crucial role in this research.

In this experiment, BHK cells were co-incubated with BAPC-GFP mRNA complexes at various PLL w/w ratio from 0 to 1.2 for 4 hours in a culture medium with reduced serum (**Figure2.5**). For the negative control and positive control, non-treated cells (**Figure2.5A**) and Lipofectamine 3000 treated cells (**Figure2.5B**) were used, respectively. Optimal in vitro transfection parameters for BAPCs, including incubation times and transfection media, had been previously optimized. The BAPCs-mRNA-heparin complex did not yield significant GFP expression in the cells (**Figure 2.5C**), with only a slight enhancement observed. In the PLL-treated group from **Figure 2.5D to 2.5H**, BHK cells began to exhibit notable increase of GFP protein expression. Notably, cells treated with PLL w/w ratio 0.8 (**Figure 2.5F**) showed almost all cells are green fluorescent shown. Surprisingly, it was observed that the groups with higher

positive charge from PLL treatment (**Figure 2.5G**) did not exhibit significant differences, suggesting that excessive PLL treatment had minimal to no discernible effects.

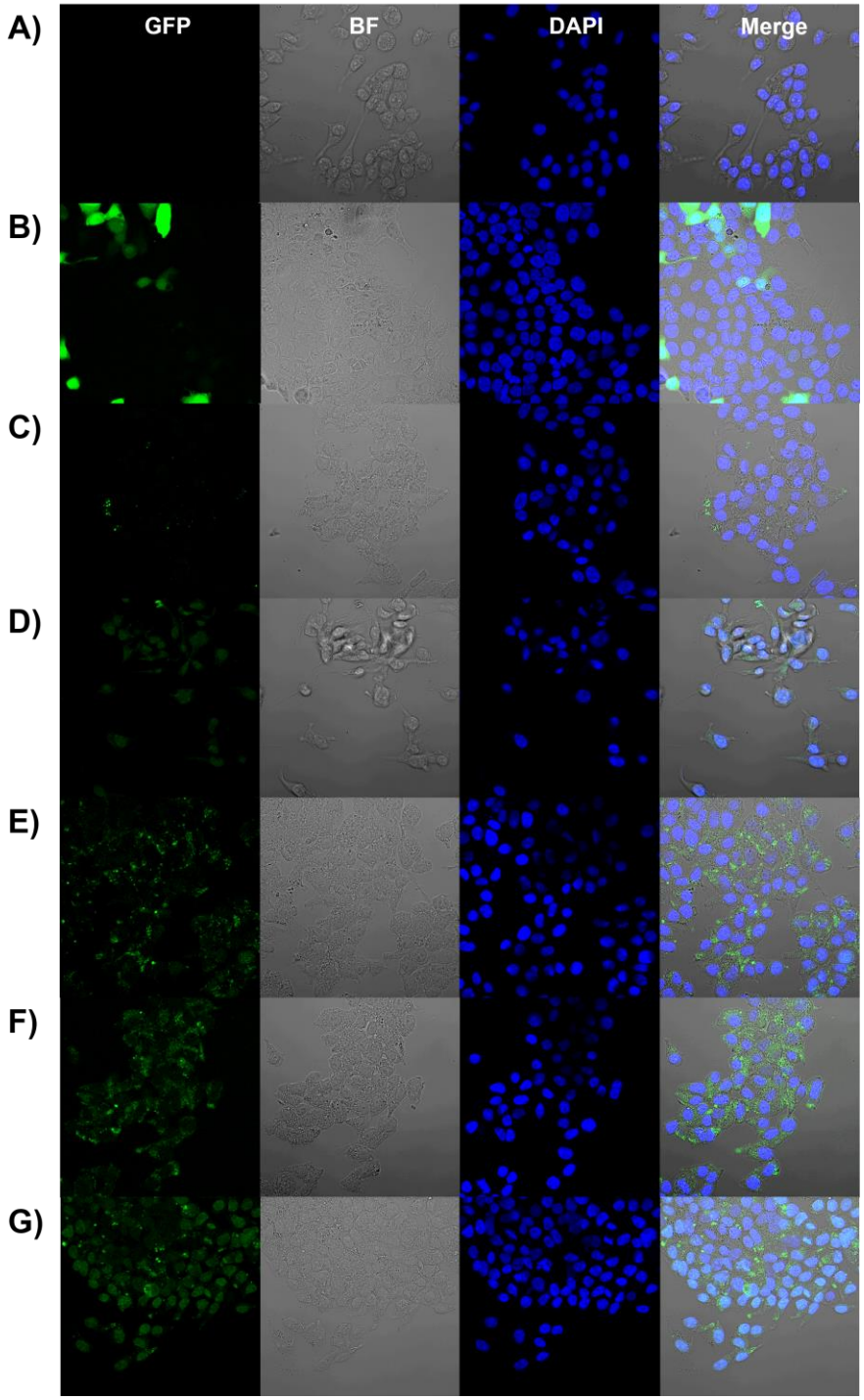

Figure 2.5. In vitro GFP mRNA transfection efficiency of BAPC-GFP mRNA complexes at varying PLL w/w ratios

A) Non-treated (negative control), B) lipofectamine 3000, and BAPCs:mRNA:Heparin:PLL (w/w) ratio 9:1:0.24: C) 0, D) 0.1, E) 0.4, F) 0.8 and G) 1.2.

Furthermore, the influence of BAPCs on cell viability was assessed using a Trypan blue exclusion assay. Both the standard amount and a doubled dose of BAPCs were applied to the cells, and cell viability was assessed at 24 and 48 h post-treatment. Remarkably, neither the standard nor the increased dose of BAPCs resulted in a decrease in cell viability during the specified time frames (**Figure 2.6**). Conversely, when cells were treated with Lipofectamine™ and MessengerMAX™ Lipofectamine™ 3000, commonly used transfection reagents, a significant reduction in cell viability was observed, particularly at the 48-hour mark. These

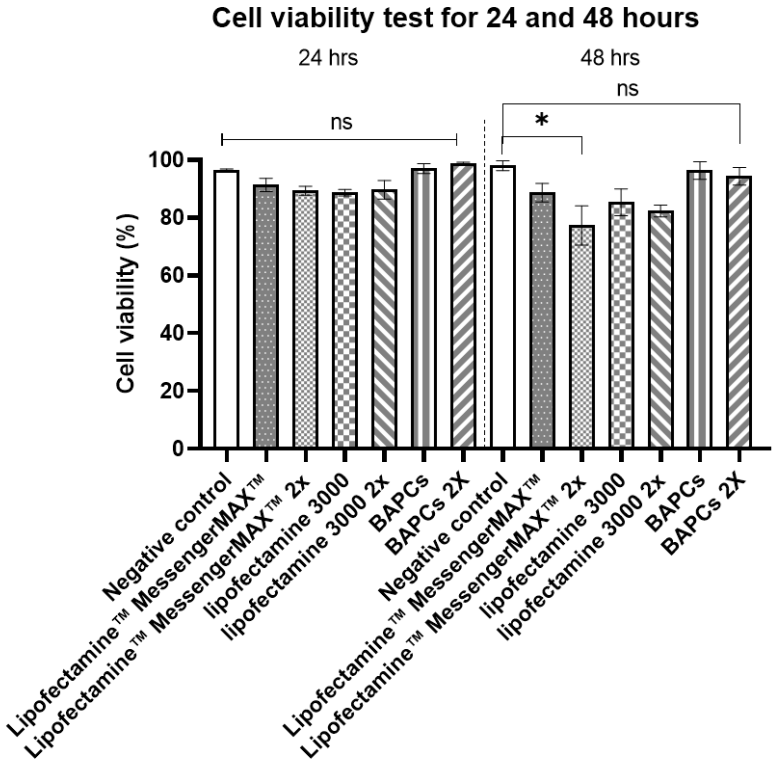

Figure 2.6. Trypan blue exclusion test of cell viability

Evaluation of BAPCs at standard and doubled doses over 24 and 48 hours. no adverse impact on cell viability, in stark contrast to Lipofectamine™ MessengerMAX™ and Lipofectamine™ 3000, which significantly reduced cell viability after 48 hours, highlighting the biocompatibility and safety of BAPCs for cell-based applications. Kruskal-Wallis test with subsequent Dunn's multiple comparison was performed using GraphPad Prism version 8.0.2, with a sample size of n = 3. Significance levels are indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

findings emphasize the biocompatibility and safety of BAPCs in contrast to lipofectamine, shedding light on their potential advantages in cell-based applications. This research contributes crucial insights to the field of cell manipulation, guiding the choice of reagents for preserving cell health.

In vivo luciferase mRNA delivery and expression

The transition from gene expression investigations within controlled in vitro settings to those within the complex in vivo environment signifies a critical and high-stakes stage in the progression and examination of gene delivery systems, holding considerable significance for translational medicine. While in vitro research provides valuable insights into cellular reactions and the fundamental processes, in vivo experiments are instrumental in bridging the gap between laboratory discoveries and potential clinical uses. In the forthcoming chapter, we commence an in vivo exploration of luciferase mRNA expression, with the primary aim of appraising the efficacy and safety of BAPCs as a vehicle for gene delivery. Luciferase, a light-emitting enzyme, has been widely employed as a reporter gene for in vivo studies due to its non-invasiveness and real-time quantifiability.[31] Measuring luciferase mRNA expression enables the evaluation of gene delivery vectors in terms of their efficiency, specificity, and duration of transgene expression within the physiological context.[31]

This chapter builds upon the promising in vitro results discussed previously, exploring the capabilities of our gene delivery system in an in vivo setting. By investigating luciferase mRNA expression, we aim to provide insights into the translational potential of our approach and its suitability for future therapeutic applications. The findings from this study contribute to the growing body of knowledge in gene therapy and hold promise for the development of novel treatments. The subsequent stage of our inquiry revolves around the evaluation of in vivo

luciferase expression by BAPCs for gene delivery evaluation. In this context, we categorized groups into BAPCs-mRNA, BAPCs-mRNA with the inclusion of poly I:C adjuvant, and BAPCs-mRNA with a positively charged surface facilitated by lysine surface coating, with and without Polyinosinic:polycytidylic acid (Poly I:C). The inclusion of poly I:C in vivo tests was deliberate. Poly I:C, a synthetic double-stranded RNA, possesses immunostimulatory properties and can act as a potent adjuvant.[34] In **Figure 2.7A**, the group treated with BAPCs and mRNA demonstrated luciferase expression in all three mice. In **Figure 2.7B**, the addition of the immunostimulant poly I:C to the BAPCs and mRNA regimen resulted in diverse responses. One mouse exhibited robust luciferase expression, while the other two displayed a weaker yet discernible level of expression. This variation underscores the dynamic interplay between the components involved in gene delivery and the immunostimulant, influencing the resulting in vivo gene expression patterns. Interestingly, unlike the significant impact observed in in vitro GFP expression (as shown in **Figure 2.5**), where positive surface modification through lysine coating was evident, **Figure 2.7C** and **2.7D** demonstrated that this positive surface charge did not induce luciferase expression, regardless of the presence or absence of the poly I:C adjuvant.

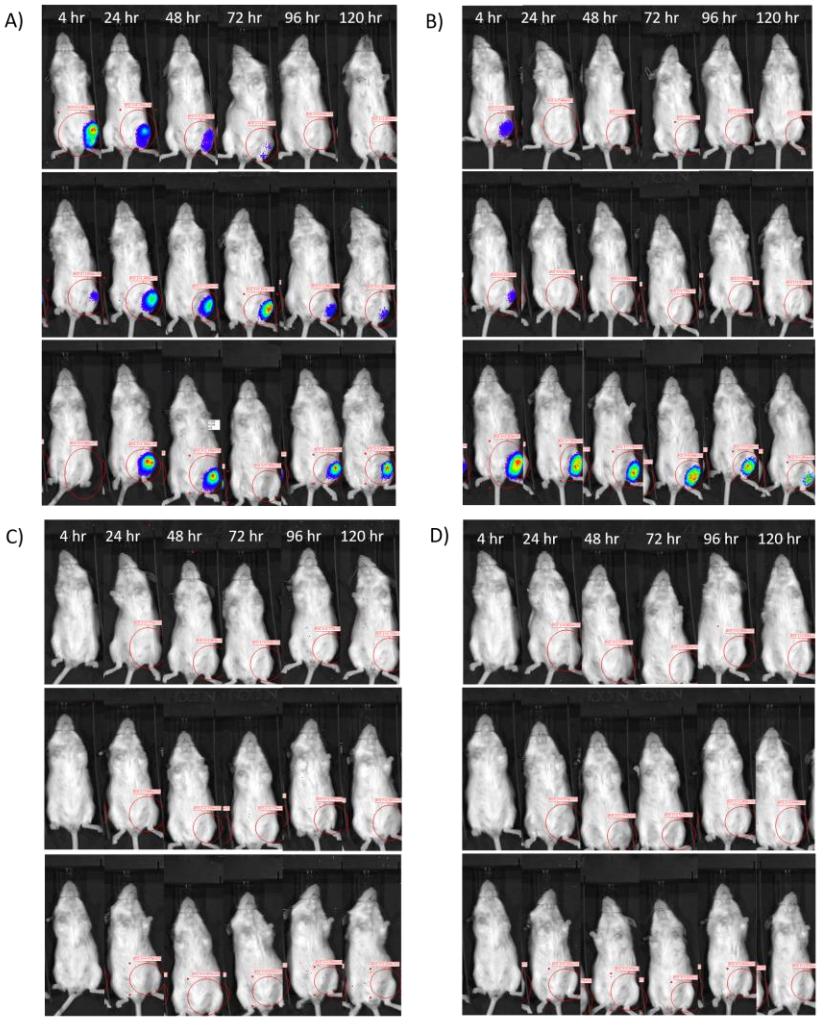

Figure 2.7. In vivo luciferase expression in response to BAPCs and Poly I:C

A) BAPCs-mRNA complex treated group (no positive surface charge). The group treated with BAPCs and mRNA displayed luciferase expression in all three mice, indicating successful gene expression. B) BAPCs-mRNA complex with poly I:C adjuvant treated group (no positive surface charge). diverse responses when the immunostimulant poly I:C was added to the BAPCs and mRNA regimen. One mouse exhibited robust luciferase expression, while the other two displayed a weaker but discernible level of expression. C) BAPCs-mRNA-Heparin-OLL_{10} (positive surface charge by lysine coating) and D) BAPCs-mRNA-Heparin-OLL_{10} with poly I:C adjuvant treated group (positive surface charge by lysine coating). positive surface modification through lysine coating did not induce luciferase expression, irrespective of the presence or absence of the poly I:C adjuvant.

Figure 2.8 illustrates the impact of poly I:C on GFP mRNA expression. **Figure 2.8A** serves as the negative control. **Figure 2.8B** shows the result for the group treated with Lipofectamine MessengerMax alone, without poly I:C. In **Figure 2.8C**, **2.8D**, and **2.8E**, cells were exposed to 10 ng, 50 ng, and 100 ng of poly I:C, respectively, while simultaneously being transfected with Lipofectamine MessengerMax. In all of these cases (**Figure 2.8C**, **D**, and **E**), there is a significant reduction in GFP expression compared to the group treated with Lipofectamine MessengerMax alone (**Figure 2.8B**). Considering the potential in vitro effect of Poly I:C on mRNA expression, shown in **Figure 2.8,** where Poly I:C significantly reduced GFP expression when delivered with Lipofectamine MessengerMax, a commonly used positive control, it becomes clear that Poly I:C has the capacity to adversely influence mRNA expression and, subsequently, protein expression. This understanding is particularly relevant when interpreting our in vivo results. In **Figure 2.7D**, where Poly I:C was used as an adjuvant, the observed lack of luciferase expression can be attributed to the potential toxicity of Poly I:C on protein expression. However, this explanation alone does not address **Figure 2.7C**, where Poly I:C adjuvant was not used, yet luciferase expression remained absent. This discrepancy underscores the complexity of our findings. The unexplained absence of luciferase expression in **Figure 2.7C** suggests that factors beyond the direct adverse effect of Poly I:C may be influencing the observed results. To gain a more comprehensive understanding of the intricate dynamics within our gene delivery system, further investigation is needed to uncover the underlying causes of this phenomenon. The interplay between various components in both in vitro and in vivo contexts introduce a layer of complexity to the optimization of gene delivery systems for in vivo applications. These observations underscore the nuanced distinctions

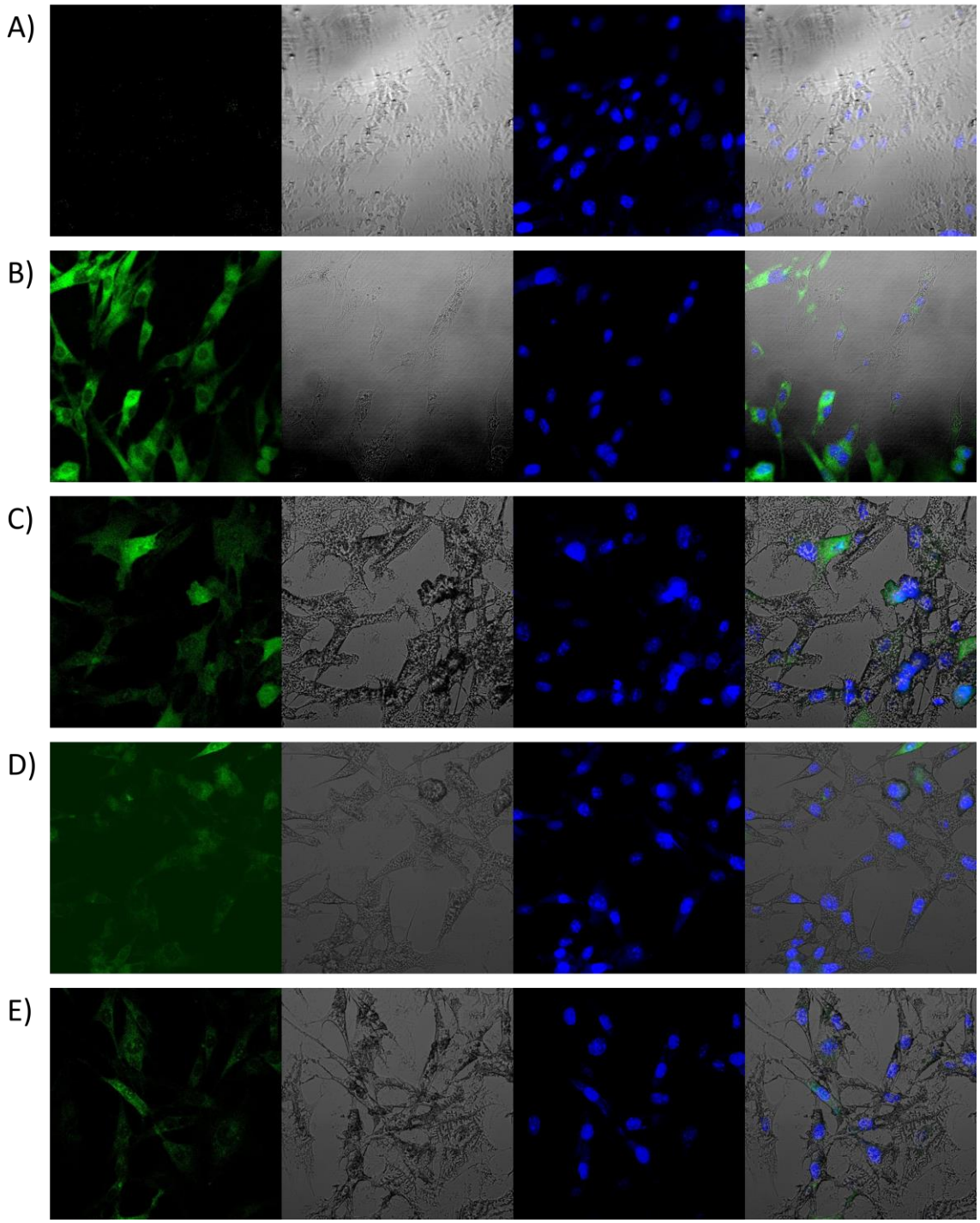

Figure 2.8 Toxic effect of poly I:C on GFP mRNA expression

A) Negative control (non-treated), B) Lipofectamine MessengerMax without Poly I:C, C) Lipofectamine MessengerMax with poly I:C (10 ng), D) Lipofectamine MessengerMax with poly I:C (50 ng), and E) Lipofectamine MessengerMax with poly I:C (100 ng).

between in vitro and in vivo responses, highlighting the intricate nature of optimizing gene delivery systems for in vivo applications. Exploring the underlying mechanisms governing these divergent outcomes calls for a more in-depth exploration. The observed discrepancies between in vitro and in vivo gene expression outcomes can be attributed to the multifaceted challenges and intricacies of the in vivo environment. In vivo settings present a complex milieu characterized by diverse cell types, dynamic immune responses, and tissue-specific microenvironments, all of which significantly influence the behavior of gene delivery vectors.

