

# **Nuclear-targeted CRISPR delivery by using aptamer-modified polymer lipid nanoparticles**

**by Zichen Xu**

Thesis submitted in fulfilment of the requirements for  
the degree of

**Research Master**

under the supervision of A/Prof. Wei Deng

## **Certificate of Original Authorship**

I, **Zichen Xu**, declare that this thesis is submitted in fulfilment of the requirements for the award of *Master of Engineering (Research)*, in the *Biomedical Engineering/Faculty of Engineering and Information Technology* at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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## **Publications included in this thesis**

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*- Incorporated as Chapters 2.*

In this publication, I made substantial contributions to the following activities.

- 1) Conception and design of the project.
- 2) Analysis and interpretation of the research data underlying the publication.
- 3) Drafting significant sections and critically reviewing the work.

2. **Zichen X**, Mohammadreza H, Niusha S, Robert M.X W, Rui S, Wei D. Nuclear-targeted CRISPR delivery by using aptamer-modified polymer lipid hybrid nanoparticles. *Nanomedicine: Nanotechnology, Biology, and Medicine*. DOI: <https://doi.org/10.1016/j.nano.2026.102928>

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## Abbreviations

|           |                                                           |
|-----------|-----------------------------------------------------------|
| AAV       | Adeno-associated viruses                                  |
| AD        | Alzheimer's disease                                       |
| APT       | Aptamer                                                   |
| APT-NPs   | Aptamer modified nanoparticle                             |
| ATP       | Adenosine triphosphate                                    |
| AuNPs     | Gold nanoparticles                                        |
| C-6       | Coumarin 6                                                |
| CRISPR    | Clustered regularly interspaced short palindromic repeats |
| crRNA     | CRISPR RNA                                                |
| DOX       | Doxorubicin                                               |
| DSB       | Double-strand break                                       |
| EE%       | Encapsulation efficiency                                  |
| FBS       | Fetal bovine serum                                        |
| FTIR      | Fourier-transform infrared                                |
| GAPDH     | Glyceraldehyde-3-phosphate dehydrogenase                  |
| GFP       | Green Fluorescent Protein                                 |
| GTP       | Guanosine triphosphate                                    |
| HDR       | Homology-directed repair                                  |
| Lcn2 gene | Lipocalin-2 gene                                          |
| mtDNA     | Mitochondrial DNA                                         |
| MC        | Mander's split coefficient                                |
| MOFs      | Metal-organic frameworks                                  |
| NES       | Nuclear export sequences                                  |
| NHEJ      | Non-homologous end joining                                |
| NLS       | Nuclear localization signals                              |
| NPs       | Nanoparticle                                              |
| NPC       | Nuclear pore complex                                      |
| NUC       | Nuclease domain                                           |
| PAM       | Protospacer Adjacent Motif                                |
| PC        | Pearson's correlation coefficient                         |
| PD        | Parkinson's disease                                       |
| PDI       | Polydispersity index                                      |
| PDT       | Photodynamic therapy                                      |
| PLGA      | Poly (lactic-co-glycolic acid)                            |
| PTT       | Photothermal therapy                                      |

|          |                                                 |
|----------|-------------------------------------------------|
| REC      | Recognition domain                              |
| RISC     | RNA-induced silencing complex                   |
| RT-qPCR  | Real-time polymerase chain reaction             |
| ROS      | Reactive oxygen species                         |
| SDT      | Sonodynamic treatment                           |
| SEM      | Scanning Electron Microscopy                    |
| sgRNA    | Single guide RNA                                |
| T7E1     | T7 Endonuclease I                               |
| TAT      | Trans-activator of transcription                |
| TALENs   | Transcription activator-like effector nucleases |
| TEM      | Transmission Electron Microscopy                |
| TFOs     | Triplex-forming oligonucleotides                |
| tracrRNA | Trans-activating CRISPR RNA                     |
| UV-Vis   | Ultraviolet-visible                             |
| ZFNs     | Zinc finger nucleases                           |

## Abstract

The successful clinical translation of CRISPR/Cas9-based gene editing depends heavily on the development of safe and efficient delivery platforms, especially those capable of overcoming intracellular barriers and achieving precise nuclear localization. In this study, we designed a nuclear-targeted delivery system using aptamer-modified polymer-lipid hybrid nanoparticles to enhance the delivery efficiency and therapeutic potential of CRISPR/Cas9 in cancer treatment. The hybrid nanoparticles comprise a biodegradable PLGA core and a lipid shell functionalized with the AS1411 aptamer, a G-rich oligonucleotide known to bind nucleolin, which is overexpressed on the surface and within the nuclei of many cancer cells. This dual-function system was engineered to simultaneously promote cellular uptake and facilitate nuclear transport.

The nanoparticles were synthesized via a nanoprecipitation method and optimized for physicochemical properties including size, polydispersity, zeta potential, and encapsulation efficiency. Conjugation of the AS1411 aptamer was confirmed by FTIR and zeta potential shifts. Cellular studies demonstrated that aptamer-functionalized nanoparticles displayed significantly higher internalization in MDA-MB-231 and HCT-116 cell lines compared to non-targeted controls. Importantly, 3D confocal imaging confirmed the successful accumulation of CRISPR/Cas9 complexes within the nuclei of treated cells, indicating effective subcellular targeting.

Functionally, nuclear-targeted delivery of Cas9-sgRNA complexes resulted in enhanced genome editing efficiency. Gene knockout experiments targeting Lcn2 revealed increased indel formation as validated by T7 endonuclease I assay and RT-qPCR. Compared to non-targeted delivery systems, aptamer-functionalized nanoparticles achieved approximately 30% higher editing efficiency. Furthermore, cellular response assays such as wound healing and transwell migration demonstrated that knockdown of Lcn2 gene led to significant suppression of cancer cell migration, indicating the therapeutic potential of nuclear delivery of CRISPR.

This research addresses a major bottleneck in CRISPR/Cas9 therapeutics by overcoming the challenge of nuclear transport using a rationally designed, non-viral delivery vehicle. The AS1411-nanoparticles platform integrates structural stability, biocompatibility, and precise nuclear targeting, resulting in higher gene editing efficiency. These findings offer a promising strategy for advancing CRISPR-based gene therapies, particularly in the context of hard-to-treat malignancies such as triple-negative breast cancer. Future investigations may further

extend this platform by investigating the in vivo gene editing efficiency and subsequent therapeutic outcomes.

## **Chapter 1: Background**

## **Chapter 2: Introduction**

### **1.1. Background**

Advancements in gene editing technologies have facilitated precise genetic modifications, offering significant therapeutic potential for a wide range of genetic diseases and cancers. Among these tools, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), which was awarded the 2020 Nobel Prize in Chemistry, has emerged as a groundbreaking innovation in revolutionising biotechnology and biomedical research through its unprecedented ability to control over gene activity in nearly any organism [1-3]. CRISPR machinery can be delivered as RNA, plasmid DNA and Cas protein [4]. CRISPR/Cas format relies on two critical components: a Cas endonuclease and a gene-targeting guide RNA (gRNA). It targets the genomic DNA of cells which is a linear double-helix molecule primarily stored in the nucleus of eukaryotic cells [5]. The CRISPR/Cas mechanism works by using the Cas nuclease to precisely cut DNA at specific sites, allowing the cell's natural DNA repair pathways to make the required modifications [6, 7]. This cleavage typically results in double-strand breaks (DSBs), prompting intrinsic cellular repair pathways [8]. Repair via non-homologous end joining (NHEJ) generally introduces small insertions or deletions at the cleavage site, frequently leading to frameshift mutations and subsequent gene disruption [8]. Alternatively, in the presence of a homologous DNA donor template, homology-directed repair (HDR) can facilitate precise genetic corrections or insertions at the targeted genomic site [7]. NHEJ is commonly employed to achieve gene knockout models for research or therapeutic interventions, whereas HDR is harnessed to rectify pathogenic mutations or insert therapeutic genes [9]. Since this gene editing and the subsequent DNA repair processes primarily occur within the nucleus, the effective nuclear delivery of CRISPR/Cas is crucial for precise and efficient gene modification.

The clinical translation of CRISPR/Cas technology has progressed rapidly, marking a significant milestone with the FDA approval of the first CRISPR-based therapeutic, CASGEVY™ (CTX001), in 2023 [10]. This ex vivo therapy involves genome editing of patients' hematopoietic stem cells to disrupt a gene regulator, effectively treating hereditary disorders such as sickle cell disease and  $\beta$ -thalassemia [11]. Such landmark approval

underscores the clinical potential of CRISPR-based therapies, particularly demonstrating the feasibility and therapeutic effectiveness of ex vivo gene editing strategies.

Concurrently, substantial efforts are being directed towards developing robust in vivo CRISPR/Cas9 delivery systems to address a broader spectrum of genetic disorders. When CRISPR/Cas9 is used for in vivo therapy, it is commonly delivered either as a ribonucleoprotein complex or in the form of plasmid DNA encoding Cas9 and sgRNA. In both cases, successful gene editing requires the components to enter the cell nucleus, where the Cas9 protein can access and cleave the target DNA sequence [12, 13]. Therefore, efficient nuclear localisation is essential to ensure successful gene editing by both the Cas9/sgRNA complex or CRISPR-encoding plasmids. CRISPR/Cas9 delivery to the nucleus of live cells can be facilitated through viral vector systems, which have evolved natural mechanisms for nuclear entry [14, 15]. This is achieved through their interactions with host cell transport proteins, such as importins and exportins, which recognize nuclear localization signals on viral components and mediate their translocation into the nucleus [16, 17]. However, the use of viral vectors raises significant concerns, including the risk of inducing immune responses, tissue inflammation and limited packaging capacity [18, 19].

Nanoparticles have been explored as alternative carriers, providing a precise and safe tools of delivering CRISPR/Cas9 to target cells [20]. Various nanocarriers, including metallic, lipid and polymer nanoparticles, have been explored for CRISPR/Cas9 delivery. Compared to viral vectors, these nanocarriers present advantages such as lower immunogenicity, greater packaging capacity and an improved safety profile [21, 22]. Nuclear-targeted delivery using nanoparticles has shown promising results for the delivery of small molecule drugs. For instance, Fan et al. constructed metallic nanocarriers carry with cyclooxygenase-2 (COX-2) and doxorubicin (DOX), which consist of erythrocyte membrane-encapsulated graphene oxide quantum dots (GOQDs) functionalized with TAT and RGD peptides, have been engineered for targeting of both the nucleus and triple-negative breast cancer (TNBC) cell membranes [23].

Polymer lipid nanoparticles integrate the structural stability of polymer cores with the biomimetic fluidity and biocompatibility of lipid shells, creating a versatile platform for gene delivery. For CRISPR systems, the polymer PLGA provides controllable release and protection of Cas9 RNP against degradation, while the lipid layer enhances endosomal escape and reduces cytotoxicity. The PEG components allow surface functionalization with targeting ligands or aptamers (such as AS1411) enabling active engagement with nucleolin and promoting

perinuclear. This advantage not readily achieved by conventional lipid nanoparticles. Together, these features make PLNPs particularly suited for efficient and targeted CRISPR delivery.

Despite these advancements, the targeted delivery of genetic material specifically into the cell nucleus remains a relatively narrow and underexplored area within the broader field of gene delivery. Translating nuclear-targeted strategies to macromolecular therapeutics like CRISPR/Cas9 systems presents significant challenges. The large molecular size, complex tertiary structure and susceptibility to enzymatic degradation of CRISPR components hinder efficient intracellular trafficking, limit endosomal escape and restrict nuclear entry [24].

## **1.2. Research hypothesis and objectives**

The central hypothesis of this study is that polymer-lipid hybrid nanoparticles functionalized with a nuclear-targeting aptamer (AS1411) can significantly enhance the nuclear accumulation of CRISPR/Cas9 on cancer cells, thereby increasing gene editing efficiency and precision. The primary objective of this project is to develop and optimize a polymer-lipid hybrid nanoparticle platform for the nuclear delivery of CRISPR/Cas9 to improve in vitro transfection efficiency. By integrating rational nanoparticle design with active nuclear targeting, this study aims to fill a critical gap in the application of nanocarriers for CRISPR-based genome editing. The outcomes are expected to inform the development of more effective subcellular delivery strategies for therapeutic gene editing, with potential implications in both cancer treatment and broader applications in gene and cell therapy.

Specifically, this project aims to:

**Aim 1:** Design and synthesize polymer-lipid nanoparticles to attach AS1411 components in an efficient and stable manner

**Aim 2:** Validate the nuclear delivery efficacy of developed nanoparticles in HCT-116 and MDA-MB-231

**Aim 3:** Validate gene knockout efficacy of CRISPR/Cas9 delivered by nuclear targeted nanoparticles in cancer cell line

## **1.3. Significance and novelty**

Efficient gene editing using the Cas9/sgRNA complex requires access to genomic DNA within the nucleus, making effective nuclear entry a critical step. While viral vectors naturally facilitate nuclear delivery, their clinical use is limited by potential immune responses and risks

of off-target mutagenesis. In contrast, non-viral nanoparticles offer a safer alternative, but their limited ability to penetrate the nucleus reduces gene editing efficiency.

This project addresses this critical gap by leveraging aptamer-modified nanoparticles to enhance targeted nuclear delivery of Cas9/sgRNA complex. Although nuclear targeting has been widely studied for small-molecule drugs, its application to gene editing systems like CRISPR/Cas9 remains largely unexplored. By developing and optimising aptamer-functionalised nanocarriers for improved nuclear localisation, this work introduces a novel strategy that could significantly boost CRISPR/Cas9 editing efficiency, expanding its therapeutic applicability to a wider range of genetic disorders.

#### **1.4. Thesis outline**

The whole thesis is composed of six chapters, and each chapter is briefly introduced as following:

Chapter 1 introduces the background and motivation of this research, highlighting the urgent need for developing novel delivery systems capable of achieving subcellular targeting, particularly nuclear localization. The chapter presents the central research hypothesis, outlines the specific aims and provides an overview of the overall thesis structure.

Chapter 2 provides a comprehensive literature review on the development of gene therapy and the CRISPR/Cas9 genome editing system. It summarizes the advantages and limitations of existing delivery strategies and explores current advances in subcellular-targeted delivery, with particular emphasis on nuclear-targeting approaches. Based on the critical knowledge gaps identified, the chapter proposes the central hypothesis and scientific rationale of this study.

Chapter 3 describes the materials, reagents, and experimental methodologies employed throughout the entire research project. It includes detailed procedures for the synthesis of polymer-lipid hybrid nanoparticles, conjugation of AS1411 aptamers, and CRISPR/Cas9 loading. The chapter also covers the methods for physicochemical characterization, cell culture, transfection protocols, and nuclear localization assays, as well as gene editing evaluations using T7 endonuclease I assays and fluorescence imaging. Additionally, techniques for assessing cellular responses are included. This chapter summaries the experiments and protocols, which directly related to the results in Chapters 4 and 5.

Chapter 4 focuses on the development and characterization of AS1411-functionalized polymer–lipid hybrid nanoparticles, which directly address Aim 1 and 2. It details the

fabrication process of the nanocarrier system, including aptamer conjugation via EDC/NHS chemistry and subsequent physicochemical characterization. The chapter then evaluates the cellular uptake efficiency of the nanoparticles in HCT-116 and MDA-MB-231 cancer cell lines using fluorescence microscopy and flow cytometry. Nuclear targeting efficacy is further assessed through confocal imaging and quantitative colocalization analysis to determine the extent of nanoparticle accumulation within the nucleus. These results confirm the successful design of a nucleus-targeted delivery platform and provide a basis for evaluating its functional gene editing capability in the following chapter.

Chapter 5 presents the functional validation of CRISPR/Cas9 delivery using the nucleus-targeted nanoparticle system, addressing Aim 3. This chapter first investigates the gene knockout efficiency of the hybrid nanoparticles in GFP-expressing cells as a model system. It then explores the therapeutic relevance by targeting the *Lcn2* gene in MDA-MB-231 cells. Editing efficiency is quantified using molecular assays, and the biological effects of *Lcn2* knockdown on cellular responses are further examined. Comparative analyses between nuclear-targeted and non-targeted nanoparticles are conducted to demonstrate the advantage of nuclear localization in enhancing gene editing outcomes.

Chapter 6 summarizes the major findings of the research, highlighting the contributions made toward the development of a nucleus-targeted CRISPR/Cas9 delivery system. The chapter also discusses limitations and proposes future research directions aimed at advancing subcellular-targeted gene therapy for translational applications.

## Chapter 2: Literature review

### 2.1. Introduction

Advances in gene editing technology have enabled precise gene modification, providing potential therapeutic effects for various gene-related diseases, thus revolutionizing the study of the human genome. Today, the most novel and most watched gene editing method is the CRISPR/Cas system. The CRISPR/Cas9 system is adapted from the bacterial immune mechanism. These microorganisms save a piece of viral DNA inserted into their own CRISPR sequences. When encountering the virus again, these sequences are transcribed into RNA, guiding the Cas protein to destroy the virus by pairing with the invader's DNA sequence and cutting them [25]. Jennifer Doudna and Emmanuelle Charpentier first used this mechanism to develop and create this advanced genome editing tool in 2012 [26]. The single guide RNA (sgRNA) in the CRISPR/Cas9 system can recognize specific target genomic sites and guide the Cas9 protein to cut the target DNA sequence [27]. Then, it can not only repair the double-strand break (DSB) by inducing non-homologous end joining (NHEJ), leading to gene insertion or gene knockout. And it can also repair the homology-directed repair (HDR) for specific gene modification [28, 29]. CRISPR/Cas9 is the new future of gene editing technology, opening a new era of precise gene manipulation.

Recently, CRISPR-Cas9 delivery still remains challenge. CRISPR/Cas9 will be degraded by biological enzymes in organisms, and endosomal escape is also one of the key challenges for the effective delivery of CRISPR/Cas9 components to target cells or tissues [30].