The subsequent stage of our inquiry revolves around the evaluation of luciferase expression in an in vivo setting subsequent to the implementation of the spray drying (SD) technique. The SD method, known for its adaptability as a drying approach, has showcased its effectiveness in preserving a diverse array of experimental vaccines. This preservation ability allows these vaccines to be storable at room temperature, obviating the necessity for refrigeration. It is worth noting that this method holds promise in developing non-viral dry powder systems designed for the delivery of genes via the respiratory route, presenting a host of advantageous features. Transitioning gene delivery systems from liquid to dry powder formulations presents a compelling rationale. Dry formulations enhance the stability, shelf life, and ease of handling compared to their liquid counterparts. Furthermore, they enable the development of inhalable gene delivery systems, which are particularly advantageous for targeted delivery to specific organs or tissues.[33] The ability to generate stable, efficient, and potentially respirable formulations through SD underscores its significance in advancing gene therapy and RNA-based therapeutics. In this phase of our investigation, we aim to explore the impact of SD on BAPCs' mRNA gene delivery, particularly in terms of in vivo luciferase expression. These endeavors hold promise for extending the utility and practicality of gene

delivery technologies in clinical applications. In our experiment, we conducted three distinct groups of investigations.

Figure 2.9A represents the control group without BAPCs, consisting solely of mRNA injection. In this context, little luciferase expression was detected in two mice at the 24-hour time point. Importantly, this expression was not sustained, and one mouse in this control group did not exhibit any luciferase expression during the entire observation period. Conversely, in **Figure 2.9B**, where mannitol was utilized in the SD process, one mouse demonstrated consistent luciferase expression throughout the entire 120-hour observation period, while two others displayed relatively lower but notable levels of expression. In **Figure 2.9C**, involving the lactose group, all three mice consistently exhibited high levels of luciferase expression throughout the entire 120 hours' observation period. These findings collectively underscore the robust and enduring mRNA delivery potential of BAPCs in the context of in vivo applications, even under conditions of SD and rehydration. This knowledge holds the potential to advance the field of gene therapy and RNA-based therapeutics, offering valuable insights into the optimization of gene delivery systems for clinical applications.

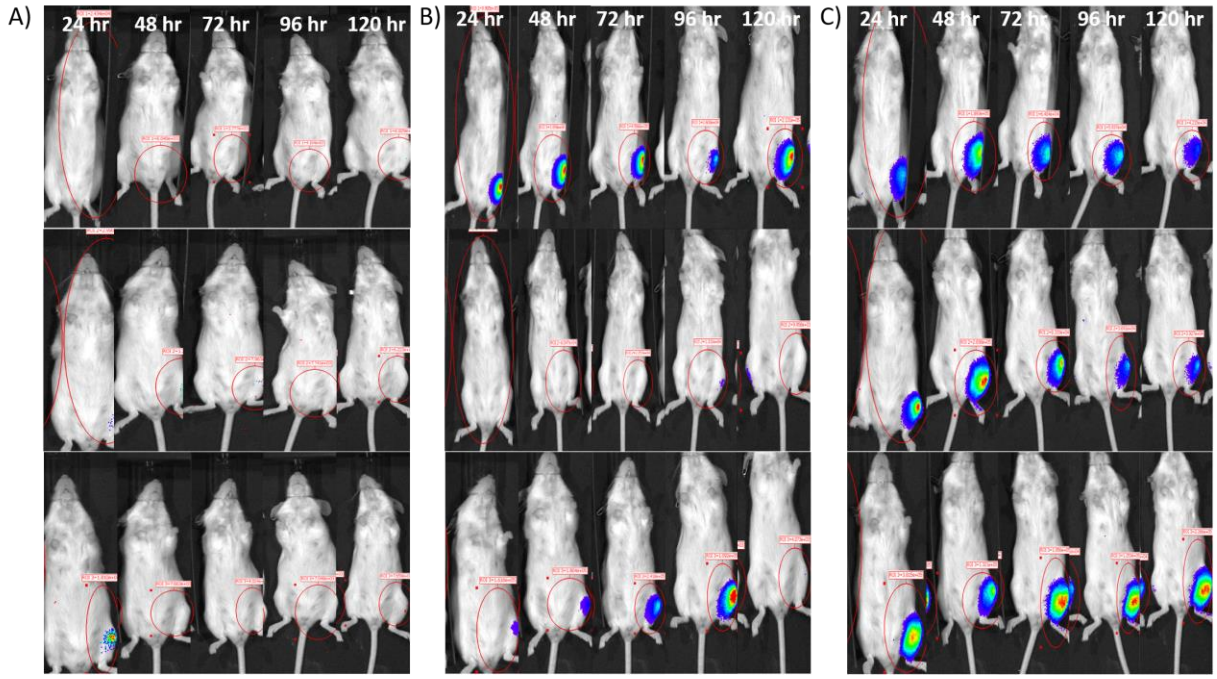

Figure 2.9. In vivo mRNA delivery using BAPCs with Spray Drying (SD)

A) Control group received mRNA injection without BAPCs. Little luciferase expression was detected in two mice at the 24-hour time point, which was not sustained, with one mouse showing no luciferase expression throughout the observation period. B) Utilizing the spray drying (SD) method with mannitol, one mouse consistently exhibited luciferase expression over the entire 120-hour observation period, while two other mice displayed relatively lower but notable levels of expression. C SD with lactose, showing all three mice consistently displayed high levels of luciferase expression throughout the entire 120-hour observation period.

The immune system plays a pivotal role in this context. Positively charged surface modifications introduced by additional layers, such as additional lysine outmost surface coating, can inadvertently trigger immune responses in vivo. The immune system's interaction with these vectors can lead to unexpected fluctuations in transgene expression, making it challenging to predict and control the performance of gene delivery systems. Furthermore, gene delivery vectors must navigate various tissue barriers within the living organism to reach their target cells, adding another layer of complexity. These barriers can impact the distribution, retention, and

overall effectiveness of the vectors. Lastly, the genetic variability among individuals in in vivo studies introduces a level of unpredictability. The genetic makeup of the subjects can influence the response to gene delivery vectors, potentially resulting in disparities in transgene expression. The dynamic and intricate nature of the in vivo environment, characterized by immune responses, tissue barriers, and genetic diversity, contributes to the observed differences between in vitro and in vivo gene expression outcomes. Addressing these complexities is essential for the successful translation of gene delivery strategies to clinical applications.

Conclusions

This study delves into the biophysical characterization and functional assessment of BAPCs, offering valuable insights into their potential for gene delivery applications. Through a comprehensive series of experiments and analyses, we explored the capabilities of BAPCs in delivering mRNA, addressing pivotal aspects in the realm of gene therapy and mRNA-based therapeutics. We employed NTA to determine the size distribution of BAPCs particles in a liquid suspension, revealing their unique capsule shapes and consistent size distribution. Importantly, we established that BAPCs are both biocompatible and safe for use in cell-based applications, surpassing conventional transfection reagents like Lipofectamine™. These investigation delved into the binding kinetics and saturation point of BAPCs with mRNA, leading to the discovery of optimal binding ratios. We also explored surface charge modification by using lysine coating as a promising strategy to achieve a desired positive charge in a dose-dependent manner. Furthermore, we examined the efficacy of the spray drying method for mRNA gene delivery, demonstrating promising results in terms of in vivo luciferase expression. The transition from in vitro to in vivo investigations shed light on the intricate nature of optimizing gene delivery systems for clinical applications. Notably, our research highlighted the role of immunostimulants and surface charge modifications in influencing in vivo gene expression, underscoring the complexities of the in vivo environment. These findings contribute to the growing body of knowledge in gene therapy, offering a path towards more effective and tailored gene-based therapeutics.

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Chapter 3 - Fine-tuning mRNA delivery: Exploring the power of histidine-modified Bilayer Amphiphilic Peptide Capsules (BAPCs)

Introduction

During the course of in vitro and in vivo experiments employing BAPCs as vectors for gene delivery, the lab encountered promising initial results. Most cells displayed GFP expression, a clear indicator of successful cellular uptake. However, upon closer examination, a notable discrepancy became evident when comparing our results to those of the positive control group. Unlike the positive control (LipofectamineTM system), I did not consistently observe the overexpression of the delivered GFP mRNA. Additionally, our in vitro luciferase experiments with BAPCs did not produce discernible expression results. Furthermore, we explored the potential of layer-by-layer coating of lysine onto the BAPCs complex to enhance gene delivery. Regrettably, this approach did not yield luciferase expression in vivo, potentially due to an immune response triggered by the additional lysine surface layer coating. These compelling observations have led us to consider modifications to the original BAPCs sequence without the layer-by-layer additional coating. The goal is to enhance our gene delivery capabilities and address the discrepancies I observed.

Endosomal escape is a formidable challenge that gene delivery vehicles must overcome to achieve effective transfection of target cells.[1-5] This process unfolds within the cellular landscape following cellular uptake, typically through endocytosis. These vehicles often become trapped within endosomes, which are membrane-bound compartments crucial for the cell's waste disposal and recycling functions.[6-8] While endosomes are crucial for cellular functions, their acidic environment can lead to conformational changes in biomolecules and potential degradation of the genetic cargo, mainly through lysosomal degradation. Lysosomes are

organelles specialized in cellular waste disposal, containing enzymes that break down biomolecules, including genetic material.[9, 10] When gene delivery vehicles are trapped within endosomes and fuse with lysosomes, the risk of cargo degradation rises.[11, 12] Thus, it is vital to develop strategies for escaping the endosomal-lysosomal pathway to ensure successful gene delivery. Successful transfection requires an escape from these endosomes,[1, 13-15] and this is where the concept of endosomal escape becomes pivotal. Effective endosomal escape mechanisms involve the disruption or permeabilization of the endosomal membrane. Once in the cytoplasm, the genetic cargo remains functional, facilitating its incorporation into the host cell's machinery. Ensuring that the genetic cargo reaches its intended destination and remains functional is a key challenge in gene therapy and drug delivery.

In parallel, I recognize the unique behavior of histidine, a pivotal amino acid with a specific pKa value, approximately 6.0. Its side chain possesses the remarkable ability to transition between neutral and positive charges, contingent upon the surrounding physiological pH conditions. As the pH in the late endosome environment typically falls below the histidine pKa, reaching levels around 5.0,[16] the histidine side chain becomes positively charged. This intriguing behavior aligns with the "proton sponge effect," wherein the positively charged histidine side chain attracts and entraps protons, which triggers a cascade of events involving chloride ions and subsequent water influx within the late endosome.[17-19] The initial influx of protons and subsequent chloride ions creates an osmotic imbalance, drawing water into the endosome and causing it to expand and eventually burst, facilitating the release of the gene delivery vehicles (Figure 3.1).[19, 20] These compelling observations have steered us towards a pivotal consideration—modifications to the original BAPCs sequence, aimed at striking a delicate balance with histidine content within the peptide structure. The overarching goal is to

enhance stability while facilitating endosomal escape post-endocytosis, ultimately improving transfection efficiency in the context of gene delivery.

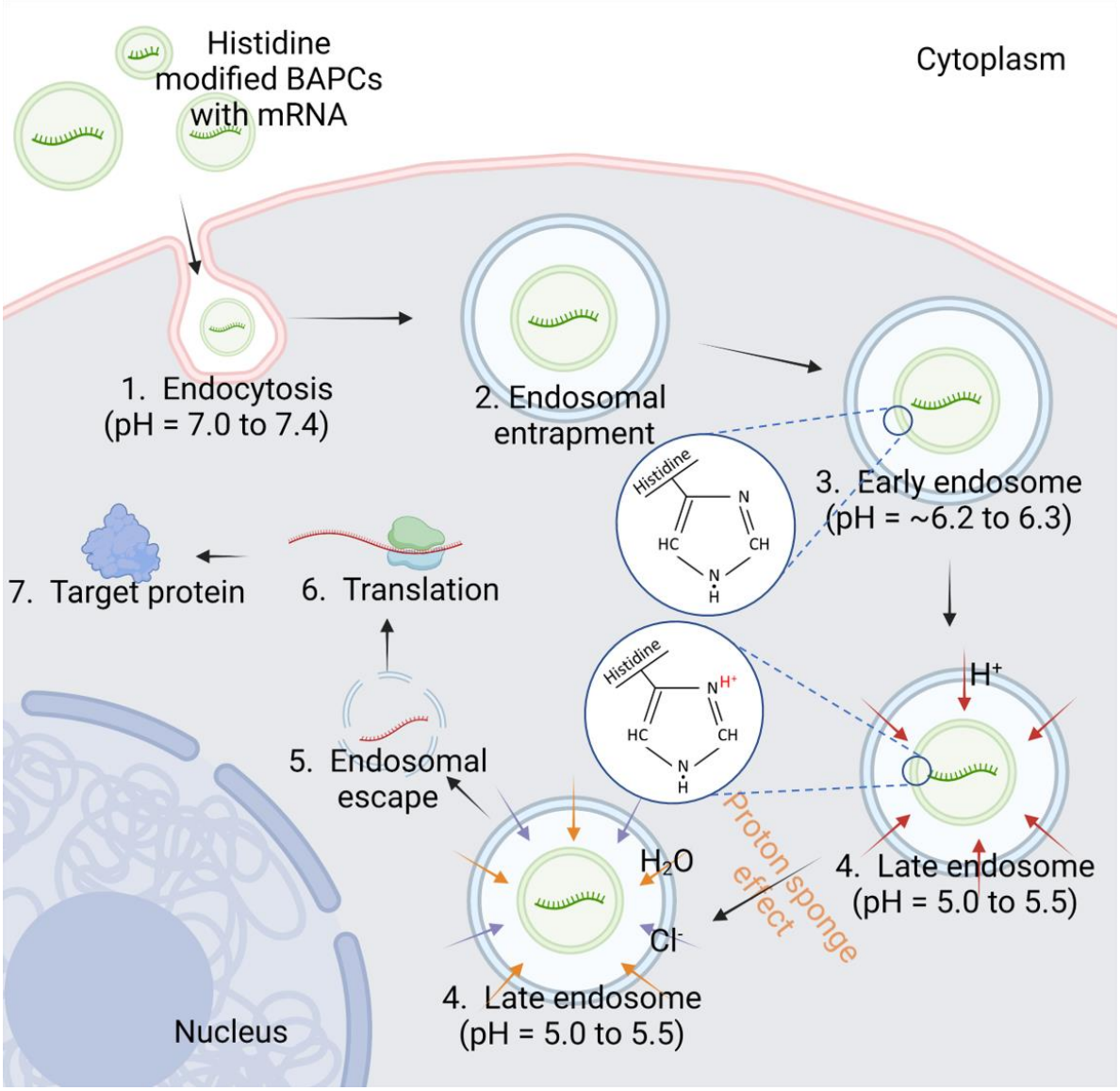

Figure 3.1. Schematic illustration of endosomal escape of histidine modified BAPCs by proton sponge effect

Endosome maturation involves a gradual pH decrease from early to late stages, promoting protonation of histidine residues with a lower pKa. In histidine-modified peptides, such as h5h and h9h, these protonated residues are pivotal in capturing protons, chloride ions, and water, triggering osmotic swelling and compartment rupture. (Schematic created with BioRender.com)

Within the intricate architecture of BAPCs, an array of forces and interactions contributes to its remarkable stability. These include hydrogen bonding, as well as inter- and intra-molecular pi-stacking (π–π) interactions between the aromatic rings of phenylalanine in the peptide sequences.[21] Yet, one of the fundamental forces binding BAPCs is the hydrophobic interaction between the two peptide sequences.[21] This interaction closely resembles the behavior of liposomes and plays a pivotal role in forming the capsules.[22] In light of these structural considerations, the introduction of an excessive number of hydrophilic amino acids, such as histidine, into the original BAPCs peptide sequence poses a risk. It could potentially hinder bilayer formation or lead to instability due to the reduced hydrophobic character. Conversely, incorporating too few histidine residues may not offer a substantial advantage in terms of facilitating endosomal escape post-cellular uptake. Our central hypothesis revolves around achieving the optimal modification and quantity of histidine in the BAPCs peptide sequence. The goal is to strike a delicate balance, allowing us to preserve the stability of the BAPCs capsules while harnessing the potential of histidine to aid in endosomal escape post-endocytosis following cellular uptake (**Figure 3.1**).

In light of the considerations described above, I embarked on redesigning the capsules by introducing histidine into the original h_5 and h_9 peptides. These newly designed sequences were referred as h_5h and h_9h, representing histidine-modified peptides for BAPC formation. To comprehensively assess their capsule-forming capacity, I combined the original peptides h_5 and h_9 with the histidine-modified peptides, h_5h and h_9h, creating a spectrum of possible number of cases and scenarios for evaluation. The evaluation of histidine-modified BAPCs' capsule-forming ability was conducted using Nano Tracking Analysis (NTA). This technique provided valuable insights into the size and distribution of the capsules, shedding light on their structural

integrity. To gage the gene delivery capabilities of these modified BAPCs, I employed in vitro assays, including the assessment of Green Fluorescent Protein (GFP) expression. The Green Fluorescent Protein (GFP) expression assay allowed us to visualize the extent to which histidine-modified BAPCs successfully delivered and expressed the GFP gene within target cells. The intensity and distribution of green fluorescence offered direct insights into the transfection efficiency of these modified peptides. The results of this assay helped us gauge the effectiveness of histidine-modified BAPCs as gene carriers. Additionally, I conducted in vitro luciferase assays, a widely recognized technique for quantifying the expression of a luciferase reporter gene. The luciferase assays were an essential component of our study, as they provided a means to measure the delivery and expression of the luciferase gene. Unlike the in vitro GFP expression assay, the original BAPCs experiment did not exhibit significant luciferase expression, making this aspect of our research particularly significant. The results of the luciferase assays further illuminated the transfection efficiency of histidine-modified BAPCs, enhancing our understanding of their potential as a nucleic acid carrier.

This strategic approach of modifying BAPCs with histidine residues, coupled with rigorous testing through NTA, GFP expression, and luciferase assays, represents a critical step towards comprehending the viability and promise of these innovative peptide sequences in the fields of gene delivery. It underscores the meticulous and methodical approach I have undertaken to assess the capabilities of histidine-modified BAPCs. These findings hold great potential and contribute to the advancement of gene therapy and drug delivery systems, offering the prospect of more efficient therapeutic interventions for a wide range of genetic and acquired diseases.

Materials and methods

Histidine modified peptide syntheses

The synthesis of histidine-modified peptides, specifically h_5 and h_9, involved the incorporation of histidine residues into the original peptide sequences, h_5 bis(FLIVI)-K-KKKK and h_9 bis(FLIVIGSII)-K-KKKK. In the case of the h_5 peptide modification, four histidines were added, with two histidines in each of the branched peptide tails (referred to as h_5h). For the h_9 peptide modification, six histidines were introduced, with three histidines in each branched peptide tail (referred to as h_9h). The solid-phase peptide synthesis, as detailed in the Materials and Methods section of the previous Chapter 2, involved the synthesis of linear peptides on clear amide resin (Peptides International, Louisville, KY). Briefly, this synthesis process was carried out at room temperature using a solid-phase peptide synthesis method. Fmoc-protected amino acids were employed, and the synthesis was carried out on the PS3 peptide synthesizer (Gyros Protein Technologies, Uppsala, Sweden). In each cartridge of the synthesizer, 0.5 mmol of the required amino acid was loaded, along with 0.068 grams of 1-Hydroxy-7-azabenzotriazole (HOAt) and 0.190 grams of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU). Reagents for the peptide synthesis, including dimethylformamide (DMF), 20% piperidine, and 0.4 M N-methylmorpholine (NMP) from Thermo Fisher Scientific in Waltham, MA, were utilized. The synthesis was conducted under a nitrogen atmosphere. After the reaction, the resin was vacuum-dried with dichloromethane (DCM), and its weight was determined before cleavage. Cleavage of the peptides from the resin was achieved at room temperature using a mixture of 98% trifluoroacetic acid (TFA) and 2% distilled, deionized water. This solution was then filtered and transferred into ice-cold diethyl ether, followed by three additional diethyl ether washes. The peptides were subsequently

lyophilized by suspension in deionized water, dried under vacuum, and stored as solids at room temperature.