To address this challenge, researchers are exploring new strategies to improve the nuclear targeting efficiency of CRISPR/Cas9. One approach is to target the cell nucleus. By utilizing CRISPR/Cas9 proteins with nuclear localization signals (NLS), it is easier to enter the cell nucleus through nuclear pores [31]. Some studies have also focused on improving carrier systems, such as using cationic polymers or lipid nanoparticles that can efficiently escape endosomes, thereby increasing the intracellular delivery efficiency of CRISPR components[32].

Unfortunately, current targeted therapeutic strategies remain limited, with only a small fraction of drugs showing the ability to effectively concentrate within the desired tissue [33]. Work published in 2016 showed that only 0.7% of nanomaterials successfully accumulated near the intended target, leaving a large portion to be absorbed by normal tissues and produce toxic side effects in patients [34]. In addition, the effective targets of CRISPR are mainly located in the cell nucleus. These facts emphasize the urgent need to develop effective targeted therapies.

## 2.2. Evolution of gene therapy technology

As early as the 1970s, researchers first completed genetic modification and gene transfer in an in vitro environment through bacterial cells, endonucleases and exogenous DNA [35-37]. Since this milestone, gene therapy has developed rapidly. Gene therapy began to develop significantly at the same time as cancer treatment. In 1990, what is generally considered to be the first successful human gene therapy case was born in the United States. Scientists performed gene therapy on a little girl suffering from adenosine deaminase deficiency (ADA-SCID) [38]. Genome editing is characterized by the precise manipulation of cellular genetic sequences to change cellular characteristics and organismal characteristics. Common gene editing technologies mainly include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) systems [39-41].

**Zinc finger nucleases (ZFNs)** are an early-development genome editing technology (**Figure 2.1**). It mainly consists of two parts: one is the zinc finger protein domain that recognizes specific DNA sequences, and the other is the nuclease responsible for specifically recognizing and cutting DNA (usually the catalytic domain of FokI enzyme) [42]. Each ZFN recognizes a specific DNA sequence, and the two work in pairs to introduce DSBs on the target sequence [43]. The cell's self-repair mechanism will repair these breaks, allowing gene repair or editing [42]. However, ZFN has inevitable problems, namely complex design and structure. Each zinc finger protein must specifically recognize a DNA sequence of 3-4 base pairs, so a combination of multiple zinc finger modules is required to recognize longer target sequences [44]. Moreover, the targets of ZFN are limited by the sequences that zinc finger proteins can recognize, and different customized parameter optimization is required for different targets [45]. This significantly increases the complexity of the design and the difficulty of wide-scale application.

**Transcription activator-like effector nucleases (TALENs)** developed to improve the complexity of ZFNs (**Figure 2.1**). Similar to ZFN, it consists of two parts but uses a transcription activator-like effector (TALE) instead of a zinc finger protein [46]. Each TALEN protein contains a series of repeatedly repeated TALE modules that accurately recognize and bind target DNA sequences [46]. Then DSB is also introduced for editing. The TALE module recognizes a single nucleotide and therefore has higher specificity and lower off-target effects than ZFN [47]. However, although the design process is simpler than that of TFN, the production of TALEN is still complex and costly.

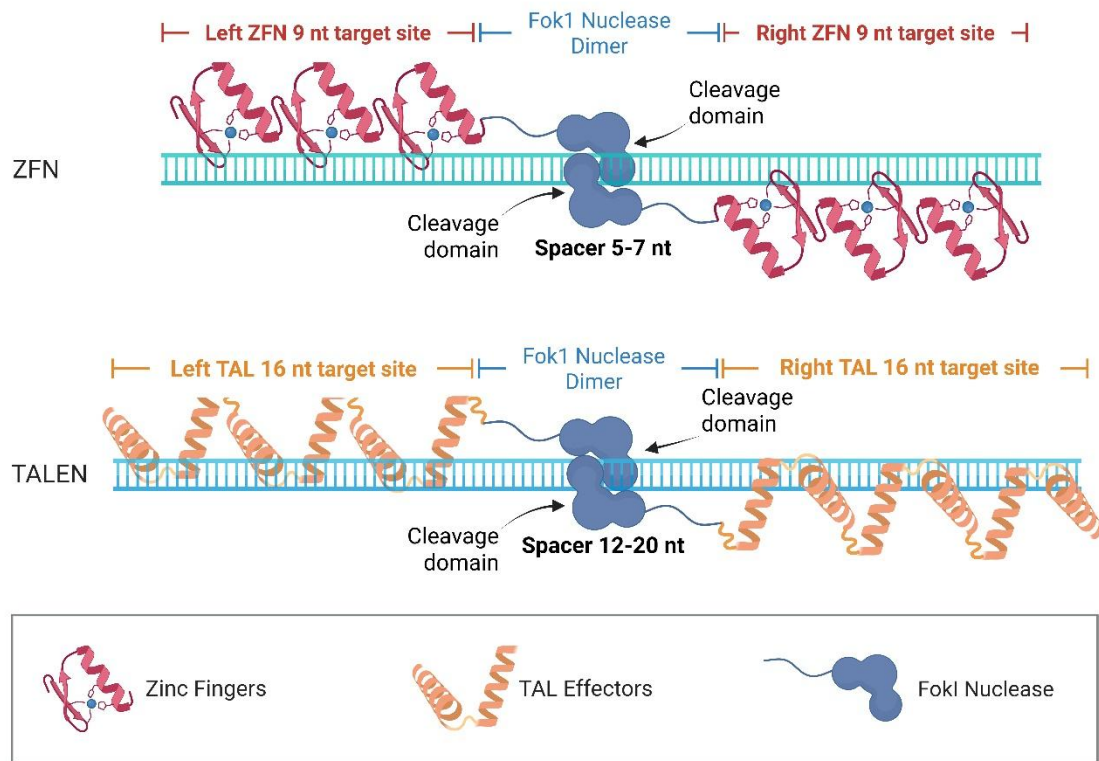


**Figure 2.1.** *Structural Comparison of ZFN and TALEN DNA-Binding and Cleavage Mechanisms.* This schematic illustrates the design and DNA recognition mechanisms of Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs). Created in BioRender. Xu (2025) <https://BioRender.com>.

**Clustered Regularly Interspaced Short Palindromic Repeat- associated protein system (CRISPR/CAS9)** can be traced back to the 1980s, but it was not until the early 21st century that it was used as one of the gene editing tools. The development of its related Cas system has completely changed traditional gene editing technology [48]. In 2012, the team of Jennifer Doudna and Emmanuelle Charpentier first proposed the possibility of using the CRISPR/Cas9 system for genome editing, and won the Nobel Prize in Chemistry in 2020 for their pioneering contributions to CRISPR/Cas9 technology [49]. The mechanism of CRISPR relies on Cas9 protein (nuclease) and guide RNA (gRNA) [50]. However, because the CRISPR system relies on base pairing of guide RNA (gRNA) with target DNA, this pairing may lead to the accidental recognition and cleavage of similar but non-target sequences in the genome, resulting in off-target effects [51]. ZFNs and TALENs rely on protein-DNA interactions, which are usually complex and precise, so their off-target risks are relatively low [47]. Although there is a problem of off-target, compared with ZFN and TALEN, the design is simpler, the cost is lower, the operation is more convenient, and the scalability is stronger [44].

## 2.3. CRISPR and mechanism

The CRISPR/Cas system is a groundbreaking advancement compared to traditional genetic engineering methods, offering a promising alternative to conventional gene editing technologies. It opens new pathways for gene therapy and cellular editing in the treatment of human diseases.

### 2.3.1. History of CRISPR gene editing technology

The CRISPR/Cas system is more novel than traditional genetic engineering and is a promising alternative to traditional gene editing technology. It paves the way for gene therapy and cell editing of human diseases. Initially, when studying the genome sequence of *Escherichia coli*, several regularly spaced repetitive sequences were noticed. The function of these repetitive sequences was unclear at the time, so they were called "CRISPR" [48]. In 2000, researchers found that these repetitive sequences were adjacent to DNA fragments of certain viruses. In 2005, geneticists Francisco Mojica and Ruud Jansen first described these systems as an immune mechanism of prokaryotes [25, 52]. As shown in **Figure 2.2**. In 2007, experiments by Rodolphe Barrangou and Philippe Horvath from the Danish Food Research Center first confirmed the role of the CRISPR/Cas system in bacterial immunity: once bacteria are attacked by bacteriophages, the CRISPR system will remember the DNA of the invading virus and identify and cut it when it appears again to prevent infection. This discovery laid the foundation for the development and application of CRISPR technology [53]. Nowadays, the CRISPR/Cas system has been adapted for precise editing of mammalian genomes.

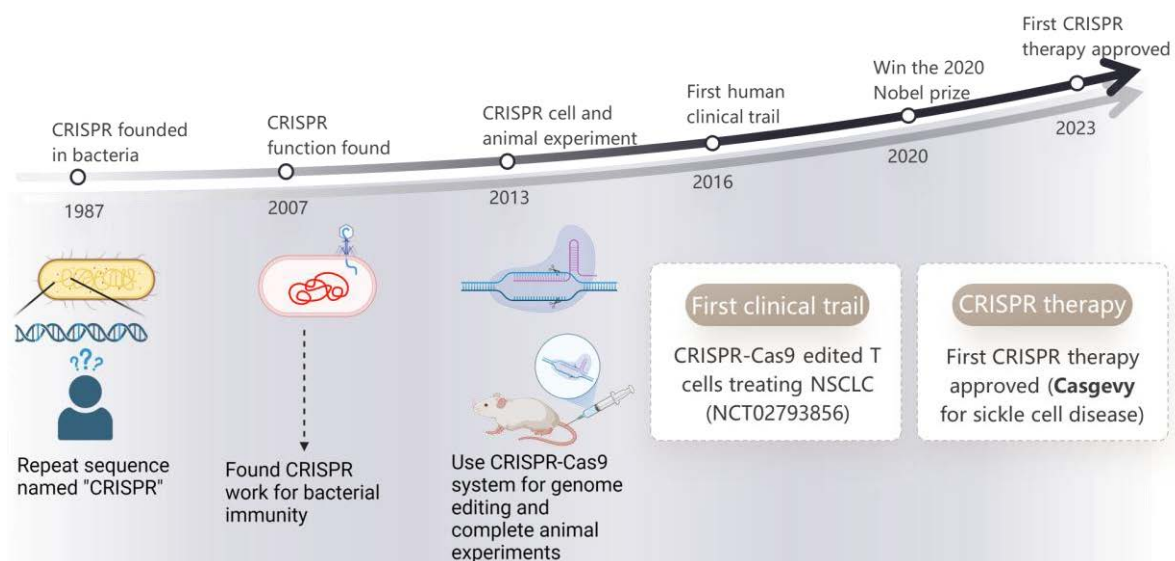


**Figure 2.2.** *Timeline of Major Milestones in CRISPR Development and Clinical Translation.* This figure illustrates the key historical advancements in CRISPR research and its clinical applications. Created in BioRender. Xu (2025) <https://BioRender.com>.

### **2.3.2. CRISPR gene editing structure and classification**

The CRISPR/Cas gene editing system is unique and consists of two main components that are delivered together: the Cas protein and sgRNA.

sgRNA is a synthetic RNA sequence that combines CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) [54]. Its primary role is to act as a guide for the Cas protein, directing it to target specific DNA sequences for editing. The crRNA part contains repeat sequences and spacer sequences. Repeat sequences are short, conserved, and directly repeated sequences, while spacer sequences are situated between repeat sequences and are derived from the DNA of invading viruses or plasmids [55]. tracrRNA includes a region that binds to crRNA, creating an RNA complex that guides the Cas protein to specific DNA sequences. tracrRNA also contains a region that binds to the Cas protein, promoting its activation [55].

The Cas protein is a multifunctional endonuclease comprising a recognition domain (REC) and a nuclease domain (NUC) [56]. The REC domain forms the bulk of the Cas protein. It contains three main subdomains responsible for recognizing and binding the crRNA-tracrRNA guide complex and the target DNA: REC1, REC2, and Bridge Helix [56, 57]. REC1 and REC2 are mainly responsible for binding RNA and providing a stable framework for the RNA-DNA hybrid sequence. REC1 is located at the core of the protein, forming multiple key molecular interactions with the crRNA sequence [57]. The Bridge Helix primarily participates in signal transduction, activating cutting activity upon target DNA recognition [58].

The NUC domain mainly includes the RuvC, HNH, and PAM-Interacting (PI) subdomains, which play a critical role in DNA cleavage during gene editing [6]. RuvC is responsible for cleaving the non-complementary strand of the target DNA. Additionally, RuvC endonuclease contains metal ions that are crucial for maintaining the CAS protein's structure and function [59]. HNH is tasked with cutting the complementary strand of the target DNA [60]. The PI domain primarily recognizes the PAM (Protospacer Adjacent Motif) sequence near the guide RNA binding site [59, 60]. PAM is a short, conserved sequence in target DNA that is essential for the Cas protein to bind and cleave DNA. It determines the accuracy of Cas9's binding site on DNA [61-63].

CRISPR can be categorized into two major classes (Class 1 and Class 2) and several subtypes (Type I-VI) based on the composition and structural differences of the Cas proteins:

Class 1 systems consist of multi-protein complexes, primarily including Types I and III [64].

- Type I is typically composed of Cas3 protein, known for its excellent target efficiency and specificity. However, its complex protein structure makes designing delivery systems challenging [65].
- Type III commonly consists of Cas10 protein, which has dual targeting capabilities, able to target and degrade both DNA and RNA. Despite its potential, this protein's complex structure remains under active research [66].

Class 2 systems are built around a large, single-effector protein structure, including Types II, V, and VI [64, 67, 68].

- Type II usually comprises Cas9 protein, which efficiently targets and cuts DNA sequences and is widely applied in genetic editing. Despite numerous improvements, it still presents potential off-target effects and immune rejection risks [69, 70].
- Type V is commonly composed of Cas12 protein, which offers a flexible targeting mechanism capable of RNA-guided targeting and cleaving of DNA. Additionally, it can activate additional nuclease degradation after target cutting. Although not as established as the Cas9 toolset, it holds significant potential in gene editing [64, 68].
- Type VI typically includes Cas13 protein, specialized in RNA targeting and degradation, aiding in RNA function research. However, its ability to focus solely on RNA targets limits its application in genome editing [64, 71].

Additionally, Type IV lacks the typical ability to recognize and cut DNA, making it challenging to apply in gene editing; its specific biological functions and applications are yet to be discovered [72].

### **2.3.3. Mechanism of CRISPR gene editing**

The CRISPR/Cas9 system consists of the Cas9 endonuclease and guide RNA, directing Cas9 to genomic sites to achieve precise DNA cleavage [73]. Cas9 possesses a complex single-protein core, incorporating a recognition domain, hinge region, two nuclease domains, a bridge helix, a protospacer adjacent motif (PAM) interaction domain, and an RNA binding site [54]. This system holds tremendous potential in genetic and biomedical applications and is widely researched and applied in gene therapy. Compared to traditional gene editing methods, this

powerful tool significantly enhances precision. It not only enables genetic correction and gene knockout but also allows the insertion of new genetic information for gene knock-in.

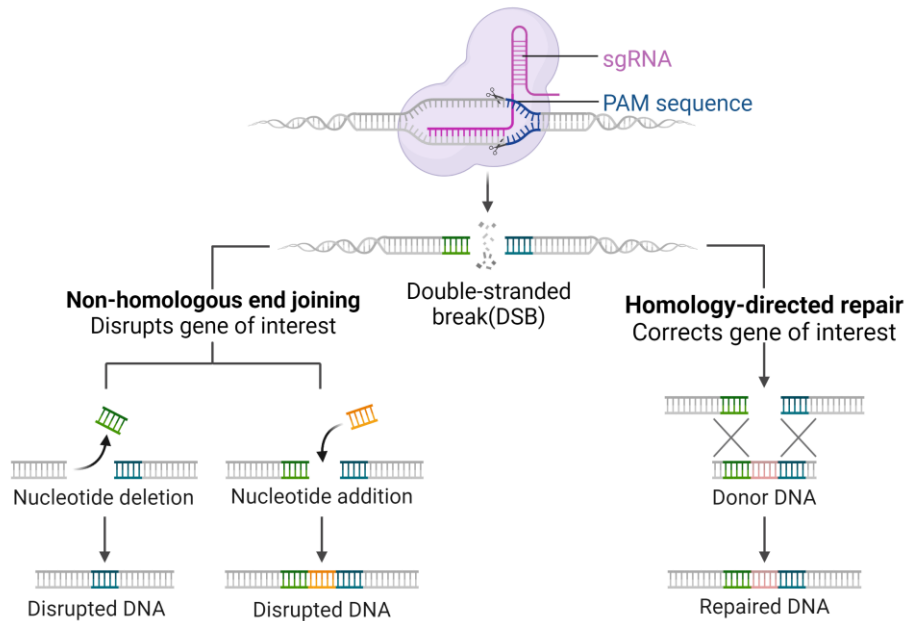


**Figure 2.3.** Mechanism of CRISPR/Cas9-Mediated Genome Editing and DNA Repair Pathways. The CRISPR/Cas9 system introduces targeted double-strand breaks in genomic DNA by guiding the Cas9 endonuclease to a complementary DNA sequence adjacent to a protospacer adjacent motif (PAM). Following cleavage, cells repair the DSBs through two main pathways. Created in BioRender. Xu (2025) <https://BioRender.com>.

The CRISPR/Cas9 system operates by exploiting the cell's inherent DNA repair mechanisms, resulting in two main outcomes of genetic manipulation: gene knockout and gene knock-in [73]. As shown in **Figure 2.3**, the guide RNA (sgRNA) plays a crucial role in the editing process, identifying the target's PAM site and guiding the entire system to the appropriate location before introducing a double-strand break (DSB) via the Cas9 endonuclease [49]. These breaks trigger the cell's repair mechanism known as non-homologous end joining (NHEJ) [28]. This process attempts to rejoin the DSB, but the gene specified by the sgRNA has already been inactivated before completion, effectively disrupting its function [28, 74]. For gene knock-in, the CRISPR/Cas9 system follows a slightly different path. Although it still induces a double-strand break in the target gene DNA like the knockout model, a repair DNA template is introduced into the target cells along with Cas9 and gRNA [29, 75]. The cellular repair

mechanism is now guided by the repair template to integrate the desired changes into the gene, thus enabling precise gene modification [75].