Peptide combination experiments

Following peptide synthesis and cleavage, the dried peptides were reconstituted in pure 2,2,2-trifluoroethanol (TFE). To determine the peptide concentration, I employed a CARY 50 Bio UV-visible spectrometer (Agilent Technologies, Santa Clara, CA) and measured the absorbance of phenylalanine at 257.5 nm. The calculation of peptide concentration in the solution was based on Beer's Law (A = absorbance, ε = molar extinction coefficient, c = concentration, l = path length), with a path length of 0.3 cm. It's worth noting that this calculation was adjusted by dividing by 2 due to the presence of one phenylalanine in each branched peptide tails. The molar extinction coefficient for phenylalanine at 257.5 nm (195 M^{-1} cm^{-1}) was utilized for these concentration determinations. Once the concentrations of the individual peptides were determined, I proceeded to explore various combinations of h_5, h_9, h_5h, and h_9h peptides. This exploration led to the identification of seven distinct cases, each denoted as follows: h_5h alone (case 1), h_9h alone (case 2), h_5h combined with h_5 (case 3), h_5h combined with h_9 (case 4), h_5h combined with h_9h (case 5), h_9h paired with h_5 (case 6), and h_9h combined with h_9 (case 7). In cases 3 to 7, where two peptides were combined, I used equal molar concentrations of any two peptides from the h_5, h_9, h_5h, and h_9h set. The combined molecular weight was then employed to calculate the overall concentration for these combinations.

Circular (CD) measurement

CD spectra were acquired using a Jasco-815 spectrophotometer (Jasco Analytical Instruments, Easton, MD). Peptides modified with histidine were prepared according to the materials and methods, with variations in pH levels (pH 4, pH 5.5, pH 7.2) using Milli-Q (MQ) water. For pH 4 and 5.5, 0.2 M HCl was used to adjust the acidic pH, while pH 7.2 samples utilized NaOH buffer adjusted to pH 7.2. To assess mRNA encapsulation, peptides from each case were meticulously dried using a vacuum evaporation system to achieve a final concentration of 0.1 mM upon rehydration with mRNA in H_2O. The mRNA concentration was adjusted according to the amount of peptides in each case to achieve a targeted encapsulation ratio of 50:1 (w/w). Nitrogen gas was passed through the CD spectrophotometer during sample runs. Spectra were collected using 1 mm path length quartz cuvettes (Starna Cells Inc., Atascadero, CA), scanning from 260 nm to 190 nm at a rate of 50 nm/min with 1 nm step intervals. Each sample underwent five scans, which were then averaged. Ellipticity was measured in millidegrees. A Savitsky-Golay filter provided by the manufacturer was applied for spectrum smoothing. Data correction was carried out using TFE and MQ water based on the sample solvent.

Nanoparticle Tracking Analysis (NTA) measurement

To evaluate the size distribution and capsule-forming ability of histidine-modified BAPCs, they were prepared at 1 mg/mL concentration methods described in Peptide combination design. For mRNA encapsulation assessment, 200 µg/mL concentration of mRNA in H_2O was used to rehydrate dried peptides to achieve the 50:1 (w/w) ratio encapsulation. NTA analysis employed a NanoSight LM14 instrument (Malvern Panalytical, UK) with a 405 nm

laser. Samples were introduced using sterile syringes, and NTA 3.3 software analyzed data for 60 secs at 25.0 frames per sec.

Dynamic Light Scattering (DLS) and zeta potential measurement

Zeta potential evaluations for each sample were conducted using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Westborough, MA). The measurements were conducted with the power set to a maximum of 200 V, encompassing a total of 340 measurements per sample, and the resulting average zeta potential was recorded. For particle size and polydispersity index (PDI) assessments, a cuvette was utilized to contain the sample, with the Zetasizer temperature set at 25℃. Each measurement involved 25 consecutive runs, yielding standard deviations alongside the particle sizes. Post-measurement, the provided results encompassed size distribution profiles and PDI.

In vitro GFP mRNA expression with histidine modified BAPCs

The GFP mRNA was encapsulated within the histidine-modified BAPCs during the rehydration process, wherein the designated amount of each case was dried using a vacuum evaporation system. Briefly, GFP mRNA was both encapsulated and surface bound in histidine-modified BAPCs by hydrating dried peptide form previously dissolved in TFE with nuclease-free water together with the nucleic acid and then incubated for 10 min at room temperature. In the assessment of GFP mRNA expression, an 8-well chambered slide was employed. Each chamber was seeded with $1x10^5$ CHO cells, and on the following day, transfection was carried out using a transfection reagent with histidine-modified BAPCs in Opti-MEM media. This transfection process lasted for 2 hours. Subsequently, DMEM containing 10% FBS was

introduced, and the cells were further incubated for an additional 48 hours. For the evaluation of GFP expression, 200 ng of mRNA was used per each chamber. Following 48 hours of incubation, nuclear counterstaining was performed using Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) for 15 minutes at room temperature, followed by PBS wash. Fixation was done using 4% paraformaldehyde (PFA) in PBS and washed by PBS three times. Subsequently, the chamber was removed, and a glass coverslip was sealed onto the cell tray using clear nail polish.

Confocal laser scanning microscopy analysis

After 48 hours of incubation, GFP transfection efficiency of cells treated with histidine-modified BAPC-mRNA complexes was evaluated using an LSM 880 laser-scanning microscope (Carl Zeiss, Gottingen, Germany), with ZEN 3.5 software used for image analysis.

In vitro luciferase mRNA expression with histidine modified BAPCs

Luciferase nucleic mRNA vitro delivery was carried out as described above for the GFP transfections. The luciferase assay was performed 48 hr later using the Pierce™ Firefly Luciferase Glow Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Briefly, cells were seeded on 24 well plate with 150,000 cells/well in 1 mL of complete medium. Upon completion of the transfection process, the media from the cells was aspirated, followed by a single rinse using 100 µL of DPBS buffer per well. Then, 100 µL of cell lysis buffer into each well and allow incubation until complete lysis is achieved. Subsequently, 20 µL of the cell lysate was introduced into the assigned well of a white 96-well plate and combined with 50 µL of Pierce™ Firefly Luciferase Glow Assay substrate. Following

an incubation period of 10 minutes at room temperature, luminescence was recorded using a Synergy™ H1 (BioTek, Winooski, VT). The reported luminescence measurements were obtained as the average of three replicate readings.

Results and discussion

Assessment of capsule formation with combinations of histidine-modified BAPCs and original BAPCs

The original BAPCs are constructed from an equimolar ratio of acetylated h_9 bis(FLIVIGSII)-K-KKKK and h_5 bis(FLIVI)-K-KKKK sequences. To develop histidine-modified BAPCs, I introduced specific modifications to both the h_5 or h_9 peptide sequences. For the h_5 modification, two histidine residues were placed in each arm of the branched peptide sequence, resulting in a total addition of four histidine residues, and I added one more lysine residue to balance between preserving hydrophobic interactions and hydrophilic outer shell, crucial for capsule formation. This modified variant is denoted as h_5h. For the h_9 modification, three histidine residues were added to each arm of the branched peptide, resulting in the addition of six histidine residues. This altered form is referred to as h_9h (**Figure 3.2**) (**Table 3.1**).

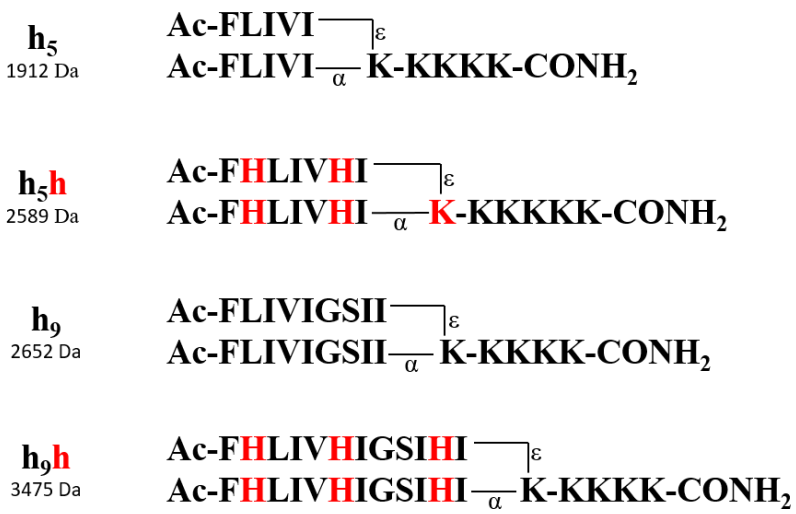

Figure 3.2. The sequences of four distinct peptides: h_5, h_5h, h_9, and h_9h, along with their corresponding molecular weights

These sequences represent the self-assembling branched peptides, including the original h_5 and h_9 peptides and the histidine-modified h_5h and h_9h peptides. The point of branching is situated at the indicated lysine residue, with branches extending from the amino groups on the N-terminus and the ε amine of the side chain.

Table 3.1. BAPC peptide sequences. Peptide amino acid sequences of original h_5 and h_9 and histidine modified h_5 and h_9

Peptides	Sequence	MW (g/mol)
h_5	bis(FLIVI)-K-KKKK	1912
h_5h	bis(Ac-F**H**LIV**H**I)-**K**-KKKKK-NH_2	2589
h_9	bis(FLIVIGSII)-K-KKKK	2652
h_9h	bis(Ac-F**H**LIV**H**IGSI**H**I)-K-KKKK- NH_2	3475

In this systematic exploration, I investigated various combinations of h_5, h_9, h_5h, and h_9h peptides, which led to the identification of seven distinct cases. These cases are individually denoted as h_5h on its own, h_9h alone, h_5h in combination with h_5, h_5h combined with h_9, h_5h combined with h_9h, h_9h paired with h_5, and h_9h combined with h_9. Each of these cases is assigned a corresponding number, namely, case number 1, 2, 3, 4, 5, 6, and 7, respectively (**Table 3.2**).

Table 3.2. Combinations of peptides for comprehensive BAPC forming Assessment

Case #	Combination	Case #	Combination
1	h_5h itself	2	h_9h itself
3	h_5h x h_5	4	h_5h x h_9
5	h_5h x h_9h	6	h_9h x h_5
7	h_9h x h_9		

Considering that one of the primary forces driving BAPC bilayer formation is the hydrophobic interaction among the branched peptides, primarily composed of hydrophobic amino acids, it becomes imperative to assess whether each of the seven cases (from case 1 to 7) leads to the formation of capsules. This evaluation is crucial because the modified versions of peptides, h_5h and h_9h, involve the addition of histidine, which introduces hydrophilic residues into the hydrophobic segments of the sequences. If these hydrophobic peptides are significantly

affected by the histidine modification, it could potentially hinder the formation of histidine-containing BAPCs. To assess the capsule-forming capacity of histidine-modified BAPCs, I employed the Nano Tracking Analysis (NTA) method. NTA utilizes laser light to track the Brownian motion of particles in a liquid, providing information on the size, concentration, and shape of the suspended particles. By utilizing this technology and analyzing Brownian movement, along with the application of relevant equations, I determined the concentration and shape of histidine-modified BAPCs, thus enabling us to identify which of the seven cases led to capsule formation. Among the cases from 1 to 7, the NTA data revealed that cases 1, 3, 4, and 5 displayed a distinct capsule shape along with a notably high concentration of capsules. **Figure 3.3A** displays the NTA data for case 1, illustrating a well-defined capsule shape with a mean diameter of 113.6 +/- 2.3 nm. The mode size is recorded as 88.7 +/- 1.7 nm, showcasing a notably sharp peak on the NTA data graph. In **Figure 3.3B**, the NTA data of case 3 reveals a distinctive capsule shape, characterized by a mean diameter of 192.6 +/- 3.4 nm. The mode size is observed at 156.3 +/- 8.2 nm. Moving to **Figure 3.3C**, the NTA data for case 4 demonstrates a recognizable capsule shape with an average diameter of 121.1 +/- 1.9 nm. The mode size is measured at 94.1 +/- 3.1 nm, indicating the specific characteristics of this case. Finally, in **Figure 3.3D**, the NTA data for case 5 portrays a distinctive capsule shape with a mean diameter of 127.1 +/- 1.9 nm. The mode size is found to be 76.2 +/- 1.4 nm. For unmentioned cases, such as cases 2, 6, and 7, a uniform size or distinct capsule shape was not observed. These cases, all including h_9h peptides, possibly encountered hindrance due to the presence of six histidine residues, which may have affected the hydrophobic bilayer interactions, consequently resulting in the absence of a clear capsule shape in the NTA analysis.

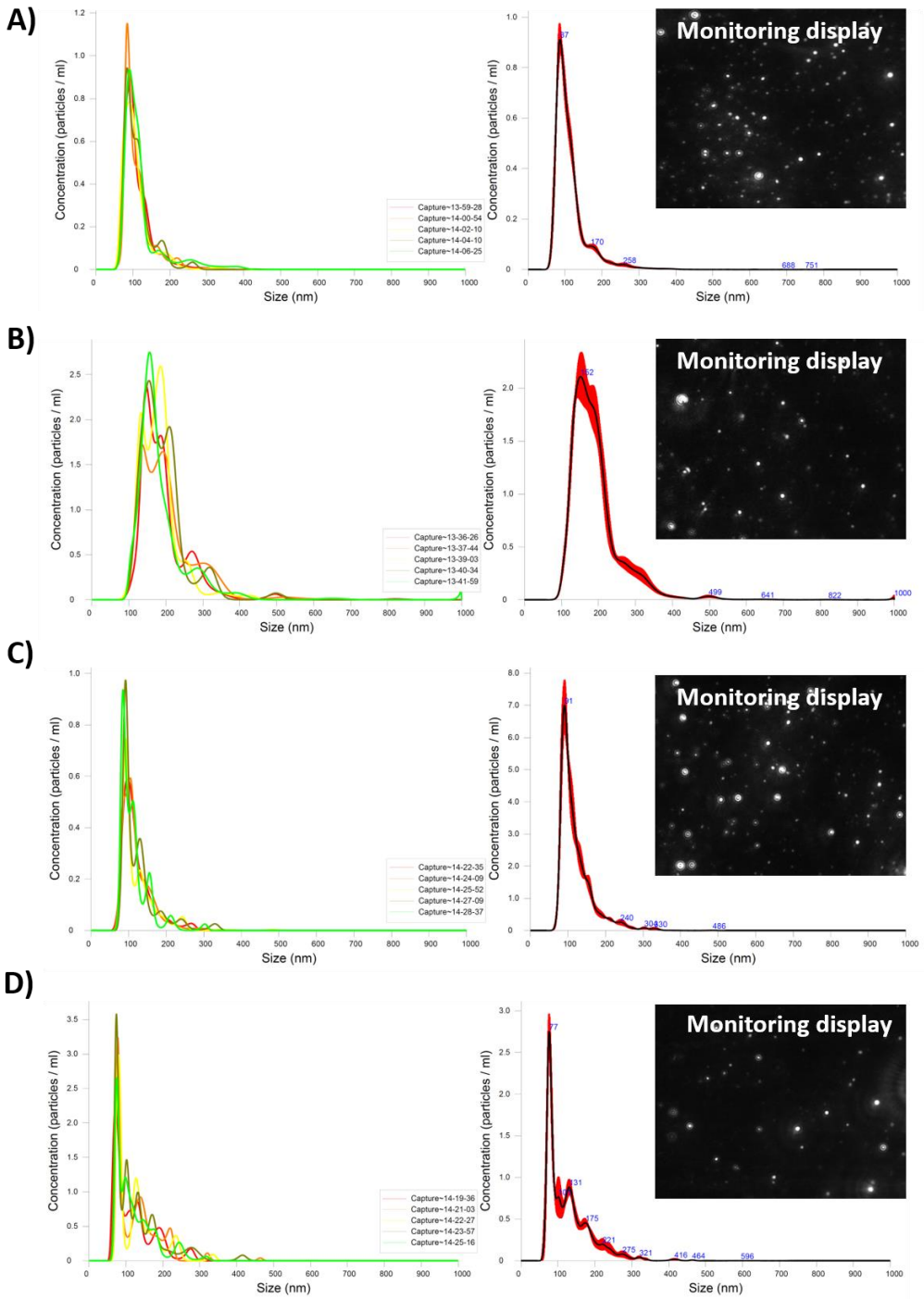

Figure 3.3. NTA analysis of size distribution profile and capsule-forming capacity of histidine-modified BAPCs

NTA assessments for different combination of histidine added peptides for BAPC formation A) case 1, B) case 3, C) case 4 and D) case 5 of histidine modified BAPCs.

Biophysical characterization of histidine-modified BAPCs under acidic and basic conditions

I hypothesized the histidine modification in the peptides would increase the gene delivery capacity and efficiency through the proton sponge effect. The "Proton Sponge Effect" posits that the introduction of a mildly basic molecule can induce endosomal rupture.[17-19] This concept, known as the proton sponge hypothesis, centers on the active intake of protons into endosomes. This proton build-up elevates the membrane potential, leading to the diffusion of extra chloride ions (Cl^-) into the endosome and this surge in ion concentrations escalates osmotic pressure, culminating in endosomal expansion and, eventually, rupture.[19, 20] This hypothesis suggests that transfection efficiency may be enhanced through the incorporation of histidine-modified peptides in the bilayer component of the delivery vehicles, potentially promoting endosomal escape at the late endosome via the proton sponge effect.

Circular dichroism (CD) spectroscopy was utilized to assess the stability and resistance of secondary structures to fluctuations in acidic and mild basic pH. This method operates on the principle of the differential absorption of left- and right-handed circularly polarized light by chiral molecules. Peptides in solution exhibit distinct CD spectra corresponding to their secondary structures. Random coils typically demonstrate a drop in CD intensity around 200 nm, followed by a plateau around 260 nm, whereas α-helices display a peak at 190 nm, decreasing around 200 and 220 nm. β-sheets show a smaller peak near 200 nm, with a subsequent decline close to 210 nm.[23, 24] These spectral features arise from the unique electronic transitions and chirality of peptide backbone conformations.

Case 1 (h_5h) peptide was selected to investigate whether the presence of additional histidine residues affects their biophysical characteristics compared to the original h_5 peptides.

Case 1 (h_5h) peptides in both TFE and BAPCs in MQ water were examined at pH 4, 5.5, and 7.2 (**Figure 3.4A-E**). **Figures 3.4A, B**, and **C** depict CD spectra of case 1 (h_5h) BAPCs at pH 4, 5.5, and 7.2, respectively, revealing a consistent minimum in CD (mdeg) near 200 nm, characteristic of a random coil secondary structure across all pH levels. In TFE (**Figure 3.4D**), case 1 (h_5h) peptides assume an α-helical secondary structure, as evidenced by a peak at 190 nm that diminishes around 200 and 210 nm. In the hydrophobic solvent TFE, these peptides remain as individual α-helical peptides instead of forming BAPCs. This highlights the pivotal role of hydrophobic interactions in shaping nano capsule structure of BAPC. These findings are in line with previous observations,[25] suggesting that the addition of 4 histidine residues does not disrupt the secondary structure observed in the original BAPCs.

In order to comprehensively understand the biophysical characteristics of histidine-added BAPCs, zeta potential experiments were conducted under varying pH conditions. Recognizing the relevance of pH dynamics in the late endosome environment, I set the acidic condition at pH 5.5 and the basic condition at pH 7.2 to mimic physiological settings of endocytosis. The rationale behind this choice stemmed from the hypothesis that histidine, being protonatable residues, might exhibit distinct behaviors under acidic and basic conditions. Specifically, I anticipated that in acidic environments, histidine would protonate, potentially influencing the zeta potential towards a more positive charge compared to basic conditions.

The results, illustrated in **Figure 3.4F**, provide intriguing insights into the behavior of histidine-modified BAPCs. At pH 5.5, resembling the late endosome environment, the zeta potential registered a positive value of +39.33 mV. Conversely, at pH 7.2, reflecting the initiation of endocytosis, the zeta potential slightly decreased to +32.3 mV.

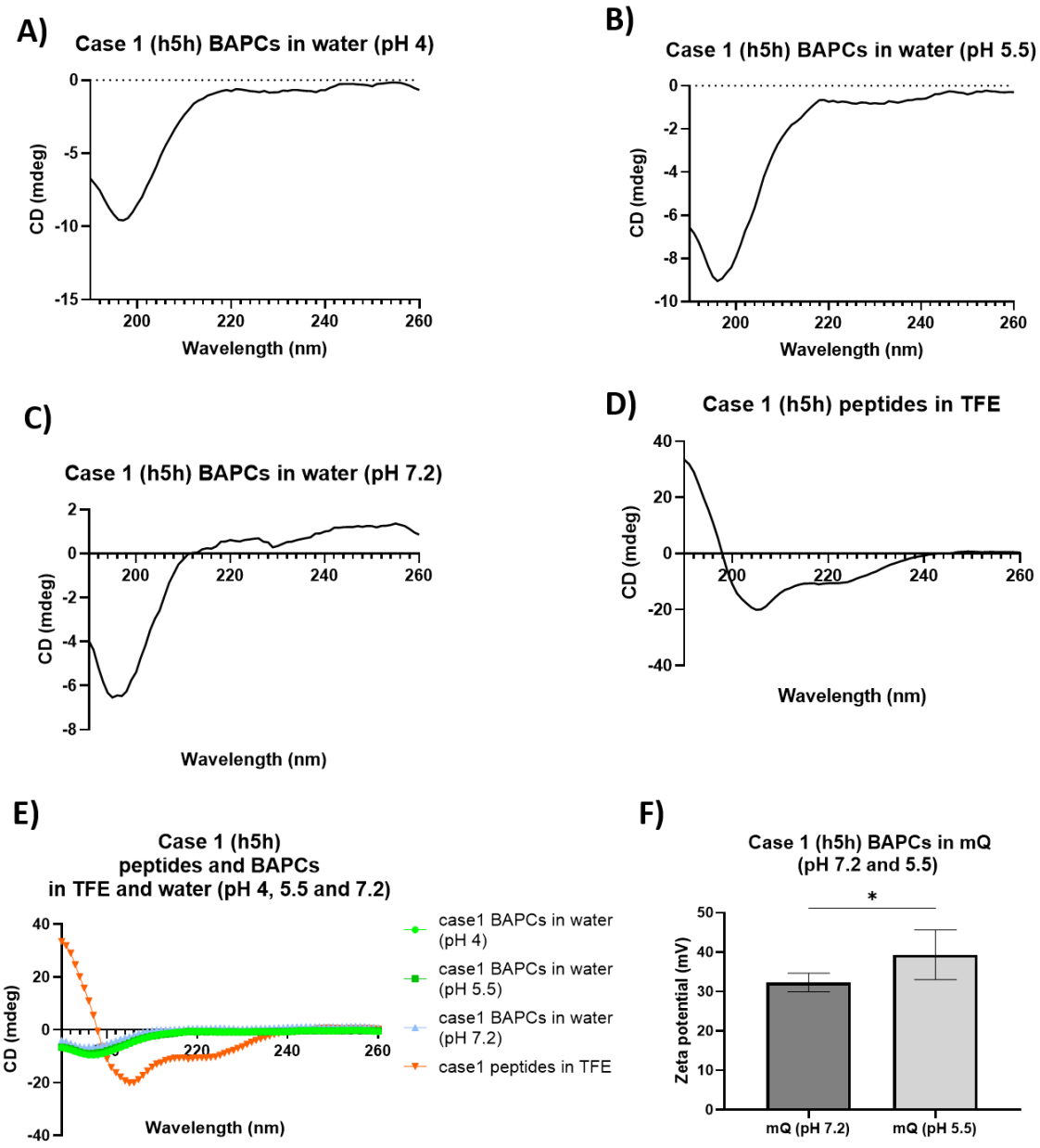

Figure 3.4 CD spectra and zeta potential measurements of case 1 (h_5h) peptides and BAPCs at various pH levels

A-C) CD spectra of h_5h BAPCs in MQ water at pH 4, pH 5.5, and pH 7.2, respectively. D) CD spectra of h_5h peptides in a TFE solution. E) Panel consolidates the total CD data into a single graph, where the vertical axis represents mean residue ellipticity and the horizontal axis denotes wavelength. F) Zeta potential data for h_5h BAPCs at pH 7.2 and pH 5.5 (n=7). Kruskal–Wallis test followed by a Dunn's multiple comparison was performed using GraphPad Prism version 8.0.2; significance determined at * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

This observed variation underscores the pH-dependent nature of histidine-modified BAPCs, highlighting their responsiveness to changes in environmental acidity. The fact that pH 5.5 falls below the pKa of histidine suggests predominant protonation of histidine residues in this acidic environment. Consequently, histidine-modified BAPCs may exhibit heightened positive charges, which potentially ensure the enhancement of their proton sponge effect. This phenomenon is pivotal in facilitating endosomal escape and may contribute to increased transfection rates. Thus, the dynamic interplay between pH and histidine protonation underscores the versatility of histidine-modified BAPCs in adapting to physiological conditions and optimizing their transfection efficiency. These findings contribute valuable information to our understanding of how histidine modifications impact the surface charge properties of BAPCs, particularly in environments mimicking intracellular compartments.

In addition, to assess the behavior of histidine-modified BAPCs encapsulating mRNA, I employed additional NTA, DLS and CD spectra analyses. Histidine-modified BAPCs of cases 1, 3, 4, and 5 were specifically examined, each encapsulating mRNA. **Figures 3.5** present the NTA data for histidine-modified BAPCs of cases 1, 3, 4, and 5, respectively. Notably, these figures showcase well-defined capsule shapes with uniform sizes. For case 1 with mRNA encapsulation, the mean diameter measured 163.1 nm ± 3.6, with a mode size of 97.3 nm ± 3.5. In the case of case 3 with mRNA encapsulation, the mean diameter was determined to be 135.4 nm ± 2.4, with a mode size of 91.4 nm ± 3.8. Similarly, for case 4 with mRNA encapsulation, the mean diameter was recorded at 160.7 nm ± 4.5, with a mode size of 137.5 nm ± 5.3. Finally, for case 5 with mRNA encapsulation, the mean diameter was measured as 167.3 nm ± 9.5, with a mode size of 101.2 nm ± 8.9. Although these sizes are relatively larger than those without

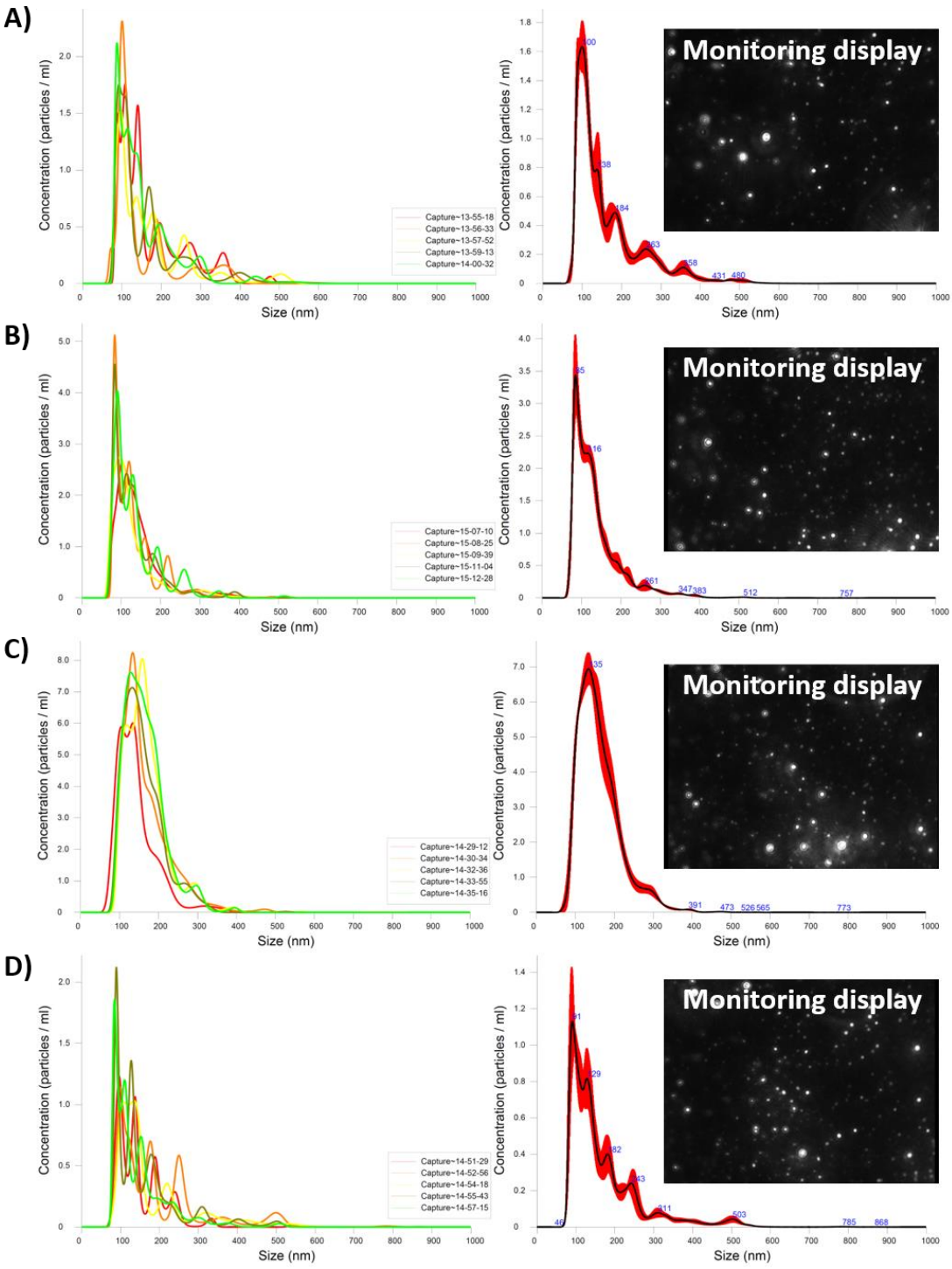

Figure 3.5. NTA analysis of the size distribution profile and capsule forming capacity of histidine modified BAPCs with encapsulated mRNA

NTA assessments for histidine added peptides for BAPC formation A) case 1, B) case 3, C) case 4 and D) case 5 of histidine modified BAPCs while mRNAs are encapsulated inner cargo of each case.

mRNA encapsulation (**Figure 3.3**), all exhibited well-defined capsule shapes with narrow size distributions while encapsulating mRNA.

Alongside NTA, Dynamic Light Scattering (DLS) was utilized to characterize histidine-modified BAPCs encapsulating mRNA. DLS employs a laser and photodetector to measure fluctuations in scattered light intensity, thereby revealing the size distribution of particles in solution.[26] This technique is particularly valuable for analyzing nanoparticles, offering insights into their size distribution profile. Additionally, DLS allows for the measurement of the Polydispersity Index (PDI), serving as a metric for the spread of particle sizes within the sample. A lower PDI indicates a more uniform distribution, while a higher PDI suggests greater heterogeneity in particle sizes. A PDI value less than 0.3 indicates the mono-dispersity or homogeneity of the suspension.[27, 28] DLS analysis revealed the following mean PDI values and hydrodynamic diameter sizes: case 1 exhibited a mean PDI value of 0.25 ± 0.06 and a hydrodynamic diameter size of 228.79 nm ± 27.35 nm; case 3 showed a mean PDI value of 0.28 ± 0.06 and a hydrodynamic diameter size of 134.72 nm ± 89.43 nm; case 4 demonstrated a mean PDI value of 0.24 ± 0.05 and a hydrodynamic diameter size of 163.59 nm ± 23.27 nm; and case 5 presented a mean PDI value of 0.24 ± 0.03 and a hydrodynamic diameter size of 124 nm ± 28.32 nm (**Table 3.3**). These results, indicating PDI values less than 0.3, are consistent with the data obtained from NTA, which indicated particle sizes ranging from 100 to 200 nm.

Table 3.3. DLS data of histidine modified BAPCs when mRNA encapsulated (BAPCs : mRNA = 50 : 1, w/w%, n=5)

Case	Size (nm)	PDI (%)
1	228.79 ± 27.35	0.25 ± 0.06
3	134.72 ± 89.43	0.28 ± 0.06
4	163.59 ± 23.27	0.24 ± 0.05
5	124.00 ± 28.32	0.24 ± 0.0

Furthermore, CD spectroscopy was conducted, revealing a consistent minimum in CD (mdeg) near 200 nm (**Figure 3.6**), characteristic of a random coil secondary structure across all histidine-modified BAPC cases while mRNA encapsulated in it like similar CD spectra patterns with bare histidine modified BAPCs (**Figure 3.4**).

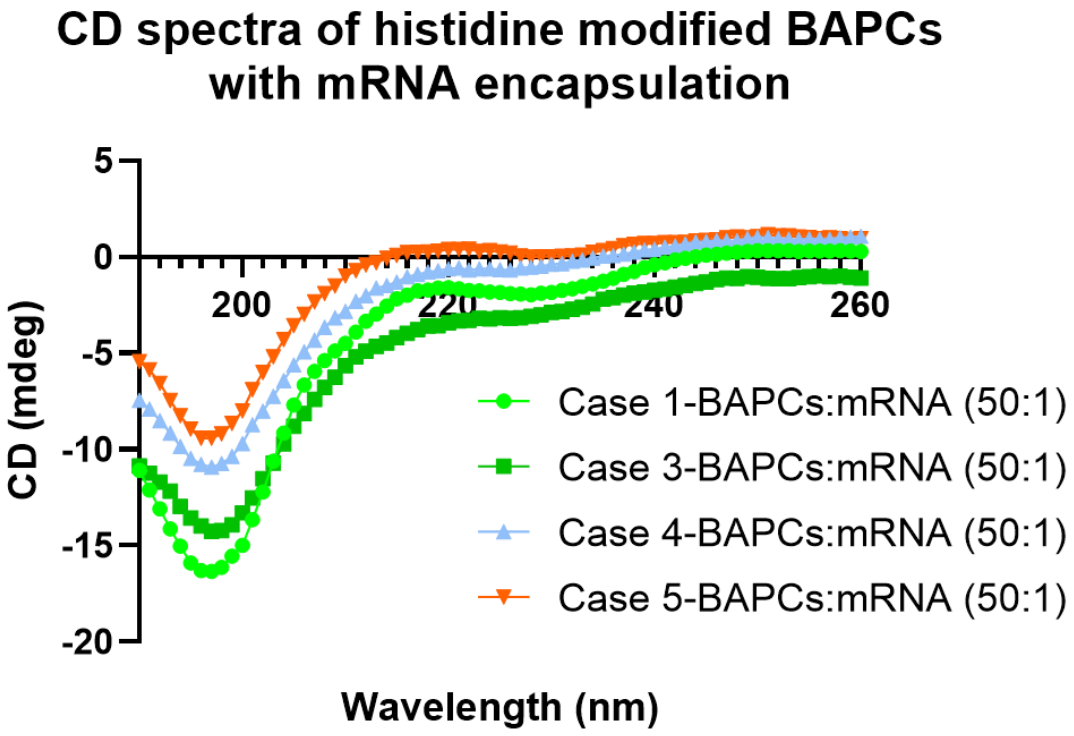

Figure 3.6. CD spectra measurements of case 1, 3, 4 and 5 histidine modified BAPCs while mRNA encapsulated

Histidine-modified BAPCs (case 1, 3, 4 and 5) encapsulating mRNA (50:1 w/w ratio) resulted in a consistent minimum in circular dichroism (CD) spectroscopy near 200 nm, indicative of a random coil conformation for the encapsulated mRNA.

In vitro GFP expression through mRNA delivery with histidine-modified BAPCs

Employing the specific combination of histidine-modified peptides, notably case 1, 3, 4, and 5 BAPCs, recognized for their successful encapsulation as supported by NTA and CD data (**Figure 3.5, 3.6 and Table 3.3**), I explored their potential for mRNA delivery. my investigation entailed an exploration of the proton sponge effect via CD spectroscopy, alongside an evaluation of their electrostatic properties through zeta potential measurements under various pH conditions, encompassing both acidic and basic environments (**Figure 3.4**). My results provided intriguing insights into the electrostatic characteristics of histidine-modified BAPCs, particularly apparent under acidic conditions resembling the late endosome environment. These conditions exhibited notable alterations in zeta potential, hinting at a potential augmentation of the proton sponge effect—the required process enabling endosomal escape of the endocytosed mRNA. Such findings underscore the dynamic interplay between pH dynamics and the electrostatic properties of histidine-modified BAPCs, crucial for enhancing their efficacy in mRNA delivery applications.

This groundwork laid the foundation for assessing their effectiveness in mRNA delivery. Leveraging GFP expression as a dependable indicator, I further evaluated their mRNA delivery capacity and efficiency, thereby enhancing the comprehensiveness of our analysis.

Figure 3.7 depicts GFP mRNA transfection using histidine-modified BAPCs across various cases. In **Figure 3.7A**, a positive control using lipofectamine 3000 is presented, while **Figure 3.7B** shows a negative control with non-treated cells. When compared to the results in **Figure 2.6** from the previous chapter, I observed a notable increase in GFP expression in **Figures 3.7C** to **3.7F**. Specifically, **Figure 3.7C** demonstrates GFP mRNA transfection for case

1, **Figure 3.7D** pertains to case 3 with histidine-modified BAPCs, **Figure 3.7E** represents case 4, and **Figure 3.7F** showcases case 5, all utilizing GFP mRNA transfection.

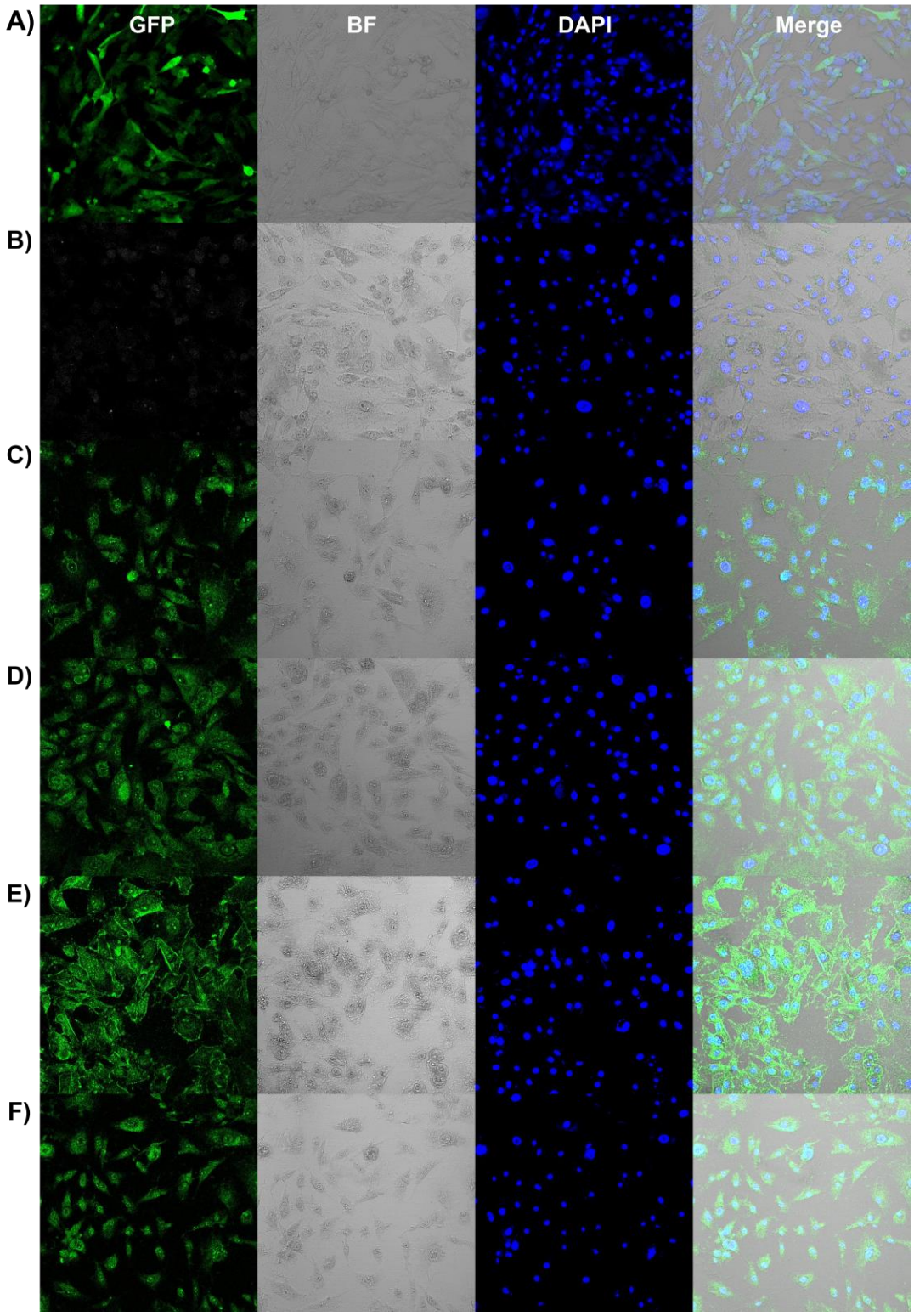

Figure 3.7. Enhanced in vitro GFP mRNA expression by histidine modified BAPCs

A) Positive control (Lipofectamine 3000), B) negative control (non-treated), C) case 1, D) case 3, E) case 4 and F) case 5 Histidine modified BAPCs were used for GFP mRNA delivery and expression.