There are a few kinds of CRISPR components (**Figure 2.4**). Plasmid delivery involves nuclear import of the vector followed by transcription of Cas9 and sgRNA within the nucleus, export to the cytosol for translation, and re-import of the Cas9-sgRNA complex into the nucleus for genome editing. mRNA delivery bypasses the need for nuclear transcription, as both Cas9 mRNA and sgRNA are translated directly in the cytosol to form the active complex. RNP delivery introduces the pre-assembled Cas9-sgRNA complex directly into the cytosol, enabling immediate nuclear import and genome editing. Among these, RNP delivery offers the fastest action and lowest risk of genomic integration, making it attractive for precise gene editing applications. Except mRNA format only needs to go into the cytosol, the Plasmid and RNP formats need to be delivered into the cell nucleus.

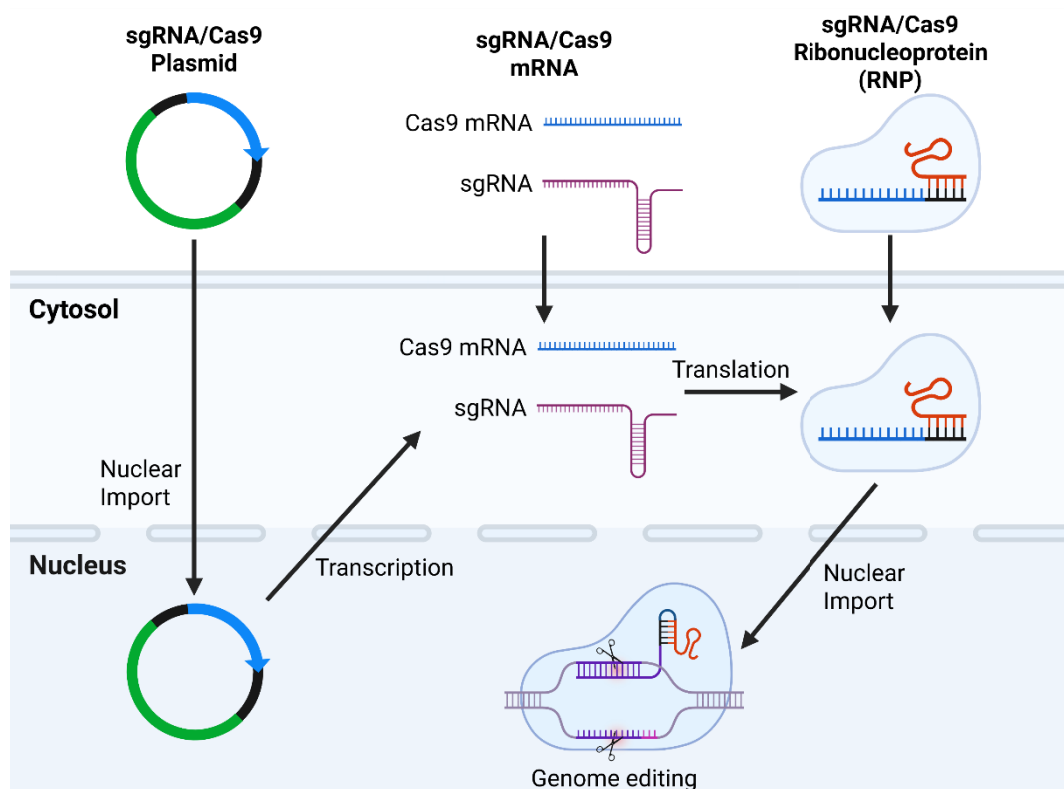


**Figure 2.4.** Intracellular Pathways of CRISPR/Cas9 Delivery Formats for Genome Editing. This schematic compares the cellular processing of three CRISPR/Cas9 delivery formats: plasmid DNA, mRNA, and ribonucleoprotein (RNP). Created in BioRender. Xu (2025) <https://BioRender.com>.

## 2.4. CRISPR delivery system

As previously mentioned, the CRISPR system has undergone transformative evolution, offering tremendous potential for treating human genetic diseases. To unlock its therapeutic capabilities, it is essential to develop innovative delivery strategies for CRISPR that can enhance targeting efficiency and reduce off-target risks. In this section, we will explore the various well-established CRISPR delivery formats currently available and then delve into the different vectors used to transport the CRISPR/Cas system to its intended cellular destinations.

### 2.4.1. Physical delivery

Microinjection is a physical technique that involves the direct injection of Cas9 and sgRNA into cells using a microscope and a fine needle [76] (**Figure 2.4**). This method minimizes the risk of off-target effects and do not need to be considered about size and weight of the Cas protein/sgRNA, with success rates potentially reaching an impressive 100% [77]. However, microinjection is a technically demanding procedure, and only a limited number of cells can be processed in each experiment. As a result, it is impractical for applications requiring the simultaneous manipulation of millions or even billions of cells. Moreover, since microinjection cannot be performed on cells within a living organism, its use is restricted to in vitro settings [77].

The hydrodynamic injection method delivers CRISPR into cells through hydrodynamic forces. CRISPR is injected into the bloodstream of an organism, achieving gene editing through systemic dispersion [78]. For example, Xue et al used hydrodynamic injection to deliver CRISPR to the liver via tail vein injection, directly targeting the tumor suppressor genes PTEN and P53, leading to their deletion, and ultimately generating a mouse model of hepatocellular carcinoma [79]. However, because this method relies on the fluid pressure within the organism, it can only be performed in vivo. Additionally, due to systemic dispersion, it inevitably exerts pressure on the heart and liver [80].

Electroporation involves applying high-voltage pulses to cells, allowing CRISPR to pass through the cell membrane and achieve delivery [81] (**Figure 2.4**). This approach boasts high efficiency and can facilitate delivery to difficult-to-transfect cells in vitro [82]. Nonetheless, due to the inability to apply high-voltage pulses directly to living organisms, it remains challenging for in vivo therapeutic applications [81, 82].

### 2.4.2. Viral delivery

The primary viral vectors used for CRISPR delivery include Adeno-associated viruses (AAV) and lentiviruses (**Figure 2.4**). Many CRISPR-based therapeutics entering clinical trials today utilize viral vectors [83]. Viral vectors display high nuclear delivery efficiency [84]. However, their lack of specificity, immunogenicity, and cytotoxicity significantly limit their clinical applications [85, 86].

AAVs are small, non-pathogenic parvoviruses, consisting of single-stranded DNA. Consequently, they are relatively safe for infecting human cells with low initial immunogenicity [87, 88]. Nevertheless, due to their small size, it is challenging for AAVs to carry the entire CRISPR system for gene editing. Additionally, AAVs pose risks of off-target mutagenesis and genomic integration [85].

Lentiviruses are a type of retrovirus, featuring a single-stranded, enveloped RNA genome [89]. They can efficiently infect both dividing and non-dividing cells, and compared to AAVs, they are capable of encapsulating larger payloads, thereby enabling the complete delivery of CRISPR complexes. Moreover, their low intrinsic immunogenicity reduces the likelihood of eliciting an immune response in the host organism. However, lentiviruses have a strong capacity for sustained gene expression, elevating the risk of genetic integration and off-target mutations [86].

### 2.4.3. Non-virus delivery

Although physical and viral delivery methods have been employed in CRISPR delivery, physical methods can cause cellular damage and are challenging to apply *in vivo*; viral delivery methods are also difficult to avoid due to immune responses and cytotoxicity. To overcome these issues, non-viral nanoparticle delivery systems present a promising option for efficient transport. Over the past decades, nanoparticle-based drug delivery systems have been widely used for the targeted delivery of therapeutic agents, reducing the non-specific side effects associated with these drugs [90]. Various types of nanocarriers, including polymeric nanoparticles, metal nanoparticles and liposomes, have been developed for drug and gene delivery [91] (**Figure 2.4**). Each type has unique characteristics in delivery applications, depending on its composition and functionality.

#### Liposomes

Liposomes are among the most extensively used drug delivery systems due to their biocompatibility and safety [92, 93]. Their amphiphilic structure allows for the simultaneous

loading of hydrophilic drugs in the aqueous compartment and hydrophobic molecules in the lipid bilayer [94, 95]. The most commonly utilised lipids in the formulation include phospholipids and cholesterol [96, 97]. These lipids offer well-established safety and biocompatibility, and their structural diversity enables tailored physicochemical properties, making them highly suitable for applications across pharmaceuticals, cosmetics and nutraceuticals. A well-known example is Doxil®, a clinically approved doxorubicin-loaded liposome used in cancer therapy. Compared with free drugs, the liposomal formulation reduced systemic toxicity while enhancing tumour accumulation via the enhanced permeability and retention effect [98, 99]. Liposomes are also utilized for gene delivery by serving as carriers. For example, plasmid DNA can be mixed with lipid solutions to form a liposomal-DNA complex, which can fuse with the cell membranes of various cell types, enabling efficient gene transfer [93, 100]. Despite their advantages, liposomes still face challenges such as drug leakage and limited targeting specificity, requesting additional surface modifications to enhance circulation time and improve tissue-specific accumulation [101]. In addition, liposomes are inherently unstable in the bloodstream. They rapidly adsorb serum proteins and are cleared by hepatic macrophages, limiting their stability and circulation time [102, 103]. While PEGylation is widely used to modify liposome surfaces and extend circulation time, it can induce immune responses (e.g., anti-PEG antibodies), thereby reducing efficacy after repeated dosing [104]. Recent advancements in liposome targeting capabilities have primarily focused on stimuli-responsive designs and modifications to lipid composition [104, 105]. For example, Paula Enzian et al designed a photosensitive bilayer liposomal system composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and DSPE-PEG(2000). The liposomes encapsulated three distinct photosensitizers: porphyrin 5,10-di(4-hydroxyphenyl)-15,20-diphenyl-21,23H-porphyrin (5,10-DiOH), its chlorinated derivative (5,10-DiOH-chlorin) and bacteriochlorin (5,10-DiOH-bacteriochlorin), each of which facilitated efficient drug release under irradiation with different wavelengths of light [106]. Upon light exposure, the system generated reactive oxygen species, inducing lipid degradation in the liposomal membrane and triggering controlled drug release. This strategy enables spatial and temporal control over drug delivery, with payload release occurring precisely at the target site upon external photoactivation. Yaroslavov et al. developed a pH-sensitive bilayer liposomal vesicle composed of egg phosphatidylcholine and a pH-sensitive “activator,” which is a derivative of lithocholic acid bearing anionic and cationic groups at opposite ends of the steroid core. This system exhibited rapid drug release, with approximately 50-60% of the encapsulated anticancer agents released within minutes upon acidification [106, 107]. pH-sensitive liposomes exploit

the acidic tumour microenvironment to destabilise the lipid bilayer structure and trigger localized drug release [108].

### **Polymeric nanocarriers**

Polymeric nanocarriers are composed of one or more types of synthetic or natural polymer units covalently bonded to achieve adjustable drug release profiles and multifunctional designs (**Figure 2.4**). They also offer simple formulations, such as poly (lactic-co-glycolic acid) (PLGA) [109, 110]. Compared to liposomes, polymers exhibit greater stability and higher cargo retention efficiency [104, 105]. This is due to their composition of block copolymers, in which the covalent bonds between blocks are more resistant to degradation than the ester bonds in phospholipids [111]. Additionally, the degradation of some polymer repeating units has a lesser impact compared to low molecular weight lipids [112], and studies have shown that polymers can remain stable for up to six months at room temperature [113]. Furthermore, the high permeability of liposomes is attributed to their high lateral fluidity, a result of their low molecular weight, which leads to poor cargo retention efficiency [109, 110]. In contrast, polymers are more thermodynamically stable, with significantly lower lateral diffusivity, allowing them to retain cargo more effectively [106, 114].

Polymer carriers play an important role in gene delivery due to their ease of synthesis and adaptable properties [111]. For example, cationic polymers form stable complexes with genetic material through electrostatic interactions at physiological pH, protecting genes from degradation and facilitating cellular uptake via endocytosis [112]. These carriers exhibit high transfection efficiency as non-viral gene delivery systems. For example, Wan et al. developed a supramolecular polymer system incorporating detachable diguanidine ligands for the delivery of Cas9 ribonucleoprotein complexes targeting mutant *KRAS* genes. Compared to non-carrier systems, this platform demonstrated improved stability and significantly enhanced transfection efficiency [115]. In another study, Li et al. designed dual-grafted trimethyl chitosan nanoparticles modified with folic acid and 2-(diisopropylamino)ethyl methacrylate for the co-delivery of doxorubicin and CRISPR/Cas9 plasmids. This formulation achieved a marked tumour regression rate of 91.0% in 4T1 tumour-bearing mice [116].

However, the potential toxicity associated with polymeric nanoparticles remains a major challenge [117]. In addition, the structural complexity of these nanoparticles presents significant barriers to large-scale manufacturing, necessitating rigorous characterisation protocols to ensure batch-to-batch consistency and safety [118, 119]. Despite these limitations,

clinically approved polymers represent meaningful progress and continue to hold strong potential for advancing the field of nanomedicine [113].

### **Metallic nanocarriers**

Metallic nanocarriers have been extensively studied for drug and gene delivery due to their unique physicochemical properties. Their small size and stability enable efficient binding with a wide range of cargo [120, 121]. In particular, gold nanoparticles (AuNPs) stand out as highly functionalized metallic carriers [122, 123]. For example, Lee et al. developed a CRISPR-AuNP vehicle for the direct delivery of Cas9 RNP and donor DNA in vivo via local administration. This system was used to correct gene alterations in an Ai9 mouse model of Duchenne muscular dystrophy [124]. Although AuNPs are generally regarded as biocompatible, concerns remain regarding their long-term toxicity and potential accumulation in the body [125].

Metal-organic frameworks (MOFs) are porous materials with high surface area, abundant pores and tuneable microenvironments [126]. These properties enable MOF to enhance drug loading capacity and achieve controlled release at targeted lesion sites [127]. MOFs can be also used for gene delivery by loading various genetic cargo such as siRNA, mRNA and DNA [128, 129]. For instance, Zhou et al developed a mesh MOF for intracellular delivery of DNzyme in human immune cells, effectively inhibiting the expression of the EGR-1 gene [130]. Pore encapsulation is a technique that integrates functional molecules within the pores of nanocarriers and is commonly used for loading therapeutic cargos into MOFs [131]. However, molecules incorporated through surface adsorption and pore encapsulation often experience gradual leakage due to weak interaction forces [127]. Covalent binding offers a solution by forming strong covalent bonds with reactive groups on the target, ensuring more stable interactions [132]. However, this approach requires complex synthetic procedures and may alter the activity of therapeutic molecules [133]. While MOFs show great promise for drug delivery applications, further research is needed to fully understand their in vivo toxicity, degradation mechanisms and pharmacokinetics [133].

### **Exosome nanocarriers**

Exosomes are small extracellular vesicles present in nearly all cell types (**Figure 2.4**). As natural transport vehicles, they can efficiently deliver various biomolecules, such as nucleic acids, proteins, and lipids, among different biological systems with high biocompatibility [134, 135]. Since exosomes are cell-derived, their innate nature makes them less likely to trigger immune responses during in vivo transport, helping to reduce potential side effects.

Additionally, specific membrane proteins on the surface of exosomes can interact with receptors on target cells, enabling targeted delivery and enhancing the efficacy of drug or gene therapy [136-139]. However, the development of exosomes is an ongoing process. Currently, there is limited development of exosomes, with complex isolation and purification processes, high costs, and limited yield. Additional research is crucial for improving their payload capacity and loading efficiency, which is essential for optimizing the future clinical use of these carriers [140].

### **Hybrid nanocarriers**

Numerous studies have demonstrated that the hybridisation of two or more nanocarrier systems can lead to the formation of novel architectures capable of integrating the complementary advantages of each component, enhancing the delivery efficiency of therapeutic agents in cancer treatments [141]. Among these, lipid-polymer hybrid nanoparticles have shown particularly promising performance in gene and drug delivery, exhibiting superior intracellular delivery and transfection efficiency compared to their single-component counterparts [142]. In this design, the lipid component enhances biocompatibility and cellular uptake, while the polymer contributes to improved structural stability, controlled drug release and tunable physicochemical properties [143]. For instance, Sedef et al developed a hybrid delivery system composed of a lipid core made from a beeswax–olive oil mixture and a biopolymer shell made from BSA/dextran Maillard conjugates for the delivery of the highly lipophilic anticancer drug paclitaxel [144]. This hybrid design exhibited optimised characteristics, including small particle size, pH stability and efficient drug encapsulation. In addition, inorganic-polymer hybrid nanocarriers also hold significant therapeutic promise. For example, Mehrab et al. developed a composite nanocarrier made of graphene oxide (GO), polyvinylpyrrolidone (PVP), and sodium alginate (SA), which significantly improved the delivery and therapeutic effectiveness of 5-fluorouracil (5-FU) against colorectal cancer cells [145]. This system offers controlled and sustained release of 5-FU at the cellular level, with the potential to improve therapeutic outcomes while minimising the systemic toxicity commonly associated with traditional chemotherapy. Despite their multifunctional potential, hybrid nanocarriers still face several translational barriers, including complex fabrication processes, structural instability, potential toxicity and regulatory uncertainty, all of which hinder their clinical implementation [146].

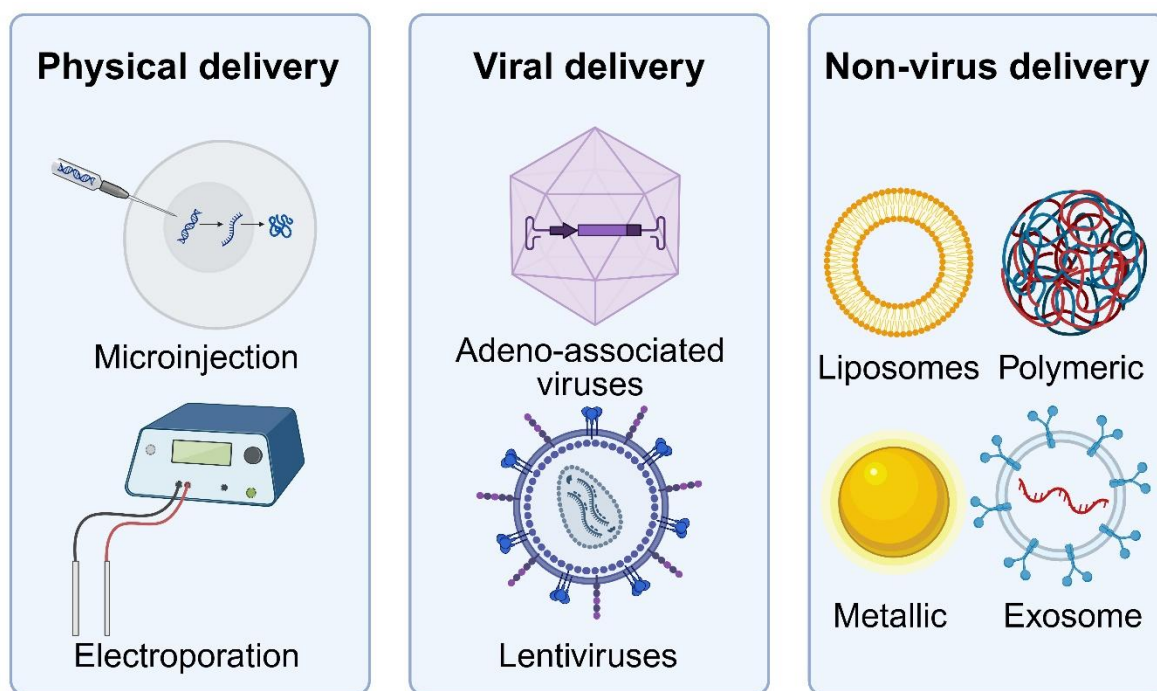


**Figure 2.5.** Classification of CRISPR/Cas9 delivery strategies based on carrier type. Gene editing components such as CRISPR/Cas9 can be delivered into cells using various strategies broadly categorized into physical, viral, and non-viral methods. Created in BioRender. Xu (2025) <https://BioRender.com>.

## 2.5. Mechanisms of targeted delivery

### 2.5.1. Passive targeting

Passive targeting strategies are predominantly based on the enhanced permeability and retention (EPR) effect, a phenomenon unique to solid tumours [147, 148]. This effect arises from the increased permeability of tumour vasculature compared to normal tissues [149, 150]. In tumours, the endothelial cell gaps range from 100 to 780 nm, depending on the cancer type, whereas in healthy tissues, these gaps are significantly smaller, approximately 5 to 10 nm [151]. Consequently, nanocarriers within an optimal size range can preferentially extravasate into tumour sites. This mechanism has been successfully exploited in clinical applications, as demonstrated by the approved nanocarrier-based formulations Doxil© and Caelyx© [152]. However, the passive targeting strategy has major limitations as the EPR effect is highly dependent on the tumour's intrinsic biological properties and varies significantly across tumour types, stages and individuals [153]. For example, metastatic liver cancer and pancreatic cancer exhibit low vascular permeability, resulting in reduced drug accumulation compared to highly vascularized tumours [153, 154]. As a result, nanocarriers relying solely on the EPR effect

often face challenges in effectively targeting these tumours, thereby limiting the overall efficacy of therapeutic drugs.

### **2.5.2. Active targeting**

To enhance the cancer cell targeting capability, active targeting strategies have been developed by incorporating specific targeting moieties, such as antibodies, aptamers and ligands, onto the surface of nanocarriers [155]. These targeting molecules have high-affinity interaction with the proteins overexpressed by the tumour cells, including folate receptors, transferrin receptors and epithelial growth factor receptors (EGFRs) [155]. Upon reaching the tumour site, targeted nanocarriers interact with cancer cells through specific affinity interactions, facilitating their accumulation within the tumour tissue. Antibodies and antibody fragments such as antigen-binding fragments (Fab) and single-chain variable fragments (scFv), play a crucial role in the development of actively targeting nanoparticle systems [156]. These molecules can selectively bind to antigens overexpressed on cancer cells, thereby facilitating enhanced cellular uptake of therapeutic agents and nanoparticle drug delivery systems [157-159]. Aptamers are single-stranded oligonucleotides where a library of random oligonucleotide sequences is exposed to the desired targeting ligand. They have emerged as promising active targeting molecules in nanocarrier systems due to their superior cancer-targeting capability [160]. Aptamers can specifically recognise disease-associated biomarkers, such as receptors overexpressed on cancer cells-for instance, nucleolin which is targeted by the AS1411 aptamer [161, 162]. One of the key advantages of aptamers is their high specificity and tunability, allowing precise recognition of target cells while minimising off-target interactions [161]. However, a major challenge is their limited in vivo stability, as unmodified aptamers are highly susceptible to nuclease degradation and rapid renal clearance [163]. Ligand-based targeting strategy utilises the fact that certain ligand molecules are specifically recognised by receptors that are overexpressed in cancer cells. Various ligand molecules such as hyaluronic acid (HA), folic acid (FA) and chitosan have been shown to largely increase the targeting capabilities of nanocarriers [164-166]. For example, FA-modified liposomal doxorubicin and paclitaxel formulations have demonstrated preclinical success, shown enhanced tumour accumulation and reduced systemic toxicity [167, 168]. While these studies showed promising results for improved cancer treatment, they did not quantify the ligand density in the nanocarrier formulations. It is necessary to evaluate the optimal ligand concentration in the liposomes to ensure effective clinical translation.

### 2.5.3. Stimuli-responsive targeting

Stimuli-responsive nanocarriers represent a transformative approach in targeted drug delivery, engineered to release therapeutic payloads in response to internal switch associated with disease microenvironments or external triggers [169]. These active delivery systems utilise disease-related factors such as tumour-specific pH gradients, elevated glutathione (GSH) levels, overexpressed enzymes or hypoxia, as well as external stimuli like light, magnetic fields or ultrasound [169]. For example, pH-sensitive liposomes become destabilised in the acidic tumour microenvironment or within endosomal/lysosomal compartments, thereby enhancing intracellular drug delivery [170]. Similarly, redox-responsive polymers break down in the high-GSH cytoplasm, improving the efficacy of encapsulated therapeutics [171]. External triggers, such as near-infrared light or ultrasound enable non-invasive control over drug release kinetics from the nanocarriers [172, 173]. Clinical trials have included thermosensitive liposomes (e.g., ThermoDox®) in combination with hyperthermia for localized cancer therapy [174]. Despite their promise, stimuli-responsive nanocarriers face some challenges. The heterogeneity of disease biomarkers, such as varying pH gradients across different tumours, can compromise the reliability of activation [170]. External stimuli like light suffer from limited tissue penetration depth, restricting their utility to superficial or accessible lesions [169]. Additionally, synthesis complexity increases the manufacturing costs of such nanocarriers and raises concerns about long-term stability [169].

## 2.6. Applications of nanocarrier-based targeted delivery

Targeted delivery using nanocarriers has been widely investigated and applied in the treatment of various diseases including genetic disorders, cardiovascular conditions, neurodegenerative diseases and cancers.

### 2.6.1. Gene disorders

A particularly promising application is in the treatment of monogenic disorders, where nanocarriers are employed to deliver genetic payloads that can modify or correct disease-causing genes [175]. For example, lipid nanoparticles functionalised with liver-targeting ligands such as GalNAc have been used to specifically deliver CRISPR-based therapies targeting the *ANGPTL3* gene to liver cells in both mouse and non-human primate models, offering a potential treatment for homozygous familial hypercholesterolemia [176]. Similarly, multifunctional lipid nanoparticles modified with all-trans-retinamine have been shown to deliver plasmid DNA to the retina in the *Rpe65*<sup>-/-</sup> mouse model for the treatment of Leber's

congenital amaurosis caused by RPE65 gene mutations [177]. In addition, a recent study reported the development of lipid nanoparticles functionalized with  $\beta$ -D-galactose-based ligands to enhance targeted delivery [178]. These nanoparticles were specifically engineered to target the asialoglycoprotein receptor (ASGPR) which is abundantly expressed on the surface of hepatocytes. By facilitating receptor-mediated endocytosis, this strategy enabled the safe and efficient delivery of messenger RNA (mRNA) to the liver. The therapeutic mRNA encoded functional Factor VIII (FVIII), aiming to restore protein expression in hemophilia A, a genetic disorder caused by mutations or deficiencies in the F8 gene that results in the absence or dysfunction of FVIII protein [178]. These targeted systems reduce off-target effects on other healthy tissue and improve therapeutic efficacy.

### **2.6.2. Neurodegenerative diseases**

Effective targeted delivery across the blood-brain barrier (BBB) is critical for treating neurodegenerative diseases [179]. Nanocarriers can be functionalised with targeting molecules such as antibodies to deliver drugs to neurons and glial cells [180]. For instance, polymer nanoparticles coated with polysorbate 80 and loaded with peptide inhibitors of polyglutamine aggregation have been used to treat Huntington's disease in both in vitro and in vivo models [181]. In another study, polymeric nanoparticles modified with rabies virus glycoprotein (RVG29) peptide was reported to successfully crossed the BBB and co-delivered a therapeutic gene (shRNA) along with epigallocatechin-3-gallate (EGCG) to brain tissue in a mouse model of Alzheimer's disease. This dual-delivery strategy effectively reduced amyloid- $\beta$  (A $\beta$ ) plaque deposition and inhibited p-tau-related fibril formation [182]. Lysosomal storage disorders (LSDs) which primarily affect the central nervous system (CNS), often cause progressive and severe neurological impairments [183]. To address this, a therapeutic strategy using transferrin receptor (TfR)-targeted delivery of a plasmid encoding  $\beta$ -glucuronidase (GUSB) has been developed. Although the resulting enzyme activity was lower than physiological levels, it was still sufficient to provide therapeutic benefit [184]. However, because this strategy depends on systemic intravenous administration, achieving sustained therapeutic benefits for chronic LSD conditions would require repeated dosing at intervals dictated by the duration of transgene expression [184]. Recent advances include the development of stimuli-responsive nanocarriers that release their therapeutic cargo in response to elevated levels of reactive oxygen species (ROS) commonly found in the microenvironment of Parkinson's disease [185].

### 2.6.3. Cardiovascular conditions

In cardiovascular therapy, nanocarriers are used to target atherosclerotic plaques, enhancing localised drug retention [186]. For example, PLGA polymer nanoparticles conjugated with the mZD7349 peptide and loaded with simvastatin have been used to target dysfunctional endothelial cells, showing significantly higher therapeutic efficiency compared to non-conjugated nanoparticles [187]. Lipid nanoparticles modified with a targeting peptide were used to specifically deliver anti-miR-712 to the endothelial surface of atherosclerotic lesions overexpressing vascular cell adhesion molecule 1 (VCAM1). In the partial carotid ligation model using *ApoE*<sup>-/-</sup> mice, this targeted delivery strategy effectively reduced atherosclerosis while minimizing off-target effects on other tissues [188]. In addition to atherosclerosis treatment, targeted nanocarriers have shown promising potential in myocardial repair and regeneration after infarction. For example, a dual-targeted lipid-based complex functionalised with the anti-myosin monoclonal antibody 2G4 (mAb 2G4) and the transactivator of transcription (TAT) peptide, has been developed to deliver therapeutic genes directly to ischemic myocardial tissue, improving localised therapeutic outcomes [189]. Beyond lipid-based systems, polymeric nanoparticles have also been investigated for targeting acute myocardial infarction (MI). For instance, a peptide-polymer conjugate system was developed using a peptide sequence identified via phage display technology, which demonstrated high affinity for infarcted myocardial tissue. Delivery of this targeted system resulted in improved cardiac function, highlighting its therapeutic promise for post-MI tissue repair [190].

### 2.6.4. Cancer therapy

Cancer therapy remains one of the most intensively investigated applications of targeted nanocarrier systems. By conjugating nanodrugs with ligands that selectively bind to receptors overexpressed on the surface of tumour cells, active targeting can be achieved with high specificity and efficiency [191, 192]. For instance, a HER2-targeted polymeric drug delivery system was developed using a star-shaped dendritic polymer (SD) conjugated with the topoisomerase I (TOP1) inhibitor SN-38 and the antigen-binding fragment of trastuzumab (HER2-Fab). This nanocarrier specifically recognises and binds to cancer cells overexpressing HER2, resulting in significantly enhanced antitumor efficacy compared to non-targeted counterparts [193]. In recent years, stimuli-responsive nanoparticles have emerged as a promising strategy for precise tumour targeting [194]. In addition to responding to endogenous tumour microenvironment cues [195], functionalised nanoparticles can also be externally activated by exogenous stimuli such as ultrasound, light illumination or radiation [196-198].

For example, a lipid-polymer hybrid nanoparticle platform has been developed to enable X-ray-induced photodynamic therapy (PDT) specifically targeting human colorectal cancer cells. This multifunctional nanocarrier co-encapsulates verteporfin, a clinically approved photosensitizer, and 5-fluorouracil, a widely used chemotherapeutic agent, within a single delivery system. Upon exposure to X-ray irradiation at 4Gy, the verteporfin generates ROS, leading to oxidative damage and apoptosis in tumour cells [198]. In cancer vaccines, targeted nanocarriers can enhance the efficiency of immunotherapy by improving antigen delivery and stimulating stronger immune responses [199, 200]. For instance, poly( $\beta$ -amino ester)-based nanoparticles functionalised with T-cell targeting anti-CD3e f(ab')<sub>2</sub> fragments were used to specifically deliver CAR-encoding plasmid DNA to T cells in C57BL/6 mice. This targeted delivery system enhanced T-cell responses and triggered a robust anti-tumour effect [201]. Iron oxide nanoparticles modified with mannose have been used to augment antitumor efficacy and enhance neoantigen vaccine through the repolarization of tumour-associated macrophages (TAMs) and improved coordination of immune cell activity [202].

## **2.7. Targeting subcellular organelles**

### **2.7.1. The biological functions of main subcellular organelles**

Cytoplasm is a semi-fluid substance within the cell that lies between the cell membrane and the nuclear membrane and fills the entire cell [203]. It is the intracellular portion that contains the organelles, including all structures outside the nucleus [203]. It consists mainly of water, ions, small-molecule metabolites, proteins and nucleic acids [203, 204]. The cytoplasm is the main site of several metabolic pathways, including glycolysis, lipid metabolism and protein synthesis [205]. Furthermore, the cytoplasm serves as the primary site of action for therapeutic miRNA/siRNA agents, allowing them to exert their effects and ultimately maximize therapeutic outcomes [206]. Once siRNA is taken up by cells, it is recognized by the RNA-induced silencing complex (RISC). This complex uses the siRNA as a guide to find and bind to specific mRNA molecules that are complementary to the siRNA sequence [207, 208]. By doing so, the RISC-siRNA complex can cut and destroy these mRNA molecules, preventing them from being translated into proteins. This process effectively reduces the expression of genes that are involved in disease development [209, 210]. Presently, a broad range of viral and non-viral vectors has been engineered to enhance the escape of siRNA from endosomes. This allows for the release of siRNA into the cytoplasm, ultimately increasing the effectiveness of gene silencing [211, 212].

Mitochondria are oval or oblong cylindrical organelles consisting of two membrane systems [213]. Unlike other organelles, mitochondria have their own DNA called mitochondrial DNA (mtDNA), which mainly encodes several proteins related to the function of mitochondria [214]. The primary role of the mitochondria is to facilitate cellular respiration and generate adenosine triphosphate (ATP), which is the key energy molecule within the cell [214, 215]. It also plays an important role in the regulation of cellular function. For instance, mitochondria have been found to be suitable subcellular targets for reactive oxygen species (ROS) generated by photodynamic therapy (PDT) [216, 217]. The ROS-induced effects are capable of disrupting cellular functions such as proliferation [218, 219], while mitochondrial DNA damage initiates diverse cell death mechanisms [220, 221]. Consequently, mitochondria-targeted PDT has proven to be more effective than a non-targeted alternative, allowing greater specificity and potentially smaller effective drug doses. In addition, mitochondrial dysfunction plays a critical role in the pathogenesis of Parkinson's disease (PD) and Alzheimer's disease (AD), targeting mitochondrial dysfunction could efficiently implicated for PD and AD treatment [222, 223].

The nucleus is a vital organelle that houses the cell's genetic material and governs the cell's growth, development and metabolism [203]. Positioned typically at the centre of the cell, the nucleus comprises a nuclear membrane and a nucleolus. It serves as the primary repository for the cell's DNA which contains the genetic instructions necessary for the functions of an organism [203]. Chemodrugs can interact with nuclear DNA through various mechanisms, such as DNA crosslinking and topoisomerase inhibition [224]. These interactions disrupt normal DNA structure and functions, leading to DNA damage and ultimately affecting cellular processes and viability (**Figure 2.6**). Additionally, compared to other strategies that target different subcellular areas or have a random distribution, nucleus-targeted PDT/photothermal therapy (PTT) is a more direct and efficient approach to kill cancer cells using light or heat [225-227]. In addition to chemotherapy and PDT/PTT delivery, it is also crucial to efficiently deliver genetic materials into the cell nucleus. This is particularly important for utilizing CRISPR/Cas gene editing tools, which hold promise for treating cancer and genetic disorders [228, 229]. To ensure successful gene editing, both the Cas9 protein and the sgRNA need to be transported into the nucleus of target cells [6, 230]. Once inside the nucleus, they can initiate the gene editing process by targeting and modifying specific DNA sequences [6]. However, this technology may have unintended off-target and cytotoxicity effects, especially when administered systemically [149, 150]. Therefore, effective nuclear targeting is essential to ensure optimal delivery and expression of therapeutic genes.

Precise targeting of the cytoplasm, mitochondria and nucleus plays a pivotal role in drug development for disease treatment. Such targeted approaches not only enhance treatment specificity and efficacy but also minimise off-target effects and associated side effects.