In vitro luciferase expression through mRNA delivery with histidine-modified BAPCs

In prior research, the original BAPCs consistently demonstrated proficient gene expression across a diverse range of genes.[29-32] However, achieving luciferase expression in vitro posed a significant challenge. The observed variations in GFP and luciferase expression between our previous and current mRNA delivery vectors can presumably be attributed to the intrinsic disparities in the proteins' half-lives and the absence of an efficient endosomal escape mechanism. In the case of our original BAPCs, which lacked an effective mechanism for endosomal escape, luciferase mRNA was predominantly sequestered within the acidic confines of the endosomes. This unfavorable environment led to rapid degradation of the luciferase mRNA, resulting in minimal luciferase expression. The situation was compounded by the short 2-hour half-life of luciferase,[33] making it particularly challenging to detect luciferase expression, especially after 24 to 48 hours of expression. In contrast, the more stable GFP, with its 26-hour half-life,[34] sustained its structural integrity and exhibited detectable expression over a longer period.

A pivotal breakthrough occurred when I introduced histidine modification to our BAPCs. This modification yielded observable luciferase mRNA expression, signifying a significant improvement in our system. Moreover, the incorporation of an endosomal escape strategy within the new vector system further enhanced the liberation of luciferase mRNA from endosomal entrapment. This facilitated translation in the cytoplasm and resulted in an elevated level of luciferase expression, addressing the previous limitations effectively.

In the context of the in vitro luciferase transfection assay, I conducted a thorough comparison of various formulations, all of which featured mRNA as the primary readout. In addition to these formulations, I utilized Lipofectamine MessengerMax as a positive control, and our findings are depicted in **Figure 3.8**.

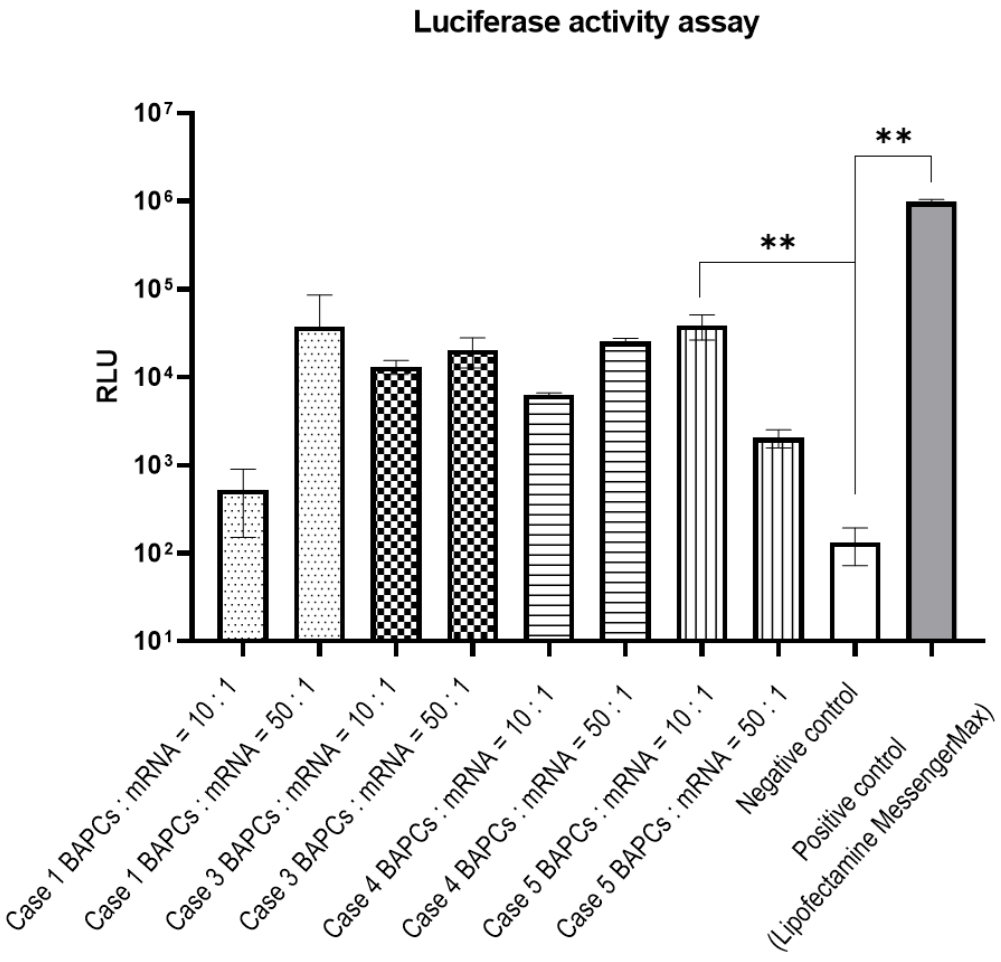

Figure 3.8. Luciferase activity assay by using luciferase mRNA encapsulated histidine modified BAPCs

Luciferase expression induced by histidine modified BAPCs, case 1,3,4 and 5 at 10:1 and 50: 1(BAPCs:mRNA w/w ratio). Data represent mean value ± SD (n = 3). Statistical differences between groups were determined using the Kruskal-Wallis test followed by Dunn's multiple comparison, performed using GraphPad Prism version 8.0.2; significance determined at * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

In the case of histidine-modified BAPCs, specifically Cases 1, 3, 4, and 5, I considered two distinct BAPCs-to-mRNA weight ratios: 10:1 and 50:1 (w/w). In each case, mRNA was encapsulated within the histidine-modified BAPCs. **Figure 3.8** reveals that in Cases 1, 3, and 4, the luciferase expression was notably higher at the 50:1 BAPCs-to-mRNA w/w ratio when compared to the 10:1 ratio. However, Case 5 exhibited the highest luciferase expression at the 10:1 ratio. It is noteworthy that in all instances involving histidine-modified BAPCs, I observed a significant increase in luciferase expression when compared to the negative control. This data holds significant importance because the original BAPCs failed to produce a discernible increase in luciferase expression, while the histidine-modified BAPCs demonstrated a noteworthy enhancement in luciferase expression.

Conclusions

Our extensive investigation into histidine-modified BAPCs has provided invaluable insights into the realm of mRNA delivery and gene expression enhancement. Throughout this study, I focused on modifying the h_5 and h_9 peptides that form the foundation of BAPCs to better understand their impact on capsule formation, mRNA delivery, and gene expression. Histidine modifications, introduced as h_5h and h_9h, were central to our research, as they were designed to leverage the proton sponge effect, a crucial factor in optimizing gene delivery. Our exploration encompassed various combinations of h_5, h_9, h_5h, and h_9h peptides, resulting in the identification of seven distinct cases, each representing a unique combination of these peptides.

Central to my study was the evaluation of the capacity of these cases to form capsules, considering the crucial role of hydrophobic interactions among branched peptides in BAPC bilayer formation. NTA emerged as a key tool to determine which cases successfully led to capsule formation. Notably, cases 1, 3, 4, and 5 exhibited distinct capsule shapes and a high concentration of capsules, clearly indicating their success in capsule formation. These results underscore the significance of histidine-modified BAPCs in enabling the creation of mRNA delivery vehicles. Furthermore, successful encapsulation of mRNA within the histidine-modified BAPCs was confirmed through NTA and CD spectroscopy. These assessments not only validated the encapsulation capacity but also provided invaluable insights into the stability of secondary structures with nucleic acids. This investigation then shifted towards enhancing gene expression through mRNA delivery using histidine-modified BAPCs. Our hypothesis was rooted in the idea that histidine modifications could enhance gene delivery efficiency by leveraging the proton sponge effect. This theory, which underscores the role of mildly basic molecules in triggering endosomal rupture through the protonation of histidine residues, guided our research.

Histidine's key role in this process is attributed to its pKa value, which aligns closely with physiological pH. This property allows histidine to effectively capture protons and chloride ions within late endosomes and lysosomes, leading to osmotic swelling and eventual rupture. This mechanism holds particular significance in gene delivery, as histidine-modified peptides facilitate the escape of gene delivery vehicles from endosomes, ensuring the therapeutic cargo reaches its intended destination within the cell. The culmination of my research centered on in vitro expression of both GFP and luciferase through mRNA delivery using histidine-modified BAPCs. The incorporation of histidine modifications marked a significant breakthrough, leading to observable luciferase mRNA expression in vitro. This remarkable improvement was attributed to the enhanced endosomal escape strategy within the new vector system, allowing for the liberation of luciferase mRNA from endosomal entrapment and subsequent translation in the cytoplasm. In vitro luciferase transfection assay further confirmed the efficacy of histidine-modified BAPCs. Cases 1, 3, 4, and 5 demonstrated significantly higher luciferase expression compared to the control. Importantly, all cases involving histidine-modified BAPCs exhibited a substantial increase in luciferase expression, highlighting their effectiveness in promoting gene expression.

This study has unveiled the potential of histidine-modified BAPCs as versatile and efficient mRNA delivery vehicles, offering substantial improvements in gene expression. The incorporation of histidine residues into BAPCs not only ensures capsule formation but also facilitates endosomal escape, making it a valuable asset in the field of gene therapy and mRNA-based treatments. These findings hold significant promise for advancing the field of nucleic acid delivery, ultimately leading to more effective and targeted gene expression in therapeutic applications.

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Chapter 4 - Evaluating stability and dual delivery efficacy of modified aqueous partitioning capsules in mRNA delivery: a surface-bound and encapsulation strategy

Introduction

Within the realm of peptide-based nano-carriers, the Aqueous Partitioning Capsules (APC) have emerged as a remarkable innovation. At the heart of this development lies a unique peptide, $K_3h_{5n}K_3$ (Ac-KKKFLIVIKKK-$COHN_2$), exhibiting remarkable versatility as a potent tool for drug delivery. The primary focus of our study was an exploration of the adhesive properties of $K_3h_{5n}K_3$,[1] with a simultaneous and comprehensive investigation into its secondary structure. This investigation unveiled a striking revelation: the secondary structure of $K_3h_{5n}K_3$ displayed distinctive variations contingent upon the pH conditions of its environment.[2] Under acidic conditions with a pH of 2, the peptide exhibited a remarkable property of self-assembling into nanospheres.[2] The nano-spheric structure displays remarkable potential for applications in drug delivery, primarily owing to its capability to offer a cationic surface for binding nucleic acid or encapsulating therapeutic agents with great efficiency. Moreover, earlier studies revealed intriguing structural differences at pH 12 that imparted adhesive properties, while at pH 7, aggregates predominated.[2] Noteworthy is the observation that even when moved from low pH values of pH 7 or greater, the nano-spheric geometry remained intact, underscoring the suitability of the peptide for stable drug delivery applications. This research underscores the remarkable adaptability of the $K_3h_{5n}K_3$ peptide, characterized by its pH-responsive secondary structures and stable nanospheres, rendering it a promising candidate for both adhesive and drug

delivery applications. This innovative approach to peptide-based nano-carriers holds substantial potential for diverse biomedical and pharmaceutical applications.

With regard to APC as a gene and drug delivery vector, the long-term stability of the capsule as well as its contents emerge as one of the key properties required for its ultimate commercialization. The significance of vector stability is underscored by its direct impact on the preservation of genetic material during transportation and delivery, ensuring the integrity and functionality of the genetic cargo within the target cells.[3-5] Furthermore, stable vectors play a pivotal role in the safe storage, handling, and transportation of these critical therapeutic agents, safeguarding their integrity against environmental factors and temperature or pH fluctuations, ultimately ensuring their arrival at their intended destinations in an optimal state. Vector stability is also central to maintaining delivery efficiency, as robust vectors effectively protect the genetic cargo throughout the delivery process, enhancing their ability to enter target cells and release their cargo with precision and reliability.[2, 6] The original APC demonstrated relatively stable behavior in aqueous conditions. However, a prior study revealed a decrease in its size and a subsequent degradation after approximately ten days from the initial production of the APC.6 This observation served as a motivating factor for me to undertake modifications to enhance the stability of the APC peptide.

One notable development in this study involved the strategic modification of the original APC peptide sequence, "KKKFLIVIKKK," resulting in the new sequence "KKKFLIVIIVILKKK." designed with an extension and mirrored reflection of a segment of the original sequence. The central portion "FLIVI" serves as the core, mirroring itself with the residues IVIL. Within this extended structure, the introduction of valine emerges as a suitable modification. Valine, with its small molecular size and hydrophobic nature, seamlessly integrates

into the peptide framework. Notably, the valine and isoleucine additions with their beta-forming tendencies potentially play a pivotal role in increasing the stability of APC. The amphipathic nature of the original sequence, with "KKK" segments forming inner and outer surfaces and "FLIVI" acting as the capsule shell, is preserved. Newly incorporated residues reinforce this property, facilitating interactions within the hydrophobic core of the capsule and enhancing the overall structural stability. The modification from ("KKKFLIVIKKK" to "KKKFLIVIIVILKKK" (**Figure 4.1**) signifies a rational extension and mirroring of the original sequence. This nuanced transformation, coupled with the strategic integration of additional valine and isoleucine, could provide an advancement in peptide-based nano-carriers. The resulting structure not only preserves amphipathic properties critical for self-assembly but also introduces elements contributing to enhanced stability. This approach holds promise for biomedical research, paving the way for improved therapeutic strategies and innovative solutions across diverse applications.

To assess the gene delivery capacity of the modified APC, my initial approach involved delivering EGFP mRNA to HEK293 cells. This modified APC demonstrated successful GFP expression through two distinct methods: surface-bound delivery, where the cationic modified APC's surface interacted with the negatively charged nucleic acid, and encapsulated delivery within the modified APCs' inner cargo. This versatile performance serves as a promising indication of the potential application of modified APC in gene delivery. Depending on the therapeutic application or the properties of the molecules being delivered, the choice between the surface-bound or encapsulation method can be tailored for optimized results.

Following the evaluation of the modified APC's gene delivery capacity, the next step involved assessing the stability of these modified APCs over an extended period. To measure

this structural integrity, Nano Tracking Analysis (NTA) was employed. With this approach, I generated data on the modified APC monitoring size, concentration, and capsule-forming capacity over time. The outcomes of this stability assessment were quite promising. Over the tested period, I did not observe any degradation or noticeable changes in size when compared to the original APC sequence. This suggests that the modified APCs maintain their structural integrity and stability, reinforcing their potential for various applications in gene and drug delivery. After assessing extended-period stability, the evaluation proceeded to examine the pH stability of the modified APCs. CD analysis revealed a consistent random coil secondary structure across various pH conditions, including pH shifting from acidic to basic, both without mRNA and with mRNA, whether encapsulated or bound to their surface. This showcases their robustness as carriers for genetic materials in diverse physiological conditions.

This research explores the transformative potential of modified APC in the realm of gene and drug delivery. Importantly, the integration of valine into the original APC structure stably showed its gene delivery capacity, accommodating both surface-bound and encapsulation methods to tailor delivery to specific needs. Additionally, a comprehensive assessment of stability, facilitated through NTA, highlights the enduring structural integrity of the modified APC. These advancements underscore the promising role of valine-isoleucine modified APCs in pioneering biomedical solutions, where gene delivery, adaptability, and stability converge to address diverse therapeutic needs.

Materials and methods

GFP mRNA expression by modified APC; surface bound and encapsulation

For experiments involving GFP mRNA transfection, both surface-bound mRNA and mRNA encapsulated were utilized in the modified APC. Various ratios of modified APC to GFP mRNA were tested, involving attachment to the surface and encapsulation within the modified APC. The tested ratios included 10:1, 20:1, and 50:1 weight-to-weight (w/w) ratio. All ratios maintained a consistent 200 ng of EGFP mRNA (TriLink Biotechnologies, San Diego, CA). The quantity of peptide varied, with 2 µg in the 10:1 ratio, 4 µg in the 20:1 ratio, and 10 µg in the 50:1 ratio. The precise volume of APC was calculated based on the concentration of the stock solution and the molecular masses of the peptides (**Table 4.1**).

HEK293 Cells were initially placed in 8-well chambers at a concentration of 80,000 cells per well, suspended in 0.5 mL of complete medium, and allowed to incubate. Following an overnight incubation, the culture medium was exchanged with Opti-MEM Reduced Serum (Thermo Fisher Scientific, Waltham, MA), incorporating modified APC-GFP mRNA complexes at various (w/w) ratios. The cells were then subjected to a 4 hr incubation at 37 ℃ with these complexes, after which they were replenished with fresh DMEM media. After a subsequent 48 hr period, cells underwent washing with phosphate-buffered saline (PBS), fixation using 200 µL of 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature, and permeabilization with 0.1% Triton X for 1 hr (Thermo Fisher Scientific, Waltham, MA). Following this, cells were rinsed with PBS, and nuclei were stained using 4', 6'-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA). Evaluation of GFP transfection efficiency resulting from the treatment with modified APC-GFP mRNA complexes was performed utilizing an LSM 880

laser-scanning microscope (Carl Zeiss, Gottingen, Germany), with subsequent image analysis conducted through ZEN 3.5.

CD spectra measurement

CD spectra were obtained using a Jasco-815 spectrophotometer (Jasco Analytical Instruments, Easton, MD). Peptides modified with valine and isoleucine were prepared according to the Materials and Methods, for acidic conditions, 0.2 N HCl was utilized to adjust the pH, while basic samples were treated with 1N NaOH buffer to achieve alkaline conditions. To evaluate mRNA encapsulation, peptides were meticulously dried using a vacuum evaporation system until reaching a final concentration of 0.1 mM upon rehydration with mRNA in H2O at a specific pH. The concentration of mRNA in H2O was adjusted based on the quantity of peptides to achieve a targeted encapsulation ratio of 50:1 (w/w). Similarly, for evaluating mRNA surface binding on modified APC, peptides underwent drying using a vacuum evaporation system to reach a final concentration of 0.1 mM upon rehydration with H2O at a specific pH. Subsequently, mRNA was added to the rehydrated modified APC and adjusted according to peptide quantity to achieve the desired ratio of 50:1 (w/w). Nitrogen gas flowed through the CD spectrophotometer during runs. Spectra were collected using 1 mm quartz cuvettes (Starna Cells Inc., Atascadero, CA), scanning from 260 nm to 190 nm at 50 nm/min. Each sample underwent five scans, averaged, and smoothed.