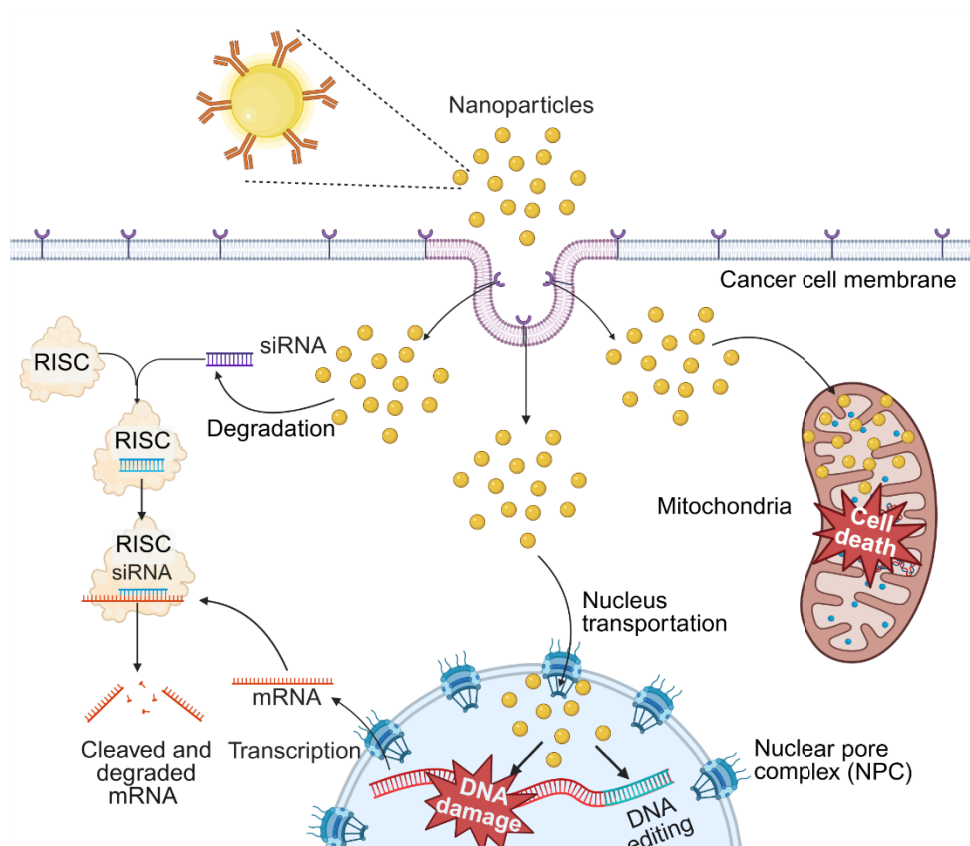


**Figure 2.6.** Schematic illustration of cytoplasm, mitochondria and nuclear targeting. Following cellular uptake, nanoparticles are trafficked to various intracellular compartments. For example, in the cytoplasm, siRNA released from the nanoparticles is incorporated into the RNA-induced silencing complex, where it guides the degradation of specific target mRNA, resulting in effective gene silencing. Nanoparticles can also be directed to the mitochondria, where they induce mitochondrial dysfunction and trigger apoptosis. In addition, some nanoparticles are transported into the nucleus via nuclear pore complexes. Created in BioRender. Xu (2025) <https://BioRender.com>.

### 2.7.2. Strategies on nuclear-targeted delivery via nanocarriers

#### Nuclear pore complex (NPC): regulator of nucleocytoplasmic transport

The NPC is a proteinaceous structure responsible for controlling the movement of various molecules, including proteins, RNA molecules and signalling molecules, between the nucleus and the cytoplasm [231-233]. It is composed of nucleoporins, a specific class of proteins that

come together to construct a channel-like structure with a central pore [234, 235]. Ions and small molecules can passively diffuse through a pore. However, larger molecules, typically those exceeding 40-60 kDa in size, require the assistance of nuclear transport receptors [236]. These receptors recognize specific signals called nuclear localization sequences (NLS) or nuclear export sequences (NES) on the molecules, allowing them to bind and facilitate their transport into or out of the nucleus [237, 238]. This process ensures that only the appropriate molecules are able to enter or exit the nucleus, maintaining cellular integrity and function.

### **Strategies for nucleus transportation**

Synthetic nanocarriers in principle need to transport drugs or genetic materials to the nucleus to exert functions for the treatment of nucleus-related diseases including cancers and genetic disorders. There are intracellular barriers for these delivery vehicles to accumulate in the nucleus during the transportation. One major intracellular challenge faced by nanocarriers is their entrapment within the endo/lysosomal compartments after being engulfed by the cell [239]. Endosomes and lysosomes are membrane-bound compartments within the cell that are involved in the degradation and recycling of cellular components [240, 241]. When nanocarriers are taken up by the cell through endocytosis, they often get trapped within these compartments, leading to their degradation and limited access to the target site, such as the nucleus. Another obstacle to successful nuclear transport is the requirement for drugs or genes to navigate through the cytosol, which is a densely packed and molecularly crowded environment at the molecular level [242]. This means that there are many molecules and cellular components present in the cytosol, making it difficult for the drugs or genes to reach the nucleus. To overcome these challenges, researchers have focused on engineering nanocarriers, which can be categorized into two types, passive and active targeting (**Figure 2.7**).

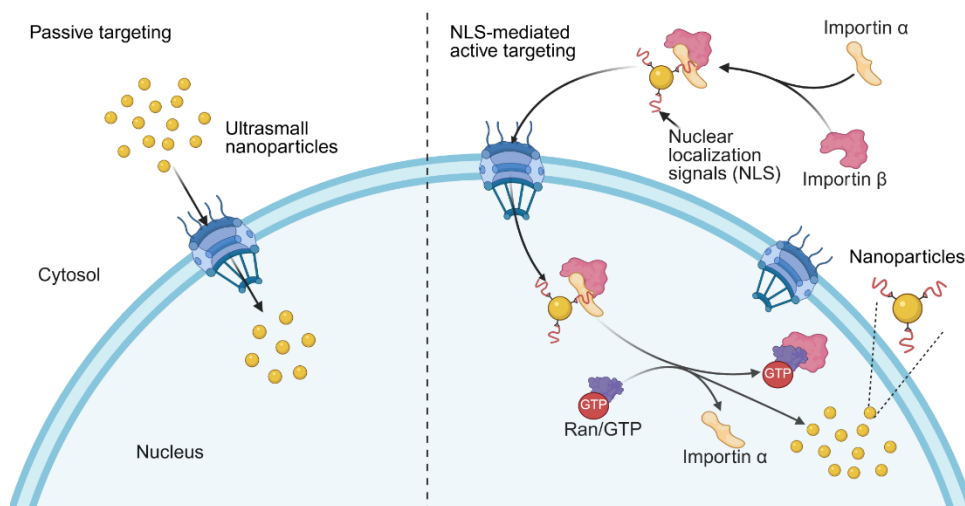


**Figure 2.7.** Schematic illustration of passive and nuclear localization signal (NLS)-mediated nuclear transport strategies. Left panel: In passive targeting, ultrasmall nanoparticles enter the nucleus by diffusing through nuclear pore complexes (NPCs) without assistance. Right panel: In active targeting, nanoparticles are functionalized with NLS peptides that bind to importin  $\alpha/\beta$  and are actively transported through NPCs. Once inside the nucleus, Ran-GTP facilitates dissociation of the importin–cargo complex, releasing the nanoparticle payload into the nucleoplasm. Created in BioRender. Xu (2025) <https://BioRender.com>.

### Passive targeting

The nucleus is enclosed by a double nuclear membrane, which limits direct interaction between the nucleus and the cytoplasm. However, it is not entirely isolated as NPCs embedded in the nuclear membrane facilitate material exchange and information transfer [243]. Passive nuclear transport is an energy-independent process driven by Brownian motion and the intrinsic affinity of molecules for nuclear components [244, 245]. Through this mechanism, small hydrophilic molecules can diffuse across NPCs into the nucleus by following concentration gradients [246].

Passive targeting involves the design of nanocarriers that can rely on the unique properties of the nucleus (e.g., size and density) to facilitate drug or gene entry into the nucleus [242]. This approach takes advantage of nanocarrier characteristics such as size, shape and surface charge to passively accumulate at the nucleus. Ultrasmall (<10 nm) nanoparticles have received particular attention for passive targeting due to their ultra-small size. In a study, researchers utilized thiopronin-coated gold nanoparticles (Au-TIOP NPs) for passive targeting of cell nuclei [247]. Thiopronin was used to prevent the coagulation of gold nanoparticles, ensuring their stability in physiological environments. The study found that only gold nanoparticles smaller than 10 nm (2 nm and 6 nm) were able to enter the nucleus, while larger ones (10 nm

and 16 nm) remained in the cytoplasm. The researchers also used 2 nm Au NPs as a carrier to directly deliver triplex-forming oligonucleotides (TFOs) into the nucleus. The results demonstrated that compared to unmodified TFOs, the conjugation of TFOs with 2 nm Au NPs significantly improved the efficiency of TFOs entering the nucleus, enhancing knocking down efficacy. Another study revealed that 2 nm gold nanoparticles were able to penetrate the nucleus, which is a crucial step for drug delivery targeting the nucleus. Furthermore, this study demonstrated that these Au NPs are capable of crossing the blood-brain barrier, which is a highly selective and protective barrier surrounding the brain [248]. This finding may offer possibilities for utilizing Au NPs in the treatment of brain-related diseases and disorders. Recently researchers have developed small metal-organic framework (MOF) nanocarriers that specifically target the nucleus for treating orthotopic pancreatic carcinoma using type I sonodynamic treatment (SDT) [249]. This MOF nanocarrier is loaded with Ti-tetrakis (4-carboxyphenyl) porphyrin (TCPP), a photosensitiser. Due to its small size (<10 nm) and charge reversal property, the MOF nanocarrier effectively enters the cells and directly targets the nuclei. This targeted approach enhances the efficiency of SDT, making it a more effective treatment option. Despite ultrasmall nanoparticles have demonstrated excellent nuclear-targeting capabilities, they face challenges in terms of stability and industrial manufacturing [250]. Another concern is their lack of gradability and biocompatibility, which causes high tissue toxicity [251, 252]. Shape and surface charge are also critical parameters influencing the nuclear uptake of nanocomposites. Hinde et al studies on the behaviour of nanoparticles with various shapes at the subcellular level [253]. The authors reported that nanoparticles with a high aspect ratio (length-to-width ratio), such as rod-shaped and worm-like nanoparticles, exhibit higher accumulation in the nucleus compared to micelle and vesicle nanoparticles. When encapsulating DOX, rods and worms delivered substantially more DOX to the nucleus compared to spherical nanoparticles. This could be attributed to the fact that nanoparticles with high aspect ratios (such as worms and rods) exhibit higher efficiency in passive diffusion into the nucleus compared to spherical nanoparticles [253]. The nuclear membrane is negatively charged, allowing positively charged nanoparticles to bind to the negatively charged nuclear membrane, thereby facilitating their entry into the nucleus [254, 255]. Junghan et al. designed positively charged quantum dots capable of directly labelling the nucleus in both fixed and live HeLa cells. In contrast, negatively charged quantum dots were confined to the cytoplasm. These findings highlight that the charge of nanoparticles is a critical factor influencing their intracellular distribution, including nuclear localization [256].

The passive targeting strategy is largely dependent on the size, shape and surface charge of nanoparticles, which constrains its versatility and broader applicability.

### ***NLS-mediated active targeting***

The active targeting strategies can be achieved by modification of the NPC itself [257] or by surface modification of nanocarriers with nuclear-targeting ligands or peptides [258]. In this section, we primarily focus on nanoparticle engineering to enhance nuclear targeting efficiency. Active targeting involves the use of specific ligands or peptides that can recognise and bind to components within the nucleus. This recognition and binding process leads to the accumulation of therapeutic molecules specifically within the nucleus. In contrast to passive targeting, which is limited by the physical properties of freely diffusing drug molecules or nanoparticles, active targeting can improve transport efficiency from the plasma membrane to the nucleus, increase the macromolecules or nanoparticles to enter the nucleus efficacy [259, 260]. These peptides have binding affinities for nuclear transport receptors located in the cell nucleus. By attaching these peptides to the surface of drugs or nanocarriers, they can effectively deliver the drugs or gene editing components into the nucleus for precise therapy. NLS are generally short peptides with signal sequences that direct proteins to the nucleus [261]. Extensive research on NLS has resulted in significant progress, enabling successful targeting of various small molecules, proteins and nucleic acids to the nucleus [262]. NLS can be categorized into two main types: classical NLS (cNLS) and non-classical NLS (ncNLS) [261]. The cNLS typically consists of positively charged amino acids, such as lysine and arginine [263]. It facilitates entry into the cell nucleus by binding to nuclear transport receptors like Importin. This signal is generally recognized and transported by the primary nuclear transport machinery, playing an important role in processes such as cell division and stress response [261, 264]. Within cNLS, there are two subtypes: monopartite (MP) and bipartite (BP). MP NLS is composed of a cluster of 4-8 basic amino acids, typically containing four or more positively charged residues [261]. Its characteristic motif requires consecutive lysine residues at the N-terminus, forming a loose consensus sequence of K(K/R)X(K/R). Only these positively charged sequences are functional in facilitating nuclear transport [265]. BP NLS is composed of two clusters of 2-3 positively charged amino acids, separated by a linker region of 9-12 amino acids [266]. These upstream and downstream clusters are interdependent and play a critical role in enabling nuclear transport. The characteristic motif of BP NLS can be represented as R/K(X)10-12KRXX [267]. The ncNLS is a non-specific combination of amino acids that is not necessarily rich in positively charged residues. Its localization mechanism often depends on the protein's folding

state or specific structural features [268, 269]. Unlike cNLS, it utilises diverse transport mechanisms and can achieve nuclear transport via complexes that rely on signalling molecules [269, 270]. This flexibility enables ncNLS to adapt its function in response to changes in the cellular environment and physiological state [271].

NLS are recognized by the karyopherin Importin  $\alpha/\beta$ , forming a complex that interacts with NPC to facilitate translocation through the nuclear pore (**Table 2.1**). This process is facilitated through the hydrolysis of guanosine triphosphate (GTP) [261, 272]. As a result, various molecules can freely transport to the nucleus. The use of NLS peptides for nanoparticle modification is a well-established strategy in the field of nuclear targeting. One of the well-known NLS peptides is the transactivator of transcription (TAT). The original TAT peptide consists of 11 amino acids (YGRKKRRQRRR) and contains a high positive charge, which is essential for its nuclear targeting ability [273, 274]. The positively charged residues allow the TAT peptide to interact with negatively charged components of the cellular membrane, facilitating its uptake into cells [261, 275]. Once inside the cell, the TAT peptide binds to importin proteins, which are responsible for transporting molecules into the nucleus [276]. This interaction allows the TAT peptide, along with the larger nanoparticles it is conjugated with, to be efficiently delivered into the nucleus [277, 278]. In a study conducted by Wan et al [227], authors developed the self-assembled nanoparticles (TID) by conjugating a photosensitiser, IR780 with TAT peptide. These targeted nanoparticles were then assembled with the chemotherapeutic drug doxorubicin (DOX). Mediated by the TAT peptide, these nanoparticles exhibited enhanced nuclear targeting capability in breast cancer cells compared to free IR780 molecules and DOX. Upon laser irradiation, the nanoparticles exhibited dual therapeutic effects of PTT and PDT. The PDT/PTT effects were achieved by damaging the genetic material within the cell nucleus and generating cytotoxic reactive oxygen species, thereby enhancing the therapeutic efficacy of the targeted nanoparticles compared to pure IR780 or DOX molecules.

NLS peptides can also be incorporated into non-viral gene carriers, such as polymer and liposomes, aiming to enhance the delivery of plasmid DNA specifically to the nucleus, improving transfection efficiency and therapeutic efficacy. In a study, chitosan (CS)/DNA complexes that are directly incorporated with NLS peptide (KPKKKRKV) efficiently deliver pEGFP-C2 plasmid DNA to Hela cells [279]. The highest transfection efficiency was significantly observed in the CS/DNA complex at the weight ratio of 8 with the addition of 120  $\mu$ g NLS, which was 74-fold higher than that in the cells transfected with the CS/DNA complex alone. In another study, Rosada et al. employed a combination of a synthetic NLS

peptide (KCRGKVPGKYGKGPKKKRKVC-amide) and a cationic liposome to deliver a plasmid DNA encoding the 65-kDa heat shock protein gene (DNAhsp65) for the treatment of tuberculosis (TB) [280]. The cationic liposome utilized in this study consisted of lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1- $\alpha$ -phosphatidylcholine (EPC). This liposome formulation aimed to provide sustained protection against TB while reducing the required dose of DNA. The NLS-mediated liposomes demonstrated superior therapeutic effects against TB compared to the cationic liposome/DNAhsp65 gene vaccine and were similar to the naked DNA treatment. These findings suggest that delivering DNA to the cell nucleus via NLS activity can enhance gene transcription, leading to an effective immune response against the relevant pathogen.

In a recent report, He et al fabricated an AS1411/TAT-functionalized polymer nano-system that can effectively deliver CRISPR/Cas9 plasmid for  $\beta$ -catenin knockout to reverse tumour immunosuppression [162]. This delivery vector was modified with two types of hyaluronic acid conjugates, one with an aptamer, AS1411 for tumour cell/nuclear targeting, and the other with a TAT-NLS peptide for cell penetration and nuclear translocation. By combining these two functionalities, this delivery system can specifically deliver the genome-editing plasmid to tumour cell nuclei. The targeted nanoparticles modified with TAT-NLS (NP-3) and AS1411 (NP-) exhibited higher nuclear-targeting efficacy compared to the non-targeted nanoparticles (NP-1 and NP-2). The combination of TAT-NLS and AS1411 modifications (NP-5) achieved the highest nuclear targeting efficiency. As a result, the Wnt/ $\beta$ -catenin pathway is inhibited, leading to the suppression of tumour cell growth. Additionally, this downregulates PD-L1 expression, restoring the anti-tumour activity of T cells.

**Table 2.1 NLS sequences used for nuclear target**

| Category                                           | Type                 | NLS Amino Acid Sequence                  | Transport protein               | Protein Source   | Reference  |
|----------------------------------------------------|----------------------|------------------------------------------|---------------------------------|------------------|------------|
| Classical nuclear localization signal (cNLS)       | Monopartite NLS      | PKLKRQ                                   | Importin $\alpha/\beta 1$       | VACM-1           | [281, 282] |
|                                                    |                      | RPRK                                     | Importin $\alpha/\beta 1$       | CXCR4            | [283, 284] |
|                                                    |                      | RRARRPRG                                 | Importin $\alpha/\beta 1$       | VP1              | [285, 286] |
|                                                    |                      | PAAKRVKLD                                | Importin $\alpha/\beta 1$       | C-MYC            | [287, 288] |
|                                                    |                      | VSRKRPRP                                 | Importin $\alpha/\beta 1$       | T-Antigen        | [289, 290] |
|                                                    |                      | QKRQK/EEKRKR                             | Importin $\alpha 3/\alpha 4$    | NF- $\kappa$ B   | [291]      |
|                                                    |                      | PKKKRKV                                  | Importin $\alpha$               | SV40             | [292, 293] |
|                                                    | Bipartite NLS        | KRPAATKKAGOAKKKK                         | Importin $\alpha/\beta 1$       | Nucleoplasmin    | [294]      |
|                                                    |                      | KGKKGRTQKEKKAARARS<br>KGKN               | Importin $\alpha/\beta 1$       | ING4             | [295, 296] |
|                                                    |                      | RKRCAAGVGGGPAGCPAPG<br>STPLKKPRR         | Importin $\alpha/\beta 1$       | IER5             | [297]      |
|                                                    |                      | RKPVTAQERQREEREKRRR<br>RQERAKEREKRRQERER | Importin 7                      | ERK5             | [298, 299] |
| Non-classical nuclear localization signals (ncNLS) | Proline tyrosine NLS | RSGGNHRRNGRGGRGGYN<br>RRNNGYHPY          | Importin $\beta 2$              | HRP1             | [300]      |
|                                                    |                      | TLLLRETMMNNLGVSDHAVL<br>SRKTPQPY         | Importin $\beta 2$              | UL79             | [301]      |
|                                                    |                      | FGPGKMDSRGEHRQDRRE<br>RPY                | Importin $\beta 2$              | FUS              | [302, 303] |
|                                                    |                      | NNOSSNFGPMKGGNFGGR<br>SSGPYG             | Importin $\beta 2$              | hnRNPA1          | [304]      |
|                                                    |                      | NQQPSNYGPMKSGNFGGS<br>RNMGGPYG           | Importin $\beta 2$              | hnRNPA2/B1       | [305]      |
|                                                    | Other ncNLS          | SANKVTKNKSNSPYLNKR<br>KGKPGPDS           | All Importin $\beta$ & RanBP5/7 | Pho4             | [306]      |
| Other                                              | Multiple NLS         | RNKKKKRKVIK                              | Importin $\alpha/\beta 1$       | NLS-RAR $\alpha$ | [307]      |

## **2.8. Current challenges in nanocarrier-based targeted delivery**

Despite extensive research on nuclear targeting, some obstacles still exist in achieving efficient delivery of therapeutic cargo to the nucleus.

### **2.8.1. Complex physiological environment**

One major obstacle is primarily related to the complex physiological environment and intricate biochemical processes involved. To ensure the sufficient nuclear accumulation, it's critical for nanocarriers to have adequate stability within the physiological environment, avoiding undesired interactions with the surrounding media from the systemic circulation [308]. Once the nanocarriers reach the blood vessels near the target sites, they encounter an additional challenge known as the epithelial tissue before entering the targeted sites. For instance, a major hurdle arises when the size of the particles exceeds 5 nm in diameter, as they face difficulties in crossing the capillary epithelial barrier [309]. Once the nanocarriers have passed through the epithelial layer, they begin to attach to the cell membrane of the target cells and are subsequently internalized into the cytosol, primarily through cellular endocytosis pathway [310, 311]. This process can vary depending on the cell type, resulting in different intracellular processes [312]. The diversity of endocytosis pathways can result in varying efficiencies of subcellular delivery, including targeting the nucleus, even when using the same delivery system. Endocytic processes can also lead to the internalization of the nanocarriers into endosomes or lysosomes [313], preventing its nuclear delivery. For instance, the nanocarriers can become trapped within endo/lysosomal compartments. These compartments are part of the cellular process responsible for breaking down cellular components. Even when nanocarriers are modified with nuclear-targeting molecules, this does not always translate into improved functional outcomes. For example, a NLS derived from the transcription factor TFIIE- $\beta$  (SKKKKTKVC) significantly enhanced nuclear uptake but failed to increase antisense activity [241, 314]. This observation highlighted that while nuclear targeting is an important step, endosomal escape remains a major barrier to achieving effective intracellular delivery and functional gene modulation [315]. To ensure therapeutic agents successfully reach the nucleus and exert their intended effects, it is crucial for the delivery system to escape from endo/lysosomal compartments promptly and avoid enzymatic degradation within lysosomes [241, 314].

### **2.8.2. Off-target effect of certain targeting molecules**

Another concern related to NLS-mediated nuclear targeting is the potential off-target effect of certain NLS such as TAT peptides, which may impede achieving the precise nuclear targeting. It may result in the accumulation of cargo in organelles other than the nucleus, such as mitochondria [316, 317]. This prevents the cargo from reaching its intended nuclear destination. Additionally, other off-target effects associated with the nanocarrier itself can disrupt normal cellular processes, potentially leading to cellular dysfunction or toxicity [318]. Therefore, it is vital to minimise off-target effects when designing strategies for nuclear target. This will ensure the efficient and specific delivery of cargo to the intended nuclear compartment.

### **2.8.3. Stability and toxicity issues of nanocarriers**

Other targeted nanocarriers also face significant challenges related to toxicity and stability. These challenges not only affect the therapeutic efficacy but also pose significant hurdles for clinical translation. Due to their unique physicochemical properties, nanocarriers can interact with biological systems in unpredictable ways, raising toxicological concerns such as non-specific protein binding and organ accumulation. The high surface reactivity of nanocarriers can result in the adsorption of plasma proteins, which may trigger an immune response and subsequent inflammation [319]. This immune activation can lead to systemic toxicity if not properly controlled. Stability is a critical factor influencing the *in vivo* performance of nanocarriers. Interactions between the nanocarriers and organs can lead to aggregation or premature degradation of the nanoparticles, reducing their circulation time and altering their biodistribution [320, 321]. This not only limits therapeutic efficacy but also increases the risk of systemic side effects.

### **2.8.4. Manufacturing complexity of targeted nanoparticles**

In addition to challenges encountered at the preclinical stage, the complexity of nanoparticle manufacturing presents significant challenges in achieving reproducibility, maintaining batch-to-batch consistency and meeting established quality benchmarks, particularly for targeting technologies that are technically intricate or difficult to replicate. For example, lipid-based nanoparticles are highly sensitive to manufacturing and storage conditions. Their triglyceride components can undergo polymorphic transitions from the metastable  $\alpha$ -form to the more thermodynamically stable  $\beta$ -form, leading to crystalline aggregation. This reduces the amorphous regions within the lipid matrix and may result in premature drug leakage [322, 323]. Furthermore, nanocarriers are highly susceptible to both physical and chemical changes during

storage, which presents substantial challenges for large-scale manufacturing. Oxidative degradation, in particular, can alter surface charge, drug release profiles and particle stability, while potentially generating toxic byproducts that reduce therapeutic efficacy [324, 325].

Nanocarrier-based therapeutics are regulated by agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), which require these systems to meet strict standards of quality, safety and efficacy prior to approval [326]. To obtain regulatory authorization, manufacturers must comply with established quality guidelines, conduct comprehensive characterization and toxicology studies and fulfill specific regulatory requirements for clinical trials.

## **2.9. Future Directions**

Some promising areas in nanocarrier research include personalised nanomedicine and the development of technologies that integrate diagnostics and therapeutics. Nanoparticle-enabled medicine offers excellent cargo flexibility, making it ideal for personalised treatments [327, 328]. For example, in cancer therapy, individual biomarkers and genomic mutations play a crucial role in guiding clinical decisions. Nanocarriers can be tailored to incorporate patient-specific molecular information, enabling personalised nanomedicine that precisely target individual patients [105]. This approach represents a significant advancement over conventional chemotherapy, which is administered non-specifically and often yields limited therapeutic efficacy across diverse patient populations. Additionally, the integration of smart technology with drug delivery systems enables real-time monitoring, allowing for treatment adjustments and leading to improved therapeutic outcomes [329].

The integration of artificial intelligence (AI) and computational modeling holds great promise for optimizing the design and development of nanocarrier-based formulations, particularly those involving lipid nanoparticles. For instance, the classical four-component lipid nanoparticle formulation framework can theoretically yield over  $10^{10}$  possible combinations, making empirical screening both time-consuming and cost-prohibitive [330]. To overcome this challenge, data from experimental formulations have been used to train predictive models, allowing for the virtual screening of large lipid libraries, such as one comprising over 40,000 candidate lipids [331]. One example is Mana.bio, an AI-based drug delivery startup that has developed a platform that combines AI with lipid nanoparticle formulation, significantly accelerating the discovery of high-performing candidates. AI-based machine learning models have also been applied to predict delivery efficiency, enabling the rapid and systematic

development of ionizable lipids with minimal experimental effort. For example, Lewis et al. constructed a library of ionizable lipids and used a trained machine learning model to predict transfection efficiency, identifying lipid tail length as the most influential chemical feature associated with delivery performance [332]. Similarly, Li et al. trained a machine learning model to screen a library of 40,000 ionizable lipid candidates [333]. The lipid nanoparticles formulated with the selected top-performing lipids demonstrated improved in vitro transfection efficiency and reduced off-target delivery [333]. Furthermore, machine learning algorithms can accelerate the clinical translation of nanomedicines by predicting the optimal physicochemical properties tailored to specific cancer subtypes. These tools offer a rational design pathway for engineering nanocarriers that align with patient-specific tumour biology and therapeutic needs, thereby supporting the development of targeted delivery systems for personalized nanomedicine [334].

### **3.0. Conclusion**

This chapter reviews the recent advances in subcellular targeting, with a particular focus on nuclear localization strategies. Although nuclear-targeted strategies have been extensively investigated, efficient delivery of CRISPR/Cas9 to the nucleus remains insufficiently addressed. Aptamer-modified nanocarriers have emerged as a promising approach for precise nuclear delivery in gene therapy. The central aim of this study is to design and optimize a polymer–lipid hybrid nanoparticle functionalized with aptamers for the delivery of CRISPR/Cas9 complexes to the nuclei of cancer cells, thereby enhancing transfection efficiency and genome editing outcomes. By integrating rational nanoparticle engineering with precise molecular targeting, this approach aims to overcome the limitations of conventional CRISPR delivery methods and advance subcellular gene delivery strategies. The outcomes of this work are expected to address a critical gap in nucleus-targeted genome editing and contribute to the future clinical translation of CRISPR-based therapies.

## Chapter 3: Materials and methodology

### Chapter summary

In this chapter, we developed a nucleus-targeted CRISPR/Cas9 delivery system based on polymer-lipid hybrid nanoparticles functionalized with the AS1411 aptamer, using a two-step process involving nanoprecipitation for nanoparticle assembly and N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) chemistry for aptamer conjugation. The work embedded in this chapter addressed all methodological aspects related to Aim 1, 2, and 3, including the design and synthesis of nuclear targeted nanoparticles and characterization (Aim 1), validation for nuclear delivery in HCT-116 and MDA-MB-231 cells (Aim 2), and CRISPR/Cas9 loaded nanoparticle for gene editing applications (Aim 3).

This chapter details the chemicals and reagents used throughout the study, outlines the key synthesis and characterization methods, such as dynamic light scattering (DLS) and zeta potential analysis for physicochemical profiling, transmission electron microscopy (TEM) for morphological evaluation, UV-Vis and FTIR spectroscopy for chemical structure confirmation, and agarose gel electrophoresis for CRISPR/Cas9 complexation efficiency. All experimental results, including cellular uptake, nuclear localization analysis, and gene knockout outcomes, along with relevant data analyses, are presented and discussed in Chapters 4 and 5.

### 3.1. Materials

For the preparation of NPs, Poly(D,L-lactide-co-glycolide) acid terminated, lactide:glycolide 50:50 (PLGA) (Product No. 719870), Poly vinyl alcohol (PVA) (Product No. P8136), L-A-Phosphatidylcholine (Lecithin) (Product No. P7443), DSPE-PEG2000-carboxylic acid (Product No. 880135P), N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDAC) (Product No. E7750), N-hydroxysuccinimide (NHS) (Product No. 130672), Chloroform (Product No. C2432), Dichloromethane (DCM) (Product No. 270997), Cas9 protein labelled with GFP (Product No. CAS9GFPPRO), Phosphate-buffered saline (PBS) (Product No. P4417 TAB), Coumarin-6 (C-6) (Product No. 442631) were purchased from Sigma-Aldrich Pty Ltd (Sydney, Australia). Truecut Cas9 protein (Product No. A36498), Lcn2 targeting sgRNA (Product No. A35533) were purchased from Thermo Fisher Scientific (NSW, Australia). AS1411 aptamer (5'- /5AmMC6/ GGTGGTGGTGGTTGTGGTGGTGGTGG - 3')

and GFP-targeting sgRNA (5'-GGGCGAGGAGCTGTTCACCG -3') were purchased from Integrated DNA Technologies (Iowa, US).

For cell culture, DAPI (Product No. R37606), Dulbecco's modified Eagle's medium (DMEM) (Product No. 11965), Opti-Minimal Essential Medium (Opti-MEM) (Product No. 31985062), Fetal bovine serum (FBS) (Product No. 10437), Trypsin (Product No. 15400054) and Dulbecco's phosphate-buffered saline (DPBS) (Product No. 14040117) were purchased from Thermo Fisher Scientific (BSW, Australia). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Product No. 475989), Dimethyl sulfoxide (DMSO) (Product No. D8418), McCoy's 5A medium (Product No. M8403) and Cell migration assay (Product No. ECM508) were purchased from Sigma-Aldrich Pty Ltd (Sydney, Australia). The RNeasy mini kit (Product No. 74104) and DNeasy blood and tissue kit (Product No. 69504) were purchased from Qiagen (Hilden, Germany). The RT-qPCR kit (Product No. SIC001) and T7 Endonuclease I (T7E1) (Product No. M0302S) were purchased from New England Biolabs (Massachusetts, US).

### **3.2. Cell culture**

Human breast cancer cell lines (MDA-MB-231, MDA-MB-231-GFP) and human colon cells (CCD-841-CoN) were grown in DMEM supplemented with 10% FBS and 1% penicillium/streptomycin antibiotic. Human HCT-116 cells were grown in McCoy's 5A medium supplemented with 10% FBS and 1% penicillium/streptomycin antibiotic. Cultures were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>. All cell lines were regularly tested for mycoplasma, and only mycoplasma-free cells were used for experiments. When the cells reached approximately 80% confluency, they were detached using trypsin and transferred to Petri dishes or well plates for various experimental purposes.

### **3.3. Preparation of various PLGA lipid nanoparticles**

Nanocarriers were synthesized through the self-assembly of liposomes and polymers by nanoprecipitation [335]. Briefly, Lecithin and DSPE-PEG2000-COOH were mixed at a molar ratio of 8.5:1.5 and gradually heated to 65°C. The mixture was heated until it became clear and transparent, ensuring complete dispersion of the lipid components in the liquid phase. The solution was gradually cooled to room temperature for further experiments. Acetonitrile solution containing PLGA (5 mg/mL) and C-6 (5 mg/mL, for assessing cellular uptake activity) was added dropwise into the lipid phase at a consistent rate. The mixture was further stirred for 2 hours at room temperature. The resulting nanoparticles were purified by centrifugation at

10,000 rpm for 3 minutes using 100 kDa centrifugal filters. The purified nanoparticles were then stored at 4°C for subsequent experiments.

To load Cas9-GFP or CRISPR component, a modified method was employed. PLGA in DCM (5 mg/mL) was mixed with Cas9-GFP (1.7 µg/µL) or Cas9 (10 µM) with sgRNA (10 µM) dispersed in DNase-free water at room temperature. The mixture was ultrasonically processed using a probe sonicator at 40% amplitude for 90 seconds to create a primary emulsion. Next, using the previous method to make 1 wt.% PVA solution composed of lecithin and DSPE-PEG2000-COOH, slowly added to the solution and ultrasonically emulsified for a second time to form a water-in-oil-in-water emulsion. The resulting solution was gently stirred for 2 hours to evaporate the DCM solvent. The final nanoparticle suspension was purified by centrifugation at 10,000 rpm for 3 minutes using a 100 kDa centrifugal filter and stored at 4°C for further experiments.

The conjugation of AS1411 aptamer to the nanocarrier surface was achieved using the EDAC/NHS reaction method. Specifically, the prepared nanocarrier suspension was mixed with 200 µL of EDAC (0.4 mol/L) and 200 µL of NHS (0.1 mol/L) at room temperature and gently agitated on a shaker for 30 minutes. Subsequently, varying volumes of AS1411 aptamer (100 nM) was slowly added to the mixture, followed by gentle shaking for 2 hours at room temperature. The final solution was purified by centrifugation at 9,000 rpm for 3 minutes to remove unbound aptamer.

### **3.4. Nanoparticle characterisation**

The size distribution and zeta potential of the prepared nanoparticles were examined using a Zetasizer (Malvern Panalytical Co.). For particle size and PDI measurement, diluted samples were placed in clear disposable cuvettes, while zeta cells were utilized for zeta potential measurement. All measurements were performed in triplicate at 25°C. The nanoparticle morphology was examined by using Transmission Electron Microscopy (TEM) imaging system (JEOL Pty. Ltd, Tokyo, Japan). For TEM imaging, nanoparticles were suspended in nuclease-free water (100 µl/ml) and sonicated for 30s. The suspension was deposited onto copper grids and allowed to air dry under ambient conditions. The fluorescence spectrum of C-6 (Ex/Em: 450 nm/505 nm) encapsulated in the nanoparticles was measured using a fluorescence spectrometer (Shimadzu RF-6000). The UV-Vis absorbance of AS1411 was measurement under a NanoDrop spectrophotometer (Thermo Fisher Scientific). For encapsulation efficiency (EE%), the amount of GFP-labelled Cas9 encapsulated in the

nanoparticles was quantified by measuring their fluorescence intensity (Ex/Em: 488 nm/508 nm) and comparing it to a standard curve generated from free Cas9-GFP. To evaluate serum stability of nanoparticles, 100  $\mu$ L of nanoparticle samples were diluted in cell culture medium with or without 10% FBS. The nanoparticle size was measured using a Zetasizer at various time points (0, 2, 6, 24, 48 hours).