Time stability studies

The stability of modified APC in aqueous solution was evaluated through Nanoparticle Tracking Analysis (NTA) measurements, following the procedures outlined in the "Nanoparticle

Tracking Analysis (NTA)" section of the Materials and Methods, as detailed in preceding chapters. The stock sample of modified APC (1 mg/mL), stored at room temperature on a laboratory benchtop (25 ℃), served as the subject of analysis. For each scheduled assessment date, 1 mL of the sample was extracted from the stock tube and subjected to the stability check. The temporal stability investigations were conducted at intervals of 1, 3, 6, 16, and 27 days subsequent to the production of the modified APC.

Results and discussion

Surface-bound and encapsulated GFP mRNA transfection using modified APC

The focus is on the examination and validation of modified APC's efficacy in mRNA delivery and transfection, employing GFP mRNA as the investigative tool. The study was methodically divided into two distinct applications to comprehensively assess the delivery dynamics. Firstly, I explored the surface-bound approach, where GFP mRNA is intricately tethered to the positively charged APC surface. In the second application, I examined an encapsulation strategy, encapsulating GFP mRNA within the aqueous core of the modified APC. (**Figure 4.1**).

Table 4.1. Original APC and modified APC peptide sequence

Peptides	Sequence	MW (g/mol)
Original APC	KKKFLIVIKKK	1413
Modified APC	KKKFLIVIIVILKKK	1853

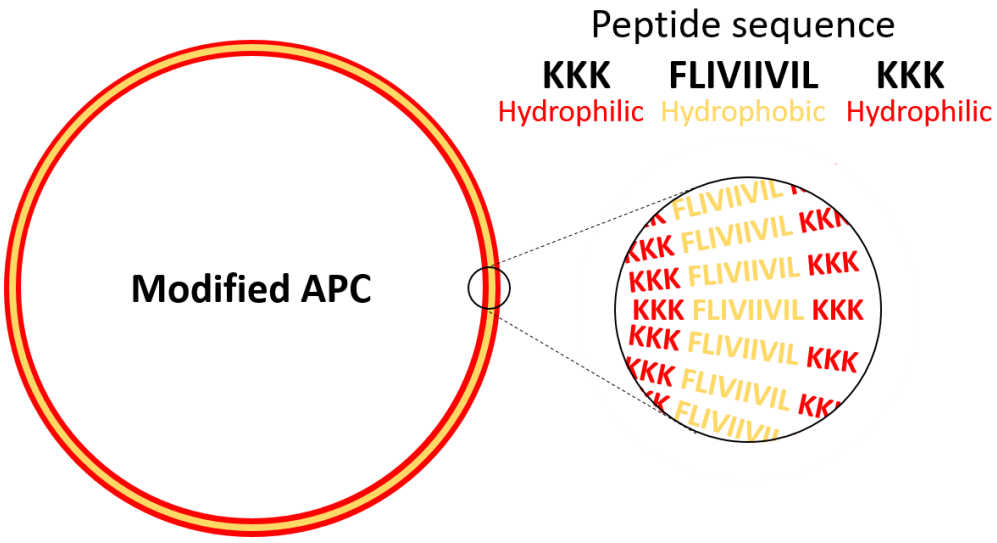

Figure 4.1. Schematic image of modified APC in aqueous solution

This dual-pronged approach not only provides a comprehensive analysis of the modified APC's mRNA delivery capabilities but also sheds light on the potential applications and limitations of surface-bound and encapsulated strategies in optimizing transfection efficiency. The results obtained from these investigations contribute valuable knowledge to the broader field of gene and drug delivery vectors.

Before assessing GFP mRNA expression with modified APCs, it's essential to evaluate their capsule forming capacity when mRNA is either encapsulated within them or bound to their surface. NTA data reveals successful capsule formation in both scenarios, when nucleic acids are

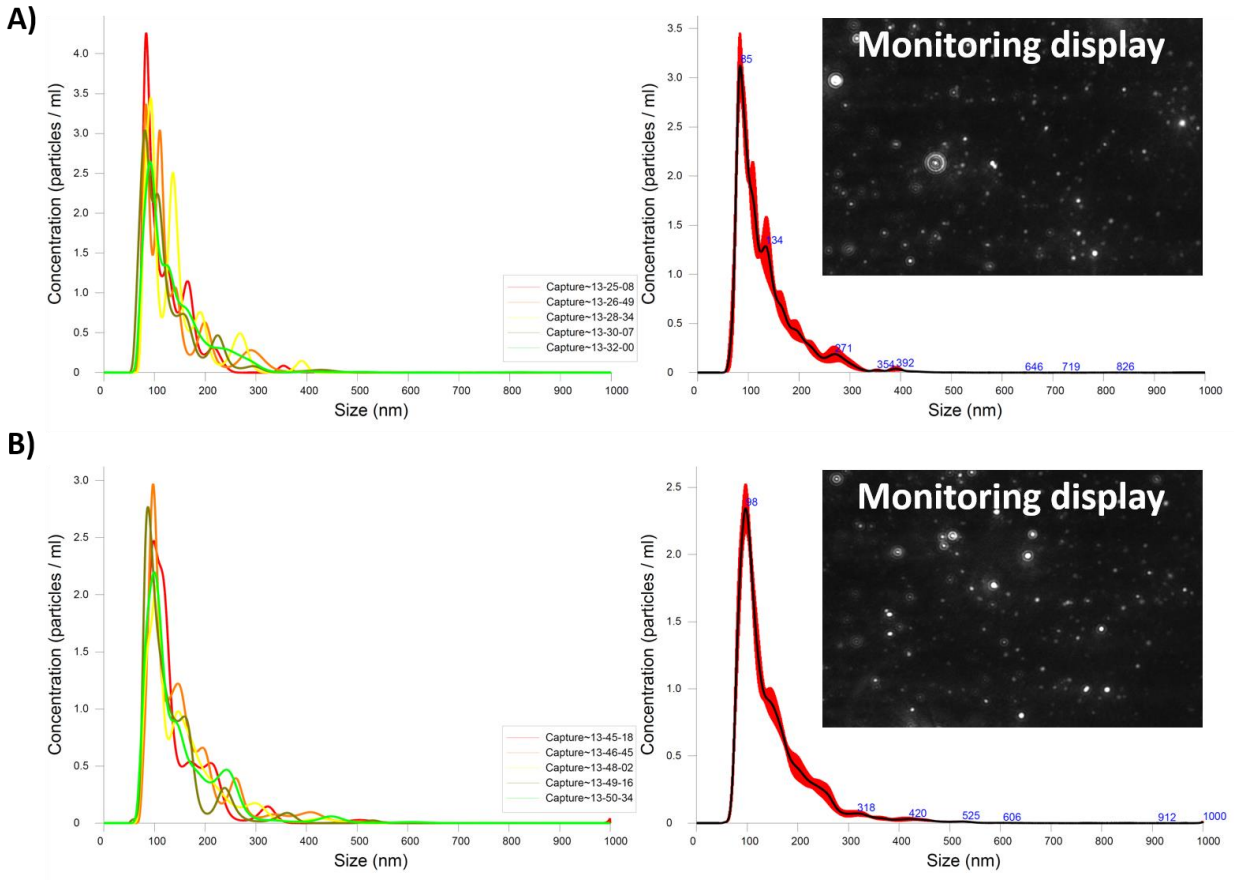

Figure 4.2 NTA data of modified APC with mRNA encapsulated and surface bound

NTA assessments for capsule forming capacity of modified APC formation with A) encapsulated in inner cargo, and B) bound on capsule surface.

encapsulated (**Figure 4.2A**) and nucleic acids are bound on surface (**Figure 4.2B**), laying the groundwork for subsequent mRNA delivery assessments.

In **Figure 4.3**, the results of surface-bound mRNA and gene transfection on HEK293 cells are displayed. **Figure 4.3A** served as the negative control, while **Figure 4.3B** shows the positive control (Lipofectamine 3000). **Figure 4.3C** demonstrated the effects at a modified APC to mRNA w/w ratio of 10:1, **Figure 4.3D** the outcome at a ratio of 20:1 and **Figure 4.3E** the experiment at a ratio of 50:1. At the 50:1 w/w ratio, a discernible increase in GFP expression was detected over the lower ratios.

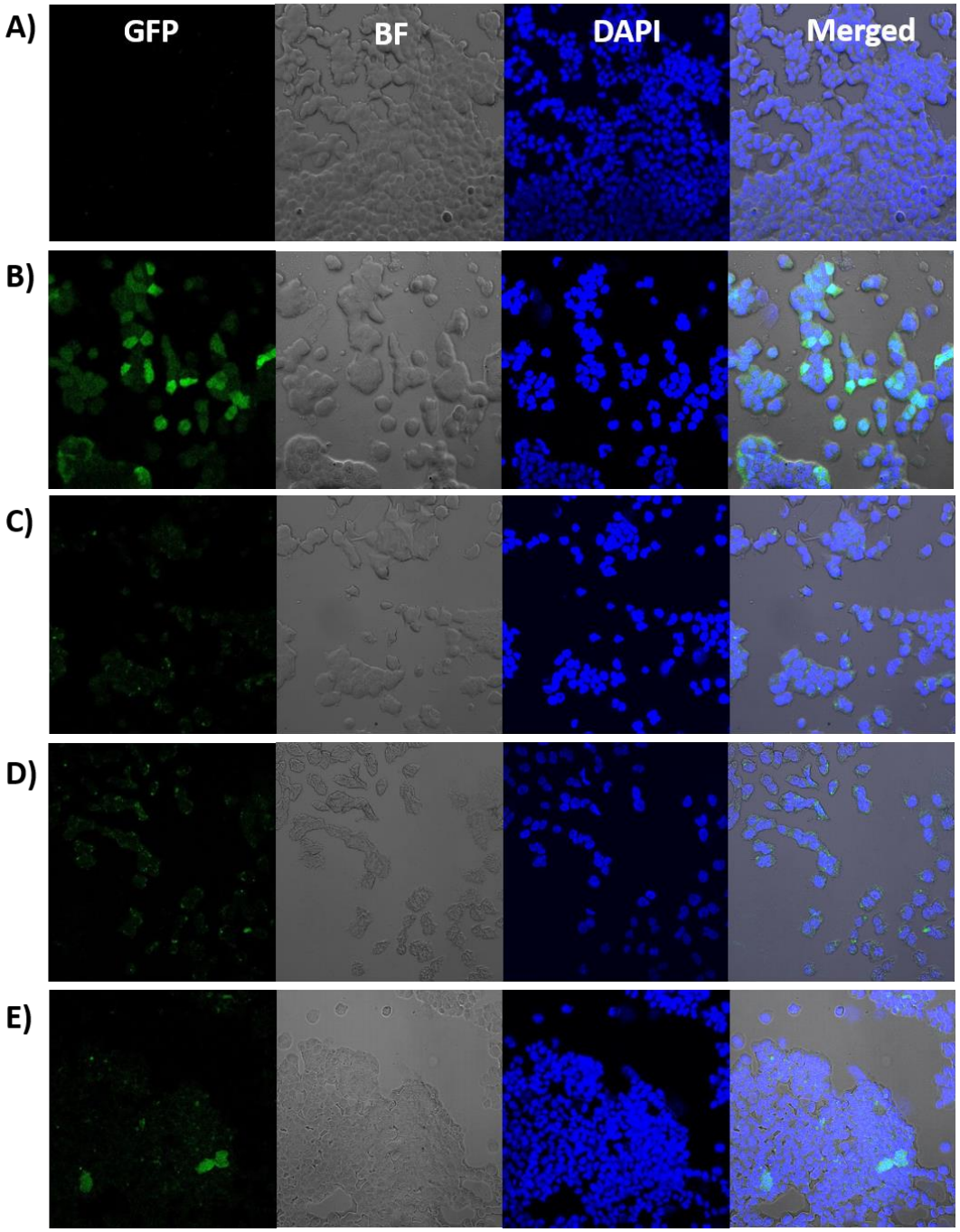

Figure 4.3. HEK293 cells treated with GFP mRNA surface-bound on modified APC with different w/w ratio

Nuclei were stained with DAPI, and the green stain represents GFP fluorescence. A) Untreated HEK293 cells. B) Positive control (Lipofectamine 3000) treated cells. C) Cells treated with modified APC and GFP mRNA at a weight-to-weight (w/w) ratio of 10:1. D) Cells treated at a 20:1 ratio. E) Cells treated at a 50:1 ratio.

Following surface-bound mRNA delivery by modified APC, I proceeded to employ encapsulated mRNA as cargo within the modified APC. As illustrated in **Figure 4.1**, the modified APC exhibited a capsule shape, with the inner capsule providing space to accommodate mRNA for cellular delivery. The divisions in this analysis mirror those of the surface-bound version, presented in **Figure 4.3**, ranging from a 10:1 to a 50:1 w/w ratio. **Figure 4.4** illustrates the GFP expression resulting from mRNA delivery encapsulated in modified APC. In **Figure 4.4A and 4.4B**, the negative control representing non-treated conditions and positive control (Lipofectamine 3000) are shown. **Figures 4.4C, D, and E** correspond to w/w ratios of 10:1, 20:1, and 50:1, respectively. This expression levels highlights the effectiveness of modified APC as a gene delivery vector, both in surface-bound and encapsulated configurations.

I analyzed various modified APC to mRNA w/w ratios, observing higher GFP expression at the 50:1 ratio. Following surface-bound mRNA delivery, we explore encapsulated mRNA within modified APC, observing significantly higher GFP expression at the 50:1 ratio. This underscores the efficacy of modified APC in gene delivery, whether surface-bound or encapsulated.

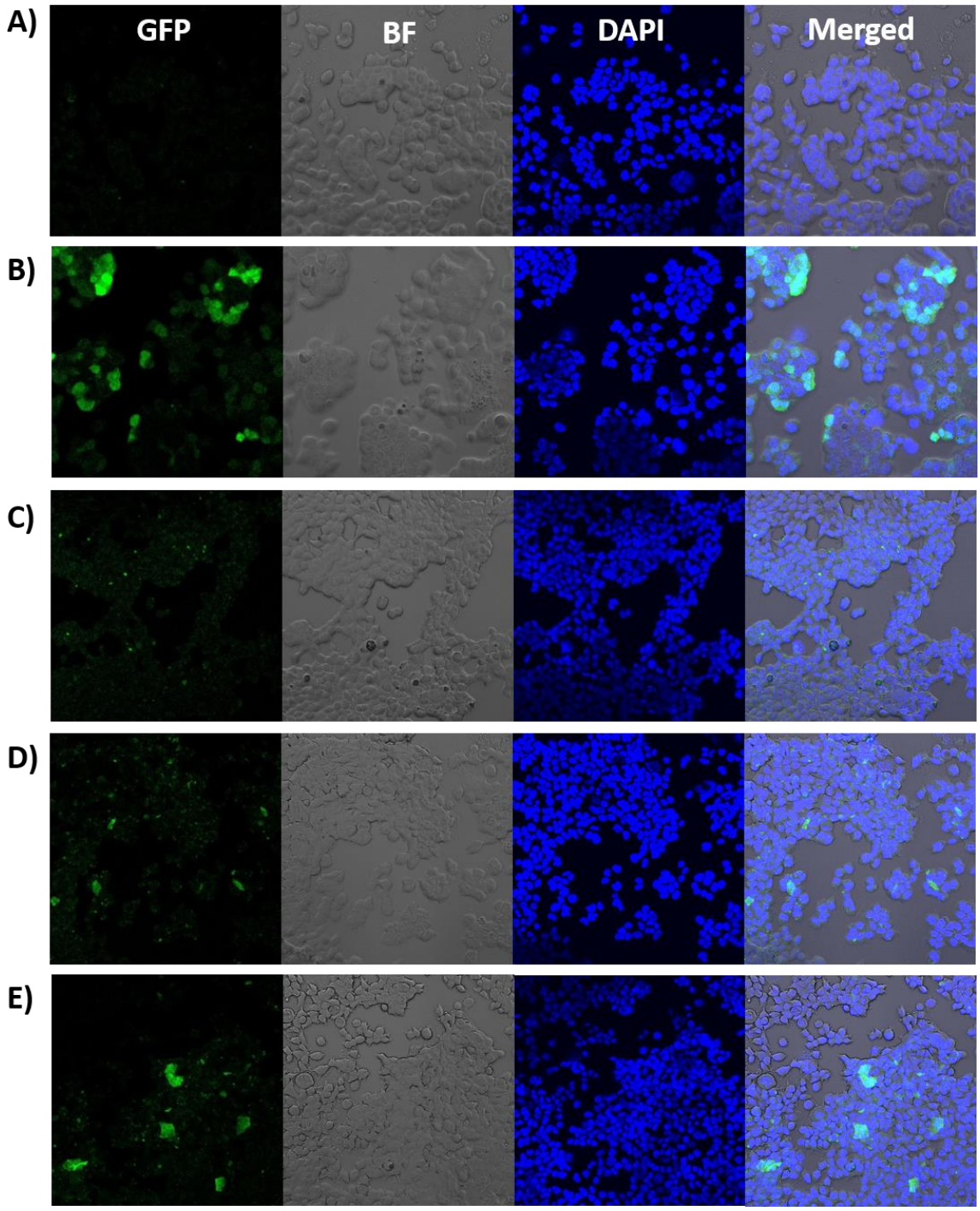

Figure 4.4. HEK293 cells treated with GFP mRNA encapsulated in modified APC with different w/w ratio

Nuclei were stained with DAPI, and the green stain represents GFP fluorescence. A) Untreated HEK293 cells. B) Cells treated with Lipofectamine 3000. C) cells treated with modified APC and GFP mRNA at a weight-to-weight (w/w) ratio of 10:1. D) Cells treated at a 20:1 ratio. E) Cells treated at a 50:1 ratio.

Assessing the aqueous stability of modified APC

An essential determinant of the effectiveness of nano-carrier delivery systems is their stability. To investigate the long-term stability of valine- isoleucine modified APC, a study was conducted examining their size characteristics employing NTA. It is worth noting that a the currently FDA-approved delivery lipid-based vectors lack temperature stability that require low temperature storage.[2, 6-8] Overcoming this limitation by developing delivery vectors that exhibit sustained stability at elevated temperatures and for extended periods would significantly enhance their utility, leading to improved product accessibility and operational efficiency. This methodology provides invaluable insights into the physical stability of nanoparticles by facilitating the evaluation of potential aggregation or degradation occurrences. This evaluation was conducted through the analysis of both the average capsule diameter and the predominant capsule size mode. Furthermore, the monitoring display provides a visual representation of the actual morphology of modified APC, enhancing our understanding of their structural characteristics and changes over time. The aggregation of APC holds significant implications for delivery efficiency, as it results in an increase in particle size. This enlargement, in turn, can render APC incompatible with efficient cellular penetration, hindering their intended therapeutic function. On the other hand, the degradation of APC particles can also substantially impact delivery efficiency, potentially leading to the disintegration of particles and the consequent inability to successfully transport cargo to target cells.

In this study, the size of the nanoparticles was systematically tracked for about a month. To ensure the ideal temperature conditions for these measurements, NTA machine was carefully configured to operate at 25 ℃ and stock samples were stored at room temperature throughout the experiment, thereby maintaining the integrity of the experimental environment and ensuring the

accuracy of the recorded data. **Figures 4.5 and 4.6** show the NTA data of modified APC and original APC with monitoring displays. In **Figure 4.5A, B, C, D, and E**, the NTA data obtained on Days 1, 3, 6, 16 Day 27 are presented. Notably, the data revealed a consistent hydrodynamic size of the modified APC over the entire 27-day observation period (**Table 4.2**). **Figures 4.6A to D** depict original APC formation and monitoring display from Day 1 to Day 25. As the x-axis represents the diameter of the capsule, the peaks of the graphs are uneven, and the size distribution is not consistent throughout the experiment duration compared to the modified APC. This stability in size strongly suggests that these modified APCs exhibit exceptional thermodynamic stability. Furthermore, the NTA monitoring display indicates minimal particle aggregation throughout the monitoring period (NTA monitoring display in **Figure 4.5**), underscoring a high degree dispersion. These combined findings strongly support the conclusion that modified APCs demonstrate outstanding colloidal stability for a duration of approximately one month at a temperature of 25 ℃. This robust stability is a highly favorable attribute for their potential applications in various therapeutic and drug delivery contexts.