### **3.5. Cellular uptake activity**

HCT-116, CCD-841-CoN and MDA-MB-231 were cultured in glass-bottom petri dishes and incubated with nanoparticle suspension (100  $\mu$ L/ml) for 2 hours. Subsequently, the cells were thoroughly washed with DPBS (pH 7.4) three times to eliminate any unbound nanoparticles at room temperature. For fluorescence imaging, DAPI was added and incubated for 10 minutes before imaging under a laser confocal microscope (Nikon A1R, Nikon Instruments Inc). All images were taken using 100 $\times$  oil objective lens. Fluorescence emission was collected using filters with band pass of 500 - 550 nm for C-6 and 425 - 475 nm for DAPI. The fluorescence images were analysed using ImageJ software and an AI segmentation-assisted approach.

The amount of coumarin-6 (C-6) encapsulated within the nanoparticles was determined by assessing the fluorescence intensity of the C-6-loaded nanoparticles and subsequently calculating its concentration using the standard curve derived from a free C-6 solution. The amount of GFP-labelled Cas9 encapsulated within the nanoparticles was determined by assessing the fluorescence intensity of the Cas9-loaded nanoparticles and subsequently calculating its concentration using the standard curve derived from a free Cas9-GFP solution. The loading efficiency was determined by measuring the fluorescence intensity of GFP-labelled Cas9 using a plate reader (Tecan Group Ltd, Männedorf).

100  $\mu$ L of nanoparticle samples were diluted in cell culture medium with or without 10% FBS. For the pH stability study, 100  $\mu$ L of nanoparticle samples were added to PBS buffer with pH 7.5, 6.0 and 5.0. The nanoparticle size was measured using a Zetasizer at various time points (0, 2, 6, 24, 48 hours). For evaluating the cellular safety profile of nanoparticles, CCD-841-CoN cells and HCT-116 cells were cultured in 96-well plates for 24 hours. After removing the old media, the cells were incubated with 100  $\mu$ L fresh media containing the nanoparticles with different concentrations for 2 hours. After incubation, the old medium was removed and fresh medium was added, followed by another 24 hours incubation. Cell viability was assessed using the MTT assay kit according to its protocol. Cell viability was calculated as the percentage of absorbance of treated cells compared to untreated cells as follows:

$$Viability \% = \frac{(A_c - A_{blank})}{(A_0 - A_{blank})} \times 100\%$$

where  $A_c$  is the absorbance of nanoparticle-treated group,  $A_0$  is the absorbance of the control group, and  $A_{blank}$  is the absorbance of cell culture medium.

### 3.6. Assessment of in vitro GFP gene and Lcn2 knockdown efficiency

For GFP knockdown, MDA-MB-231-GFP cells were seeded in petri dishes at a density of  $1.0 \times 10^6$  cells/well 1 day prior to transduction. MDA-MB-231-GFP cells were transfected with Cas9/sgRNA (GFP targeting) loaded nanoparticles for 2 hours. Subsequently, the cells were thoroughly washed with PBS three times to remove any unbound nanoparticles. At 72 h post-transfection, the cells were imaged under a laser confocal microscope (Nikon A1R, Nikon Instruments Inc). All images were taken using  $100\times$  oil objective lens. Fluorescence emission was collected using filters with band pass of 500 - 550 nm for C-6. Additionally, flow cytometry analysis was performed after the treatments. The cells were washed, trypsinized and collected, and their fluorescence was quantitatively analysed using a CytoFLEX LX flow cytometer (Beckman Coulter). The GFP fluorescence intensity data was analysed using FlowJo software.

For Lcn2 knockdown, the MDA-MB-231 cells were incubated with the nanoparticles loaded with CRISPR/Cas9 targeting the Lcn2 gene for 2 hours. Subsequently, the cells were washed with PBS three times to remove any unbound nanoparticles. Following the wash, the cells were incubated for 72 hours prior to subsequent experiments.

#### 3.6.1. T7 Endonuclease I assay (T7EI) and sanger sequencing

Genomic DNA was extracted for T7EI assay using the DNeasy Blood and Tissue Kit (Qiagen, Germany). PCR amplification was carried out using T7EI primers (Table 3.1) designed specifically for targeting the site using Q5 High Start High-Fidelity Master mix (New England Biolabs, US). The resulting products were examined through 2% agarose gel electrophoresis to confirm successful amplification and assess the product size. An annealing program was performed with an initial temperature of  $95^\circ\text{C}$  for 5 min, followed by a gradual decrease of  $2^\circ\text{C}$  per second until reaching  $85^\circ\text{C}$ , and then decrease at a rate of  $0.1^\circ\text{C}$  per second until reaching  $25^\circ\text{C}$ . After completion of the annealing step,  $1\ \mu\text{L}$  of T7EI enzyme (New England Biolabs, US) was added to the annealed product, the contents were thoroughly mixed and incubate at  $37^\circ\text{C}$  for 15 min to allow for the digestion reaction to occur. Upon completion of the reaction,  $1.5\ \mu\text{L}$  of 0.25 M EDTA was added to stop the digestion reaction. The digested products were

measured by 2% agarose gel electrophoresis. The band intensity was analysed using ImageJ software. The percentage was calculated using the formula [336]:

$$Indel \% = 100 \times \left(1 - (1 - f)^{\frac{1}{2}}\right)$$

where  $f$  represents the fraction of cleaved PCR product.

**Table 3.1. Primers for PCR amplification**

| Primer                      | Sequence                     |
|-----------------------------|------------------------------|
| <b>Lcn2 T7EI – Forward</b>  | 5'- TATGGGGAGAAGCCCACGTT -3' |
| <b>Lcn2 T7EI – Reversed</b> | 5'- GGCTGGCAGACACCACATAA -3' |

### 3.6.2. RT-qPCR

Total RNA samples were isolated by RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The cells were first centrifuged and lysed by Buffer RLT, then the lysate was transferred to a RNeasy spin column and centrifuged at  $8,000 \times g$  for 15 seconds. The column was subsequently washed with buffer RW1 and RPE, and RNA was eluted in 40  $\mu$ L of RNase-free water and stored at  $-80^{\circ}\text{C}$  until further use. The RNA concentration was measured by absorbance at 260 nm using Nanodrop spectrophotometer (Thermo Fisher Scientific, Australia). For the RT-qPCR assay, 100 ng of total RNA was used for cDNA synthesis and qPCR using the Universal One-Step RT-qPCR Kit (New England Biolabs, US) on a QuantStudio 6k FLEX real-time PCR system. Reactions were performed according to the manufacturer's protocol. Primer sequences are provided in Table 3.2.

**Table 3.2. Primers and sequences used for RT-qPCR**

| Primer                  | Sequence                       |
|-------------------------|--------------------------------|
| <b>Lcn2 – Forward</b>   | 5'-TCACGCTGGGCAACATTAAG -3'    |
| <b>Lcn2 – Reversed</b>  | 5'- TCAGGGAGGCCAGAGATTT -3'    |
| <b>GAPDH – Forward</b>  | 5'- CGCTCTCTGCTCCTCCTGTTC -3'  |
| <b>GAPDH – Reversed</b> | 5'- ATCCGTTGACTCCGACCTTCAC -3' |

### 3.7. Transwell cell migration assay

Transwell migration assays were conducted using 24-well inserts with polycarbonate membranes (8.0  $\mu\text{m}$ , Corning). After 24 hours of serum starvation, 300  $\mu\text{L}$  of MDA-MB-231 cell suspension was added to the upper chamber. Subsequently, 500  $\mu\text{L}$  medium containing 10% FBS was placed in the lower chamber as a chemoattractant. Following a 24-hour incubation, migrated cells on the lower membrane surface were detached, stained with CyQUANT GR

Dye and quantified by measuring fluorescence intensity (Ex/Em, 480/520 nm) using Tecan plate reader (Tecan, Switzerland). The relative cell migration rate was calculated by dividing the number of migrated cell numbers in treatment group by the number of cells migrated in cell control.

### 3.8. Wound healing assay

MDA-MB-231 cells were seeded at  $1 \times 10^6$  per well in 6-well tissue culture plates and cultured until they reached approximately 90% confluence. After that the wound scratch was created using a sterile pipette tip. Detached cells and cellular debris were gently removed by washing cells twice with PBS. Then, the cells with created wound were treated with targeted and non-targeted nanoparticles loaded with CRISPR/Cas9. The plates were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and wound closure was observed using an inverted microscope (4× magnification). Images were captured at different time points (0, 6, 24, 48 and 72 hours). The wound area was analysed using ImageJ software, and cell response was quantified as the percentage of wound closure relative to the initial wound area. All assays were performed in triplicate, and the results are expressed as mean  $\pm$  standard deviation. The wound closure was calculated using the equation [337]:

$$\text{Wound closure \%} = \left( \frac{A_0 - A_t}{A_0} \right) \times 100$$

where  $A_0$  is the area (pixels) of the wound calculated after scratching ( $t = 0$  h) and  $A_t$  is the area (pixels) of the unhealed wound (which is not covered by the cells) that remained at different time points after the scratching.

### 3.9. Statistical analysis

All quantitative data are shown as mean  $\pm$  SD from at least a triplicate experiment. Using Prism 10 to do the statistical analysis. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## **Chapter 4: Characterisation of prepared polymer-lipid hybrid nanoparticles and nucleus uptake activity**

### **4.1. Introduction**

Polymeric and lipid-based nanoparticles are extensively explored drug delivery systems, each with distinct advantages and limitations [338]. Polymeric nanoparticles, exemplified by poly(lactic-co-glycolic acid) (PLGA), provide excellent structural integrity, controllable degradation kinetics, and sustained drug release [339]. Lipid nanoparticles exhibit enhanced biocompatibility, efficient cellular entry mediated by membrane fusion, and generally acceptable safety profiles, but their broader application remains limited by lower mechanical robustness and constrained loading capacities for hydrophilic therapeutics [340]. To capitalize on the strengths while mitigating the limitations of both systems, polymer-lipid hybrid nanoparticles have been developed. Specifically, PLGA-Lecithin-PEG2000-COOH nanoparticles integrate the structural robustness and controlled-release characteristics of PLGA, the biocompatibility and cellular membrane affinity of lecithin [341, 342]. Moreover, carboxyl-functional groups on the nanoparticle surface facilitate versatile conjugation strategies for targeted drug delivery [341].

Targeted delivery of therapeutic agents to the cell nucleus holds considerable promise for cancer therapy and precise genome-editing applications [258]. Direct nuclear targeting can significantly enhance therapeutic efficacy by increasing payload accumulation at the site of genomic action, simultaneously reducing systemic exposure and off-target effects [258, 343]. The AS1411 aptamer, a nucleolin-binding DNA oligonucleotide, has gained attention due to its high affinity and specificity for nucleolin, which is abundantly expressed on the nuclear membrane of various tumor cells [344, 345]. Conjugating AS1411 onto nanoparticle surfaces promotes selective nuclear entry, thereby improving the specificity and effectiveness of therapeutic interventions directed at nuclear targets [345].

Nucleolin-targeting works because AS1411 binds specifically to nucleolin that is overexpressed on the surface of cancer cells, enabling selective uptake via nucleolin-mediated endocytosis. Once internalized, nucleolin shuttles between the membrane, cytoplasm, and nucleus, helping guide the attached nanoparticle toward perinuclear or nuclear compartments. MDA-MB-231 (triple-negative breast cancer) and HCT 116 (colorectal carcinoma) were

chosen because both exhibit high surface expression of nucleolin, making them suitable models for evaluating nucleolin-targeted delivery systems such as AS1411-modified nanoparticles.

In this chapter, we systematically characterized lecithin-PEG2000-COOH-coated PLGA nanoparticles conjugated with the AS1411 aptamer for enhanced nuclear targeting. To evaluate their cellular uptake and nuclear delivery capabilities, we employed coumarin 6 dye for intracellular tracking and Cas9-GFP fusion protein as a functional cargo representative of genome-editing tools. These fluorescent cargos enabled direct visualization and quantitative assessment of nanoparticle uptake and subsequent nuclear localization. These activities were highly aligned with Aim 1 and 2.

## **4.2. Result and Discussion**

### **4.2.1. Characterization of nanoparticles with different formulations**

Size and zeta potential are standard parameters to characterize the nanoparticles, which also have influences on the encapsulation efficiency, the stability of nanoparticles and the cellular uptake ability of nanoparticles [346-348].

Nanoparticles fabricated via the nanoprecipitation method have been extensively validated in previous studies as effective drug delivery carriers, offering well-controlled size distribution, high encapsulation efficiency, and reproducibility. Moreover, the effects of varying molar ratios of polymer, lipid, and surfactant components on nanoparticle properties have already been systematically investigated. To facilitate efficient intracellular delivery and nuclear uptake, nanoparticle size should be minimized to promote membrane diffusion. However, to accommodate the relatively large molecular weight of the Cas9 protein (~160 kDa), particle size cannot be excessively small.

In the initial design, nanoparticles were prepared by mixing an organic phase - comprising PLGA and the cargo dissolved in acetonitrile—with an aqueous lipid phase under controlled stirring. Upon solvent evaporation, spontaneous self-assembly occurred, yielding polymer-lipid hybrid nanoparticles. While this approach is compatible with small-molecule drugs that exhibit high chemical stability, it poses significant challenges when applied to macromolecular cargo such as Cas9 protein and single-guide RNA (sgRNA), both of which are highly sensitive to organic solvents. Prolonged exposure to acetonitrile can lead to denaturation, aggregation, or irreversible functional loss of these biomolecules. As a result, the formulation strategy was modified to a probe sonication-assisted emulsification method, which minimizes direct contact between bioactive cargo and organic solvents. This technique enables the transient formation

of nano-emulsions, allowing encapsulation of aqueous-phase Cas9-sgRNA complexes within the core while preserving their biological activity.

The nanoparticles were constructed with a PLGA core to provide mechanical stability and controlled release capability. The outer lipid layer was composed of lecithin and DOPE-PEG2000-COOH, which together enhanced biocompatibility and colloidal stability. During the nanoprecipitation process, these amphiphilic lipid components spontaneously assembled at the organic-aqueous interface, forming a hybrid lipid-polymer shell that encapsulated and stabilized the PLGA core. The terminal carboxyl groups on PEG2000 further allowed for downstream surface functionalization or ligand conjugation. The average hydrodynamic diameter and zeta potential of nanoparticles formulated with different lipid compositions were characterized by dynamic light scattering (DLS).

However, PLGA-based nanoparticles tend to aggregate during post-synthesis processing due to their intrinsic hydrophobicity and inter-particle interactions, particularly under mechanical stress such as centrifugation.

To evaluate the influence of centrifugal force on nanoparticle stability during purification, the formulated nanoparticles were subjected to centrifugal filtration using Amicon Ultra filter units (MWCO 100 kDa) at varying relative centrifugal forces (3000, 5000, 8000, 10000, 15000  $\times$  rpm). Hydrodynamic diameters of nanoparticles retained within the filters were measured using DLS at 25  $^{\circ}$ C. Prior to measurement, all samples were diluted in deionized water to minimize scattering artifacts, and triplicate measurements were conducted to ensure reliability.

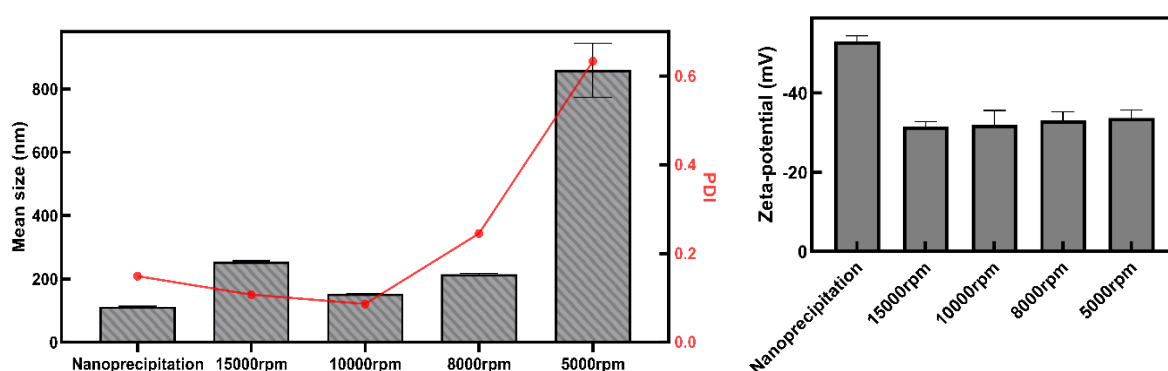


**Figure 4.1.** Nanoparticle stability under different centrifugal forces and construct method. (a) the mean sizes and PDI values of nanoparticles by nanoprecipitation and centrifugal forces; (b) the zeta potentials of nanoparticles by nanoprecipitation and centrifugal forces.

As shown in **Figure 4.1**, the hydrodynamic size of the nanoparticles gradually decreased with increasing centrifugal speed, reaching an optimal size of approximately 160 nm at 10,000 rpm, accompanied by the lowest polydispersity index (PDI). This size distribution closely aligns with that of the originally designed self-assembled formulation, indicating that moderate centrifugal force effectively removes loosely bound aggregates or free lipids without compromising particle integrity. However, when the centrifugation speed was further increased to 15,000 rpm, both the average size and PDI exhibited a marked increase. This trend is likely attributable to excessive mechanical stress leading to interparticle fusion or partial disruption of the lipid shell structure. Such conditions may result in transient destabilization of the core–shell interface or promote aggregation due to transient local concentration effects during ultrafiltration.