Table 4.2. Size stability of modified APC for 27 days at room temperature

	Hydrodynamic diameter (nm)	sd	Mode (nm)	sd
Day 1	175.8	1.6	141.8	8
Day 3	171.8	5.1	136.2	7.9
Day 6	143	2.1	116.2	7.8
Day 16	151.9	2.3	126.5	4.4
Day 27	164.7	2.2	136.9	3.3

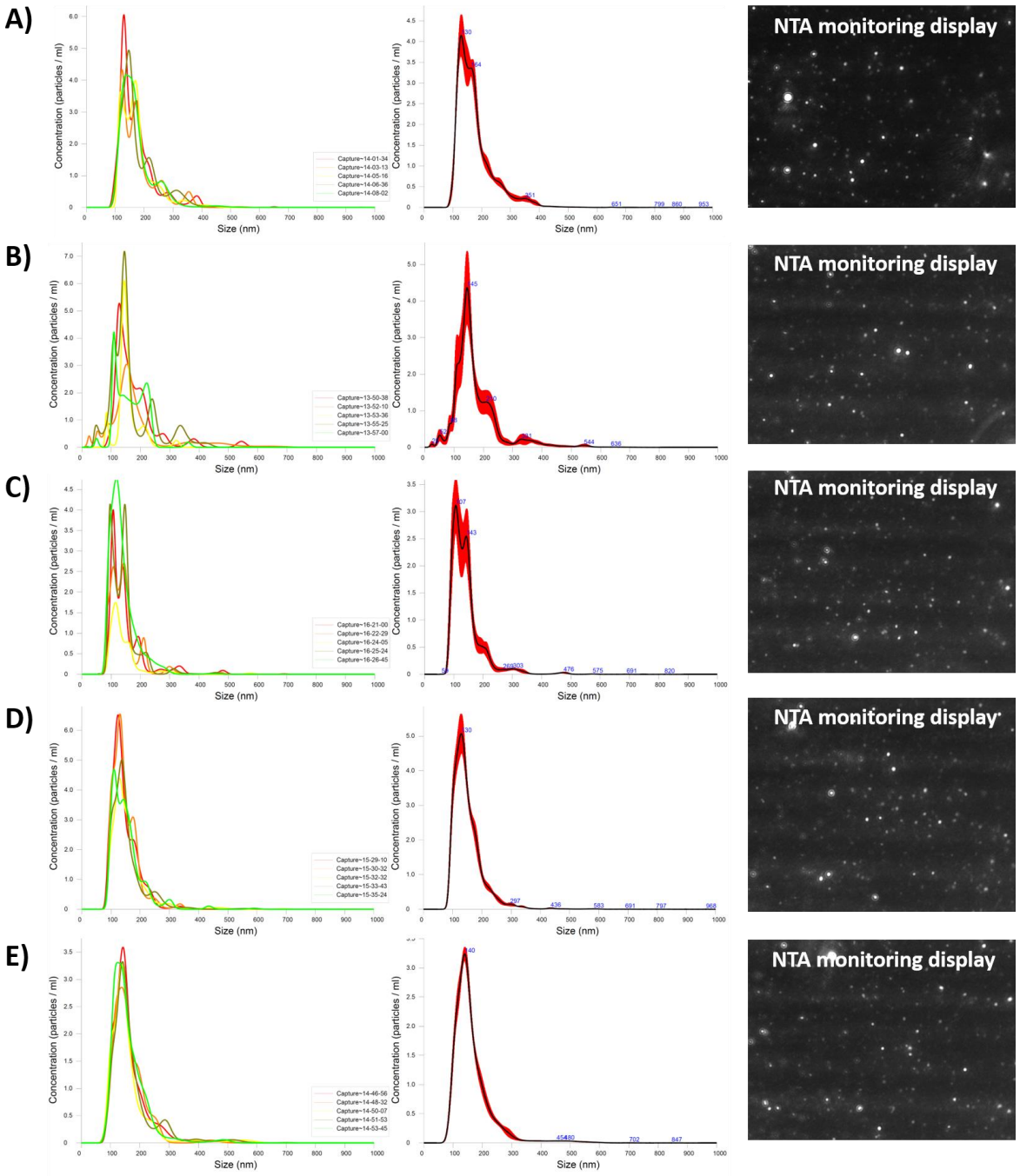

Figure 4.5. NTA tracking of modified APC size stability when stored at room temperature (25 °C) for 27 days

Modified samples stored at room temperature in an aqueous solution were monitored from day 1 to day 27. A) Nanoparticle tracking analysis (NTA) data on the first day of production. B) Data from the 3rd day after production. C) Data from the 6th day after production. D) Data from the 16th day after production. E) Data from the 27th day after production.

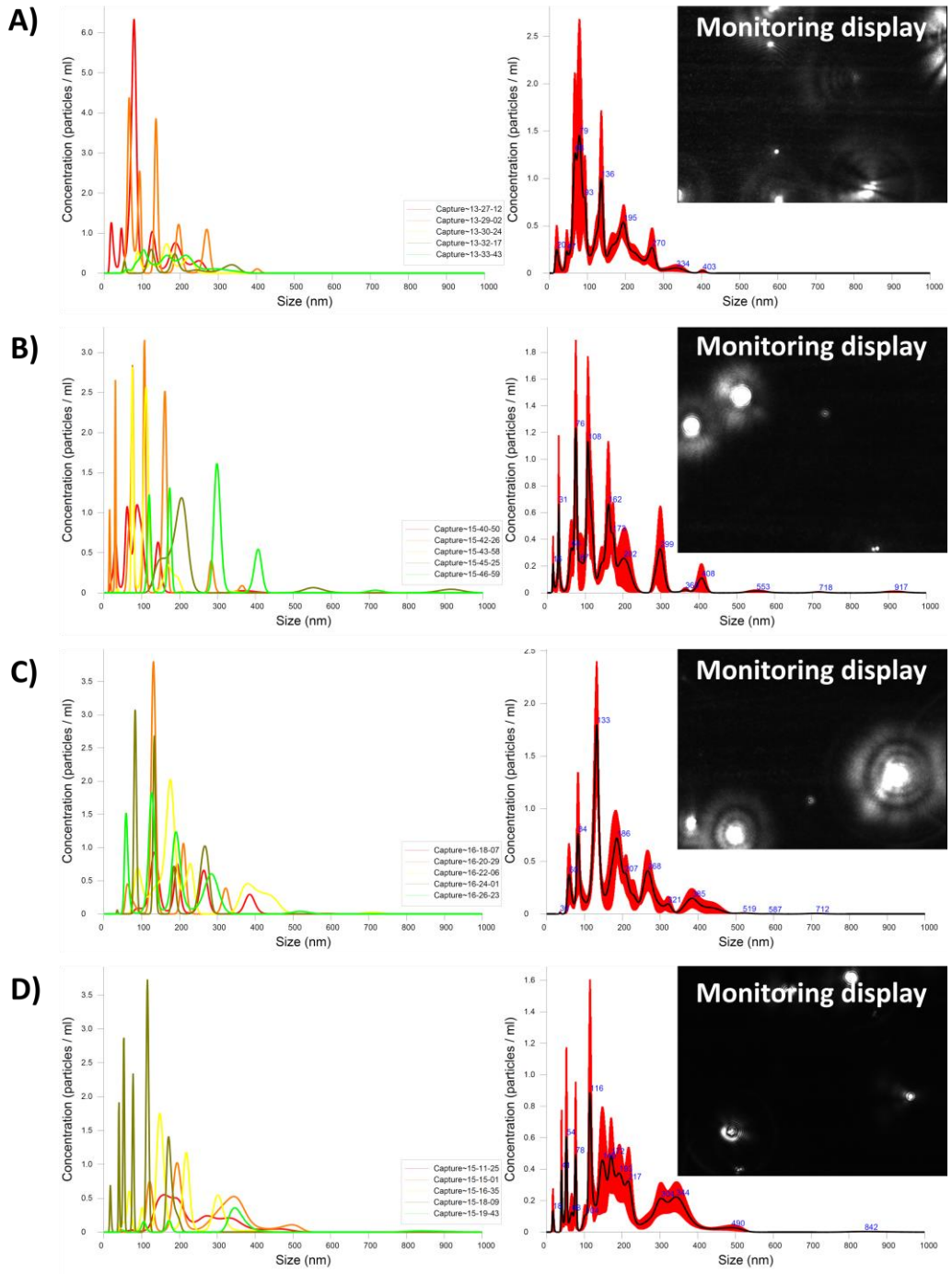

Figure 4.6. NTA tracking of original APC size stability when stored at room temperature (25 °C) for 25 days

Original samples stored at room temperature in an aqueous solution were monitored from day 1 to day 25. A) NTA data on the first day of production. B) Data from the 4th day after production. C) Data from the 14th day after production. D) Data from the 25th day after production.

After assessing and comparing the capsule-forming capacity, aggregation, and stability, long term stored modified APCs were tested for their transfection capacity. **Figure 4.7** displays the GFP expression resulting from mRNA delivery with mRNA surface-bound on modified APCs. These modified APCs were stored at room temperature in aqueous solution for approximately two months before use. In **Figures 4.7A and B**, the negative control (representing non-treated conditions) and positive control (Lipofectamine 3000) are presented, respectively. **Figures 4.7C, 4.7D, and 4.7E** correspond to w/w ratios of 10:1, 20:1, and 50:1, respectively. Similar to freshly prepared modified APCs, long-term stored modified APCs also show discernible GFP expression in HEK293 cells, especially at the 50:1 w/w ratio. These results highlight the transfection efficiency and stability of modified APCs as gene delivery vectors.

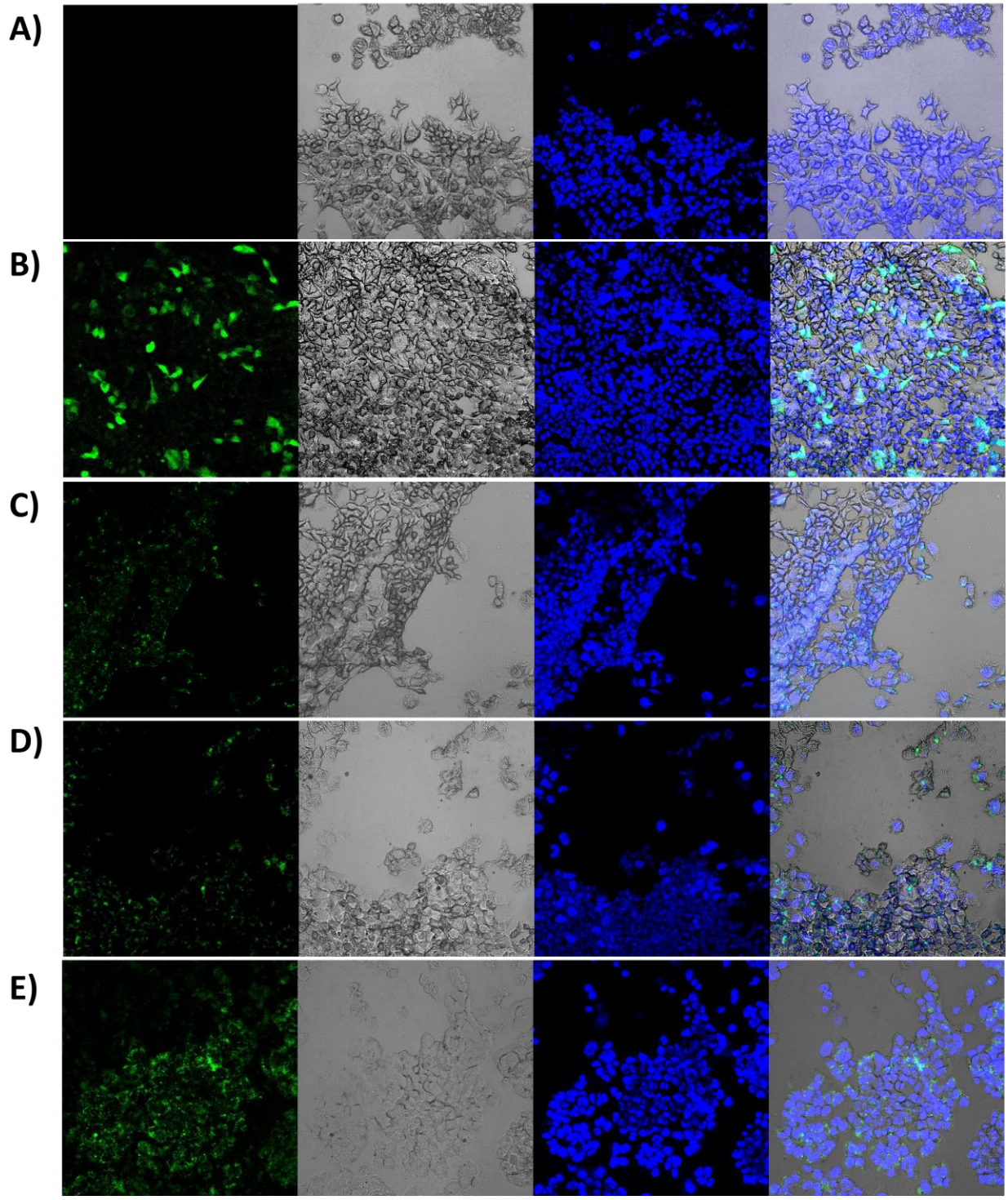

Figure 4.7. Evaluation of GFP mRNA expression using modified APCs with different w/w ratios for surface-bound attachment after long-term storage at room temperature

A) Non-treated HEK293 cells as a negative control, B) Lipofectamine 3000 treated, C) Cells treated with modified APC and GFP mRNA at a w/w ratio of 10:1, D) 20:1 ratio and E) 50:1 ratio.

In addition to long-term stability, assessing pH stability is crucial in understanding the robustness of nano-carrier delivery systems. Variations in pH can profoundly influence the behavior and integrity of nanoparticles, particularly in physiological environments. Therefore, alongside the evaluation of long-term stability, we extended our analysis to include pH stability. This comprehensive assessment provides a deeper understanding of how modified APCs respond to variations in pH, further enhancing our ability to design delivery vectors that maintain efficacy

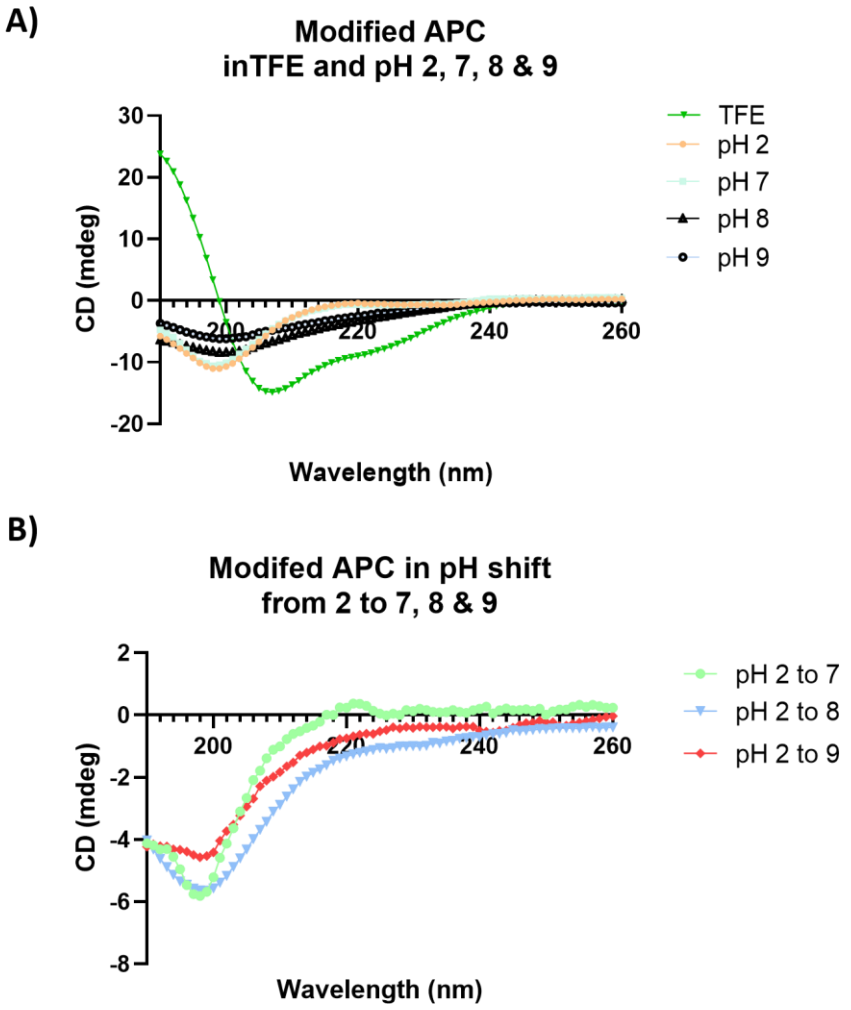

Figure 4.8. Circular dichroism data for modified APC at different pH value and pH shift

A) Beta-sheet secondary structure of modified peptide in TFE and random coil structure of modified APC in pH 2, 7, 8 and 9. B) Random coil secondary structure in pH shift (pH 2 to 7, 8 and 9).

across diverse biological conditions. CD was employed to assess the secondary structure of the modified peptides comprising APC across various pH values, as depicted in **Figure 4.8A**, including pH 2, 7, 8, and 9. At pH 2, the modified APC exhibited a random coil secondary structure, indicated by a distinct minimum in CD (mDeg) near 200 nm. Similarly, the modified APC formulated at pH 7, 8, and 9 also displayed a distinct minimum in CD (mDeg) at 200 nm, characteristic of a random coil secondary structure. Remarkably, even as the pH of the aqueous solution changed from 2 to 7, 8, and 9, transitioning from acidic to basic conditions, the random coil secondary structure was sustained (**Figure 4.8B**). it is noteworthy that despite the shift in pH from acidic to basic conditions, the secondary structure of the modified APC remained unaffected, demonstrating robustness across varying pH environments.

Following the CD spectra measurement of modified APCs under different pH conditions, subsequent analyses were performed on mRNA encapsulated within the modified APCs and mRNA bound to their surface. These analyses specifically addressed the preservation of the modified APCs' secondary structure across varying pH environments while nucleic acids were either encapsulated or surface-bound. These investigations yielded pivotal insights into the stability of them during interactions with nucleic acids.

Figure 4.9 illustrates mRNA encapsulated within modified APCs across various pH values. Remarkably, the modified APCs maintained a consistent random coil secondary structure irrespective of pH changes. This stability in secondary structure, akin to the observations without nucleic acid (**Figure 4.8**), underscores the robustness of modified APCs.

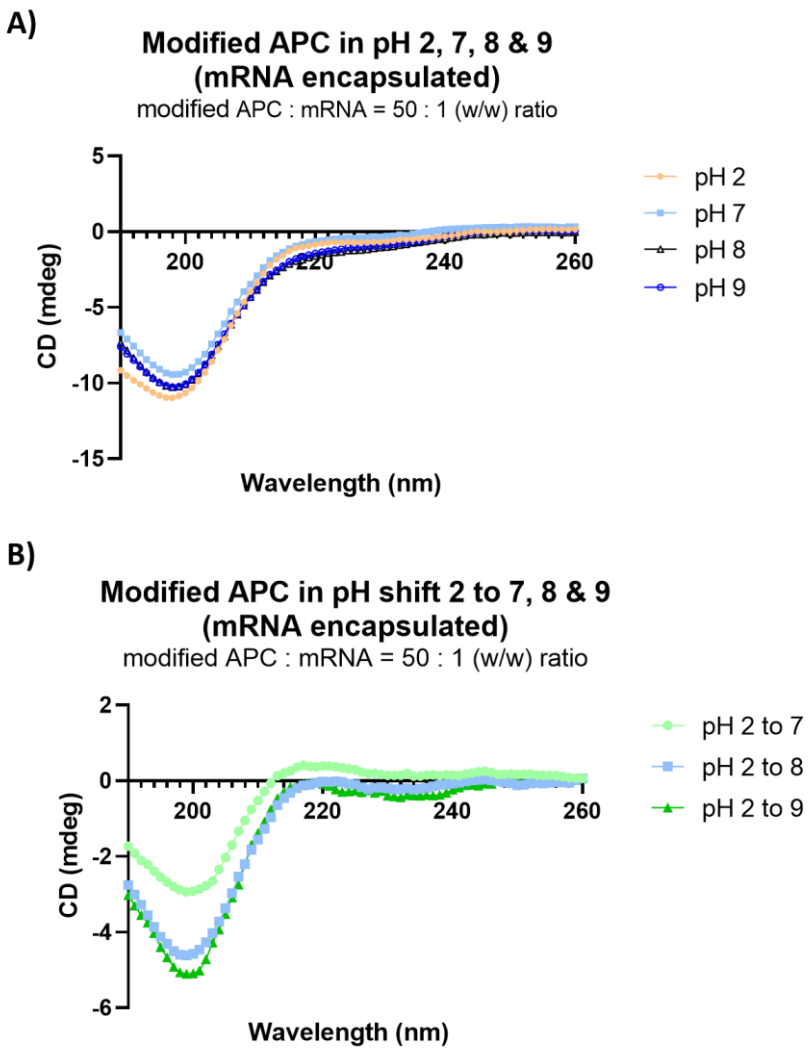

Figure 4.9. CD data for modified APC with nucleic acid encapsulated

A) Random coil structure of mRNA loaded inner cargo of modified APC in pH 2, 7, 8 and 9 and B) Random coil structure in pH shifting condition (from pH 2 to 7, 8 and 9) while mRNAs are encapsulated. Modified APC : mRNA = 50 : 1 (w/w) ratio.

Similarly, **Figure 4.10** provides additional evidence of the modified APCs' pH stability, showcasing their consistent random coil secondary structure even when mRNA is bound to their surface across different pH values. This robust maintenance of secondary structure suggests the modified APCs' resilience and suitability for effectively delivering genetic materials under diverse physiological conditions.

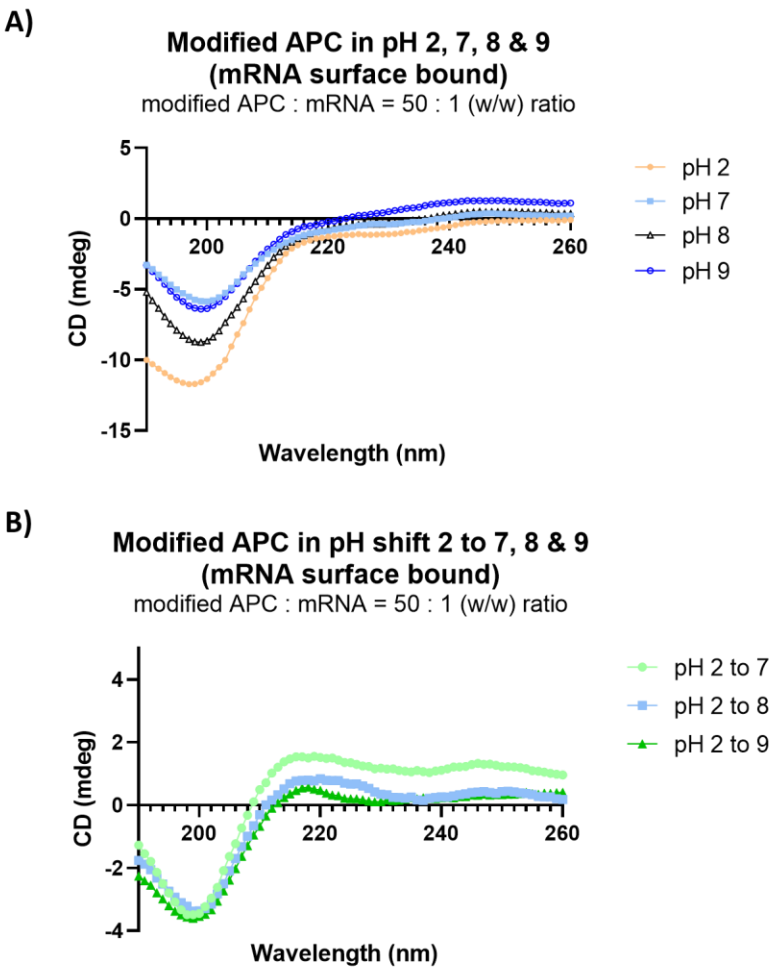

Figure 4.10. CD data for modified APC with nucleic acid surface bound

A) Random coil secondary structure of mRNA loaded on the surface of modified APC in pH 2, 7, 8 and 9 and B) random coil structure in pH shifting condition (from pH 2 to 7, 8 and 9) while mRNAs are encapsulated. Modified APC : mRNA = 50 : 1 (w/w) ratio.

Conclusions

The strategic modification of the original APC peptide sequence, “KKKFLIVIKKK” to "KKKFLIVIIVILKKK," marked by the incorporation of valine and isoleucine, represents an advancement in the of peptide-based nano-carriers developed in the Tomich laboratory. This innovative approach preserves the amphipathic properties critical for self-assembly while introducing elements that contribute to enhanced stability. The resulting structure, characterized by a mirrored and extended reflection of the original sequence, holds promise for biomedical research, offering improved therapeutic strategies and innovative solutions. The comprehensive evaluation of the modified APC's gene delivery capacity, utilizing GFP mRNA and assessing both surface-bound and encapsulated approaches, showcases its versatility in optimizing transfection efficiency. The observed increase in GFP expression from 10:1 to 50:1 w/w ratios in surface-bound applications and consistent expression levels in encapsulated mRNA across various ratios affirm the stability and efficiency of the modified APC as a gene delivery vector. Furthermore, the meticulous assessment of the aqueous stability of modified APC through NTA reveals exceptional thermodynamic and kinetic stability over a 27-day period at 25 ℃. The absence of degradation or noticeable changes in size underscores the potential applications of these nano-carriers in various therapeutic and drug delivery contexts. In addition to long-term stability, the pH stability of modified APCs was evaluated through CD analysis, demonstrating consistent random coil secondary structure across various pH conditions. This robust stability under different pH environments further highlights their suitability as carriers for genetic materials in diverse physiological and relatively extreme pH conditions. This research highlights the transformative potential of modified APCs, combining adaptability, stability, and efficient gene delivery. The nuanced modification of the peptide sequence, coupled with the strategic

integration of valine and isoleucine, positions these nano-carriers as promising candidates for addressing diverse therapeutic needs in biomedical and pharmaceutical applications. This dissertation contributes valuable insights to the evolving landscape of gene and drug delivery vectors, paving the way for future advancements in the field.

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Chapter 5 - Discussion and future directions

Discussion

The investigation conducted in this study has provided insights into the biophysical properties and functional capabilities of branched amphiphilic peptide capsules (BAPC) and aqueous partitioning capsules (APC) as potential vehicles nucleic acid delivery platforms. Through a series of experiments and analyses, various aspects crucial to understanding and optimizing the performance of these peptide nano-carriers (developed in the Tomich lab) in delivering mRNA were explored. One key aspect of my investigations involved the modification of iron oxide Magnetic Nano Beads (MNBs) with maleimide functional groups, facilitating the selective reaction with sulfhydryl groups of a custom-designed peptide. This modification enabled the formation of stable thioether linkages for monolayer formation on the MNBs' surface. This format allowed the investigation of the introduction of BAPs, which elevated MNBs' surface charge, confirming bilayer formation through π–π interactions and fluorescence quenching. This demonstrated the significance of phenylalanine aromatic ring interactions and bilayer formation. Further characterization of BAPC were conducted using Nano Tracking Analysis (NTA), which revealed distinctive capsule shapes and consistent size distribution over time.

These findings provided important insights into the capsule-forming capacity of BAPC, offering a look into their potential for targeted delivery applications. Investigations into the binding kinetics and saturation point of BAPC with mRNA shed light on optimal binding ratios, crucial for enhancing treatment efficacy while eliminating cold-chain requirements. The delicate balance between BAPC and mRNA binding capacity was explored, leading to the identification of optimal weight per weight (w/w) ratios. Moreover, the potential use of heparin for enhancing

mRNA release post-cellular uptake was investigated, emphasizing the importance of optimizing mRNA binding capacity without compromising premature release.

Surface charge modification emerged as a pivotal consideration for optimizing nucleic acid delivery vectors. The positively charged nature of surface cationic lysines presented a promising avenue for imparting a positive surface charge to BAPC and APC with mRNA complexation. Utilizing commercial poly-L-lysine (PLL) and lab-synthesized oligo-L-lysine with 10 lysine residues (OLL_{10}) demonstrated a dose-dependent increase in the positive charge of BAPC complexes, with OLL_{10} offering a more efficient and tunable approach. The ability to fine-tune the surface charge in a dose-dependent manner holds significant implications for customizing nucleic acid delivery vectors to specific applications, enhancing their effectiveness and biocompatibility. In vitro validation of positively charged BAPC coated with lysyl-oligomers revealed significant improvements in mRNA internalization and expression in mammalian cells, as evidenced by enhanced green fluorescent protein (eGFP) expression. Importantly, assessments of cell viability highlighted the biocompatibility and safety of BAPC compared to commonly used transfection reagents, underscoring their potential advantages in cell-based applications. Transitioning from in vitro investigations to in vivo studies represented a critical phase in evaluating the translational potential of BAPC for gene delivery. The exploration of luciferase mRNA expression in vivo, facilitated by various formulations including spray drying, provided promising insights into stabilizing mRNA/BAPC complexes, even under conditions of rehydration. However, challenges such as limited immune responses, tissue barriers, and genetic variability among individuals introduced complexities that influenced in vivo gene expression outcomes. The interplay between various components within the in vivo environment underscores the need for further investigation to unravel the underlying mechanisms governing

these divergent outcomes to optimize gene delivery systems for clinical applications. While exploring the potential of layer-by-layer coating of lysine onto the BAPC complex aimed to enhance gene delivery, it unfortunately did not yield luciferase expression in vivo, potentially due to an immune response triggered by the additional lysine surface layer coating. These observations led me to consider modifications to the original BAPC sequence without the layer-by-layer coating and thereby motivated the development of histidine-modified BAPC.

The exploration of histidine-modified BAPC provided promising avenues for mRNA delivery enhancement. Through systematic experimentation and analysis, this study aimed to elucidate the impact of histidine modifications on BAPC formation, mRNA encapsulation, and subsequent gene expression. The findings offer valuable insights into the potential of histidine-modified BAPC as versatile and efficient vehicles for nucleic acid delivery, with potential implications for gene therapy and mRNA-based treatments. Central to this investigation was the assessment of capsule-forming capacity among various combinations of h5, h9, h5h, and h9h peptides. NTA emerged as a crucial tool in discerning successful capsule formation, with distinct shapes and high concentrations observed in cases 1, 3, 4, and 5. These results underscore the importance of hydrophobic interactions in BAPC bilayer formation and highlight the efficacy of histidine-modified BAPC in capsule formation, laying a foundation for further exploration. Furthermore, the successful encapsulation of mRNA within histidine-modified BAPC was confirmed through NTA and Circular Dichroism (CD) spectroscopy. These assessments not only validated the encapsulation capacity but also provided insights into the stability of secondary structures with nucleic acids. The findings suggest that histidine modifications do not significantly disrupt the secondary structure observed in the original BAPC, emphasizing the compatibility of histidine-modified BAPC with mRNA encapsulation and delivery. The

incorporation of histidine residues into BAPC was motivated by the proton sponge effect hypothesis, which posits that mildly basic molecules, such as histidine, can induce endosomal rupture, thereby enhancing gene delivery efficiency. This rationale provides a seamless connection between the rationale for histidine modification and the expected outcomes in terms of nucleic acid delivery efficacy. Zeta potential experiments under varying pH conditions revealed pH-dependent behavior in histidine-modified BAPC, with notable alterations in surface charge observed. This dynamic interplay between pH dynamics and histidine protonation underscores the adaptability of histidine-modified BAPC to physiological conditions, potentially optimizing their transfection efficiency. The culmination of this research focused on evaluating the mRNA delivery capacity and efficiency of histidine-modified BAPC, particularly in promoting gene expression. Through in vitro assays utilizing GFP and luciferase as indicators, histidine-modified BAPC demonstrated a significant enhancement in gene expression. The observed increase in GFP and luciferase expression highlights the efficacy of histidine-modified BAPC in facilitating mRNA delivery and subsequent gene expression, paving the way for advancements in therapeutic applications. Notably, the incorporation of histidine modifications marked a significant breakthrough, enabling observable luciferase mRNA expression in vitro. This improvement has been attributed to the enhanced endosomal escape with the new vector system, that facilitates the liberation of mRNA from endosomal entrapment and subsequent translation in the cytoplasm. The findings underscore the potential of histidine-modified BAPC as efficient mRNA delivery vehicles, offering substantial improvements in gene expression and advancing the field of nucleic acid delivery.

Another potential peptide-based vehicle for gene delivery explored in this study is aqueous partitioning capsules (APC) and their modifications. Through extensive experimentation

and analysis, I examined the secondary structures, stability, and gene delivery capacities of these nano-carriers, illuminating their potential applications in biomedicine and pharmaceuticals. Modifications to the original APC sequence, incorporating valine and isoleucine, were strategically aimed at enhancing stability while retaining critical amphipathic properties necessary for self-assembly. The extended and mirrored reflection of the original sequence contributed to structural robustness, enabling interactions within the hydrophobic core of the capsule. Remarkably, the modified APCs demonstrated sustained stability over an extended period. Evaluation of the gene delivery capacity of modified APCs showcased their versatility in facilitating GFP mRNA expression via surface-bound and encapsulated delivery methods. The observed increase in GFP expression with varying APC to mRNA ratios underscores the flexibility of these nano-carriers in tailoring delivery strategies to specific therapeutic applications. Long-term stability in aqueous solution was crucial for the utility of modified APCs as delivery vectors. NTA measurements over 27 days showed consistent hydrodynamic size and minimal particle aggregation, indicating exceptional thermodynamic and kinetic stability. This trait preserves the integrity and functionality of therapeutic payloads during storage and transportation, enhancing accessibility and operational efficiency in biopharmaceutical contexts. Additionally, CD analysis demonstrated a consistent random coil secondary structure across diverse pH conditions, showcasing the resilience of modified APCs for delivering genetic materials, even in extreme pH environments.

Future directions

Enhancing immunogenicity reduction in BAPCs

In light of the observed immune response triggered by the additional lysine surface layer coating in BAPC, future efforts could pivot towards refining strategies to increasing immunogenicity for vaccines aimed at generating strong neutralizing antibody responses. Currently APC require the addition of adjuvants to initiate humoral responses. Without the adjuvants no inflammatory or antibody production is seen.

It is essential to recognize that immune stimulation reactions present significant hurdles to the clinical translation of gene delivery systems.[1-3] Both lipid nano-particles and attenuated virus systems trigger such responses, rendering them ineffective for repeated dosing situations. The fact that BAPC and APC do not induce inflammatory and immune responses make them a reasonable alternative to these other systems.

One promising direction involves exploring alternative surface modification methodologies or rationally designing peptide compositions aimed at improving immune recognition and activation upon administration. By judiciously engineering BAPC with surface moieties that are recognized as foreign and induce immunological responses would be useful for future mRNA vaccine development.

Advancing endosomal escape mechanism in histidine-modified BAPCs for enhanced mRNA delivery

Building upon the success of histidine-modified BAPC in facilitating mRNA delivery through the proton sponge effect, future research could aim to enhance endosomal escape mechanisms by exploring variations in the number and positioning of histidine residues within

the peptide sequence. The proton sponge effect, characterized by the osmotic swelling and rupture of endosomes induced by the influx of protons,[4-6] has proven effective in promoting the intracellular release of therapeutic payloads, such as mRNA. Leveraging this phenomenon, researchers can investigate how different configurations of histidine residues within BAPC influence endosomal escape and transfection efficiency.

Future investigations could focus on systematically varying the number and positions of histidine residues in the peptide sequence of BAPC to optimize endosomal escape mechanisms. By strategically adjusting the proton buffering capacity and membrane destabilization properties of histidine residues, BAPC formulations can be customized to improve the cytoplasmic delivery of mRNA. This approach holds promise for fine-tuning the intracellular fate of BAPC and maximizing their therapeutic potential in gene therapy and mRNA-based therapeutics.

Exploration of alternative cargo: broadening therapeutic horizons

The investigation into alternative cargoes represents a pioneering leap, steering beyond the use of mRNA. Among the forefront contenders are Small Interfering RNA (siRNA) and CRISPR-Cas9 components, cutting-edge tools in genetic modulation. SiRNA, renowned for its precision in selectively silencing specific genes[7, 8], becomes a molecular scalpel in the genetic toolkit. Meanwhile, the revolutionary CRISPR-Cas9 system emerges as a genomic editor, promising unprecedented levels of precision in gene modification. This strategic shift to incorporate alternative cargoes into the versatile realm of BAPC unfolds a new chapter in therapeutic applications. BAPC, whether positively charged with lysine coating or enriched with histidine, now stand ready to embrace siRNA and CRISPR-Cas9. The positively charged BAPC, as evidenced by the in vivo results, showcase promising outcomes in physiological

environments, highlighting their potential for navigating complex in vivo conditions. This expansion not only broadens the therapeutic horizons but also positions BAPC as agile platforms for a spectrum of genetic therapies. The ability to target specific genes with siRNA or precisely edit the genome with CRISPR-Cas9 aligns BAPC with the evolving landscape of personalized medicine. The promising outcomes from both in vivo and in vitro studies propel this gene-editing approach into the forefront of innovative and effective therapeutic strategies, marking a significant step towards translating these findings into practical clinical applications.

Integration of targeting strategies: precision in gene delivery

The investigation into the integration of targeted delivery strategies, specifically ligand-conjugated BAPC, represents a sophisticated approach to gene therapy. This strategic direction aims to enhance the specificity of gene delivery, potentially targeting specific cell types or tissues with unparalleled precision. Ligand-conjugated BAPC can be designed to recognize and bind to specific receptors on the surface of target cells, improving the accuracy of gene delivery. This level of precision holds immense promise for therapeutic applications, allowing for tailored treatments that minimize off-target effects and enhance the overall safety and efficacy of gene delivery. The integration of targeting strategies opens new avenues for the development of personalized and highly effective gene therapies. In addition to using ligand-conjugated BAPC, alternative methods for modifying the surface of gene delivery vehicles may involve incorporating stealth polymers like PEGylated polymers.[9, 10] These stealth polymers form a protective coating around the delivery vehicle, shielding it until it reaches its target cells and prolonging its time in the bloodstream. By employing PEGylated polymers, the delivery of therapeutic genes to target cells can be made more efficient, thus enhancing the overall

effectiveness of gene therapy. By combining ligand-conjugated BAPC with stealth polymer modifications, a comprehensive approach to achieving precise and effective gene delivery is possible, offering unprecedented control over treatment outcomes. This synergistic blend of targeting and stealth techniques represents a significant advancement in gene therapy, with the potential to transform the treatment landscape for various genetic disorders and diseases.

Advancing preclinical safety evaluation and regulatory compliance for clinical translation of BAPCs and APCs

Advancing towards clinical translation necessitates meticulous attention to safety and regulatory compliance, particularly in the context of BAPC and APCs for biomedical applications. The transition from preclinical research to clinical trials mandates comprehensive safety assessments to mitigate potential risks and ensure patient welfare. Future studies should prioritize rigorous preclinical evaluations, encompassing a spectrum of safety parameters ranging from immunotoxicity testing to biodistribution studies and long-term efficacy assessments. These evaluations are imperative for elucidating the safety profile and therapeutic potential of BAPC and APCs, laying the groundwork for their successful translation into clinical settings.

Comprehensive biodistribution studies are indispensable for elucidating the pharmacokinetics and tissue distribution profiles of BAPC and APCs post-administration. This knowledge not only informs dosing regimens but also aids in identifying potential off-target effects and optimizing therapeutic outcomes. Furthermore, long-term efficacy assessments are essential for gauging the durability and sustained efficacy of BAPC and APCs over extended treatment durations. These evaluations provide critical insights into the therapeutic efficacy,

safety, and long-term viability of BAPC and APCs as delivery vectors, guiding their clinical development and eventual integration into mainstream medical practice.

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