Moreover, due to the presence of carboxyl functional groups on the terminal ends of PEG2000, all nanoparticle formulations maintained a consistently negative zeta potential across conditions (Fig 1b). This surface charge contributes to electrostatic repulsion between particles, enhancing colloidal stability and supporting downstream surface conjugation processes.

To enable targeted delivery, carboxyl-terminated PEG chains on the nanoparticle surface were activated using carbodiimide chemistry for ligand conjugation. Specifically, EDC and NHS were used to activate surface-exposed –COOH groups, forming reactive NHS esters that subsequently reacted with the primary amine groups of AS1411 aptamers (or other ligands), resulting in covalent amide bond formation.

The size distribution and surface charge of various nanoparticle samples were listed in Table 4.1. The average size of pure poly-lipid nanoparticles was 113.3 nm, and with AS1411 modification, the size of the targeted nanoparticles increased to 172 nm. The zeta potential ( $\zeta$ ) was also increased slightly after aptamer modification. The polydispersity index (PDI) of all samples was between 0.1 to 0.3, indicating that these nanoparticles were close to a monodisperse state [349].

**Table 4.1.** The average size, zeta potential, PDI and encapsulation efficiency (EE%) of nanoparticles in deionized water. NPs refer to pure nanoparticles without any modification; AS1411 (10) – NPs, AS1411 (20) – NPs, AS1411 (40) – NPs represent aptamer-modified nanoparticles with AS1411 concentration of 2, 4 and 8  $\mu$ M respectively.

| Samples | Zeta potential (mV) | Size (nm) | Polydispersity | EE% of Cas9 |
|---------|---------------------|-----------|----------------|-------------|
|---------|---------------------|-----------|----------------|-------------|

|                                  |                   |                    |                   |       |
|----------------------------------|-------------------|--------------------|-------------------|-------|
| NPs                              | $-44.3 \pm 0.404$ | $113.3 \pm 1.429$  | $0.119 \pm 0.012$ |       |
| AS1411 (10) - NPs                | $-30.3 \pm 1.01$  | $172.2 \pm 0.9504$ | $0.208 \pm 0.008$ |       |
| AS1411 (20) - NPs                | $-24.4 \pm 0.651$ | $163.4 \pm 3.325$  | $0.234 \pm 0.41$  |       |
| AS1411 (40) - NPs                | $-33.3 \pm 0.577$ | $161.1 \pm 2.25$   | $0.258 \pm 0.009$ |       |
| Cas9 loaded NPs                  | $-25.0 \pm 0.379$ | $166.5 \pm 2.265$  | $0.091 \pm 0.016$ | 28.6% |
| Cas9 loaded<br>AS1411 (20) - NPs | $-3.46 \pm 0.11$  | $236.7 \pm 7.375$  | $0.288 \pm 0.026$ | 27.9% |

The morphology of liposomes was checked by SEM and TEM imaging. The SEM images of nanoparticles and aptamer-modified nanoparticles are shown in **Figure 4.2 a and b**, respectively. However, SEM is hard to capture clear and structurally representative images of the nanoparticles. This limitation is likely due to the soft, organic nature of the lipid-polymer hybrid nanoparticles, which are susceptible to deformation, collapse, or aggregation during the drying and conductive coating steps required for SEM. In particular, the dehydration process can distort the morphology of the PLGA core and lipid shell, leading to artifacts or irregular particle outlines. Furthermore, the low electron density of polymeric and lipidic materials results in poor contrast under SEM without heavy metal staining, which may further compromise structural integrity.

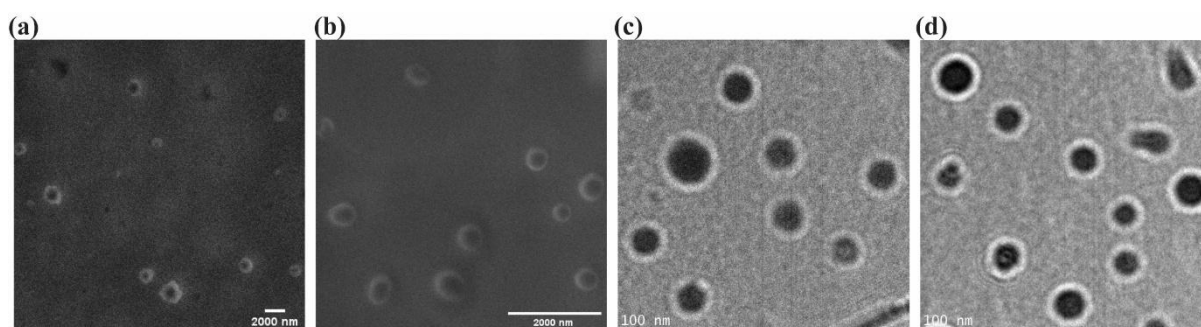


**Figure 4.2.** Electron microscopy analysis SEM images of nanoparticle. (a), modified nanoparticle (b), TEM image of nanoparticle (c), modified nanoparticle (d).

To overcome these limitations, TEM was used as an alternative technique. Unlike SEM, TEM allows for higher-resolution imaging of nanostructures with minimal structural distortion. As a result, TEM provided more accurate visualisation of the nanoparticle morphology and size distribution under near-native conditions. TEM image shown in **Figure 4.2 c and d** indicate a uniformly spherical morphology of the nanoparticles.

To evaluate the encapsulation efficiency of the nanoparticles, a standard calibration curve was established using a series of known concentrations of coumarin 6 (C-6). The fluorescence

intensity exhibited a strong linear correlation with C-6 concentration, with a coefficient of determination ( $R^2$ ) of 0.97 (**Figure 4.3**), indicating high assay reliability. Analysis of the fluorescence signal from the nanoparticle formulation confirmed that C-6 was successfully encapsulated, with an estimated encapsulation efficiency of approximately 90% (**Figure 4.4**).

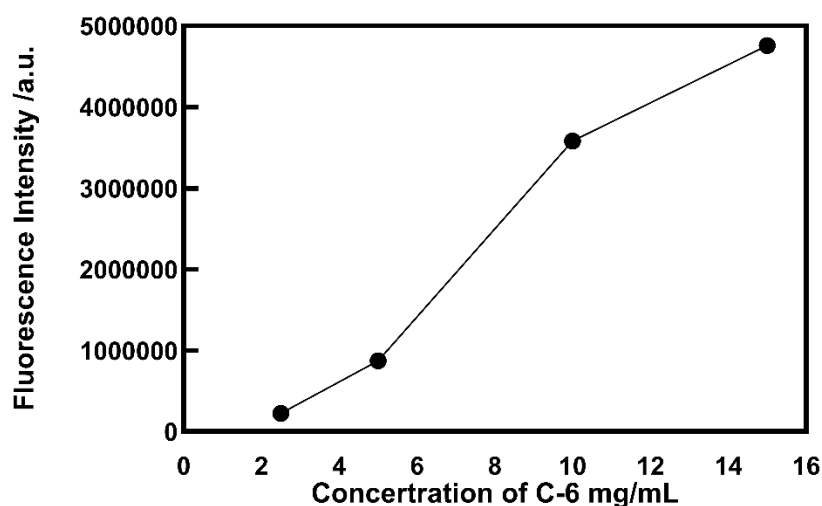


**Figure 4.3.** Standard curve of coumarin 6 (C-6) fluorescence intensity versus concentration. A calibration curve was generated by plotting the fluorescence intensity of C-6 against its concentration.

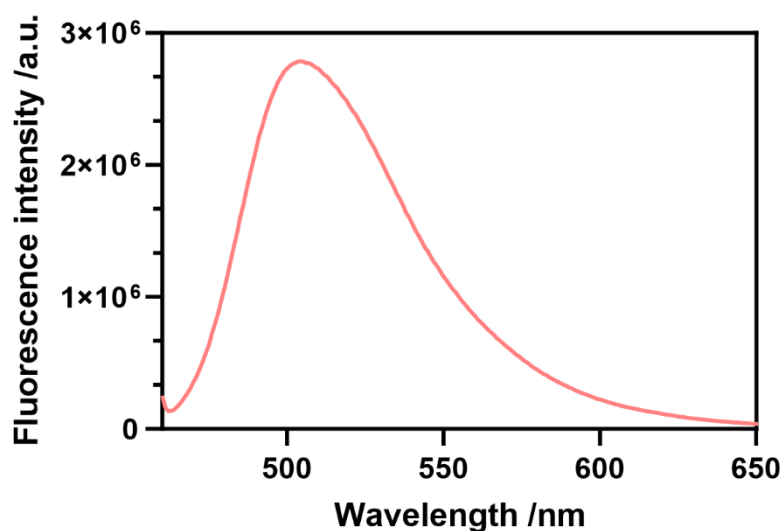


**Figure 4.4.** Fluorescence Emission Spectrum of Coumarin 6 (C-6). The fluorescence emission profile of Coumarin 6 was recorded, showing a maximum emission peak around 500 nm.

In order to confirm the successful binding between the nanoparticles and the aptamer, the Fourier-transform infrared (FTIR) was performed to assess the covalent conjugation of AS1411 to the surface of PEGylated nanoparticles. A slight shift in the absorption band near  $1600\text{ cm}^{-1}$  was observed following the EDC/NHS coupling reaction (**Figure 4.5**), which is consistent with the formation of amide bonds between the carboxyl groups on the nanoparticle surface and the amino-modified AS1411 aptamer. This spectral shift suggests successful attachment of AS1411.

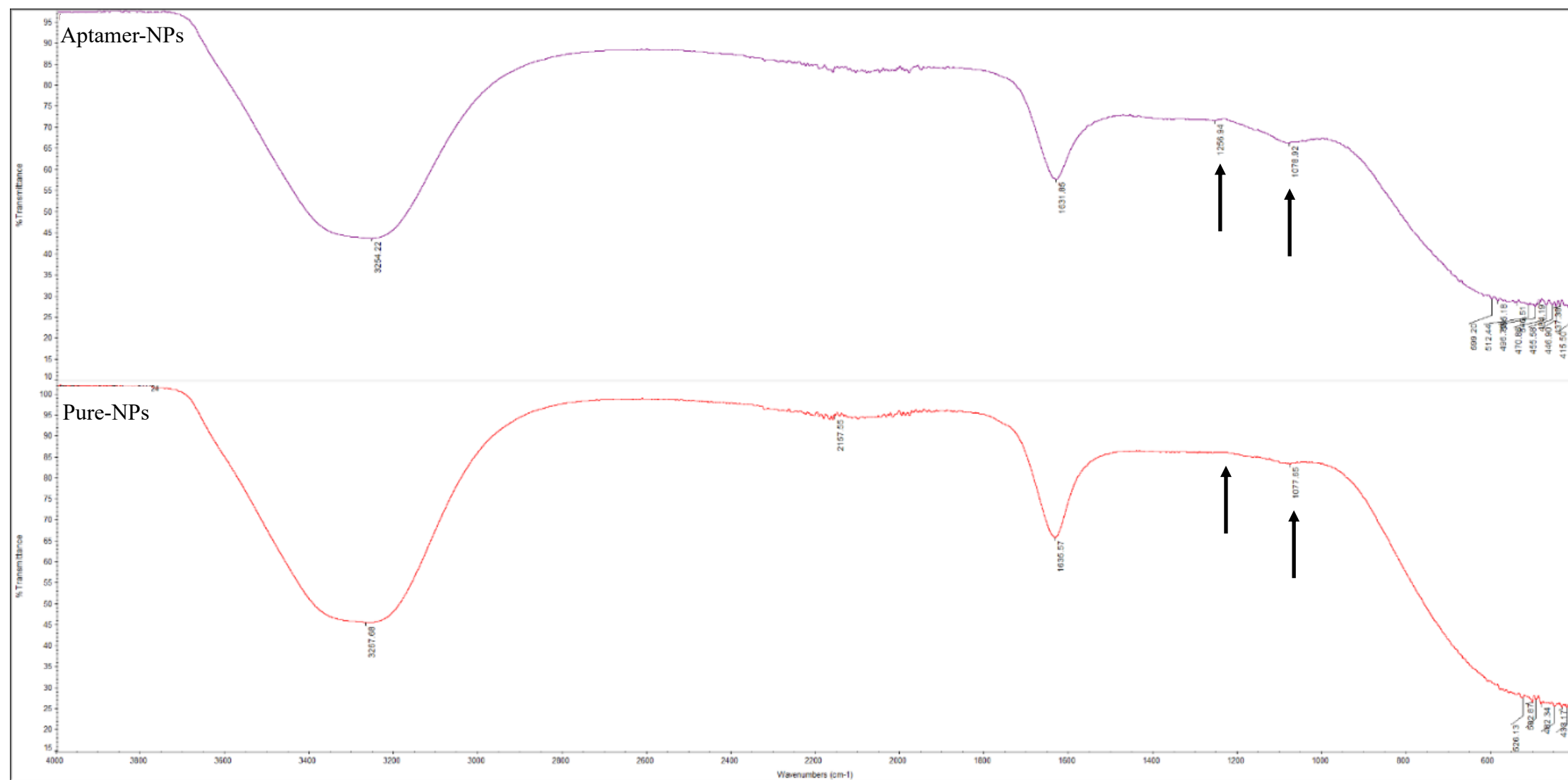


**Figure 4.5.** C-6 loaded nanoparticle fluorescence intensity (FTIR): Aptamer modified nanoparticles on the top; pure nanoparticles on the bottom.

However, due to the inherently low sensitivity of FTIR for detecting minor surface modifications, the changes were not sufficiently distinct to serve as definitive evidence. Therefore, ultraviolet-visible (UV-Vis) spectroscopy was subsequently employed to further verify the aptamer conjugation. As shown in **Figure 4.6**, pure AS1411 showed a clear characteristic absorption peak at 255nm. This characteristic peak also appeared in the targeted nanocarriers. This confirmed that the AS1411 aptamer had been successfully bound to the nanocarriers.

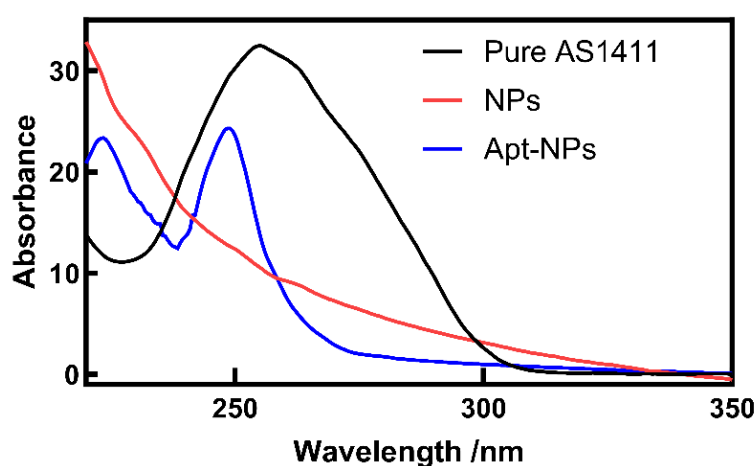


**Figure 4.6.** UV-vis absorbance spectra of pure AS1411, pure nanoparticles (NPs) and AS1411-modified nanoparticles (Apt-NPs).

Stability is a very important property of nanoparticle for cellular uptake and circulation. It is worth to investigate if different PH value and Serum everiment is stable. The DLS size change from nanoparticles with different formulations were measured for 48h. **Figure 4.7** confirmed that the nanocarriers exhibited a stable profile in the presence in the cell medium containing 10% FBS. **Figure 4.8** and **Figure 4.9** demonstrates that the nanoparticles and aptamer modified nanoparticles remained stable across pH levels ranging from 5.0 to 7.5. All these findings indicated that our nanoparticles were sufficiently stable under the current experiment conditions, without major cargo leaking out of its carriers.

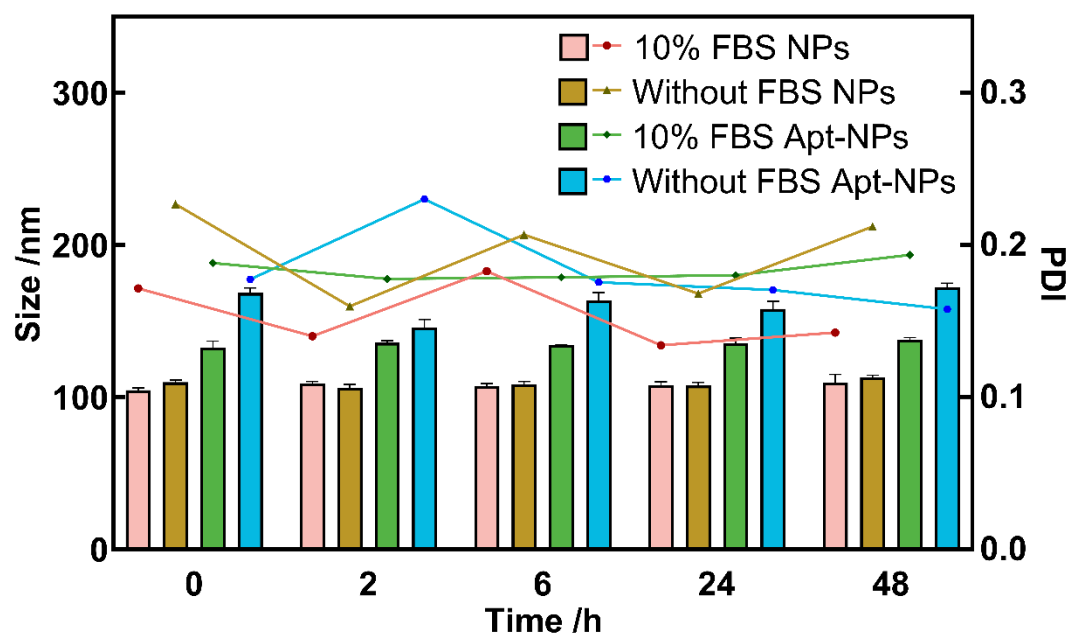


**Figure 4.7.** Serum medium stability profiles of prepared nanoparticles.

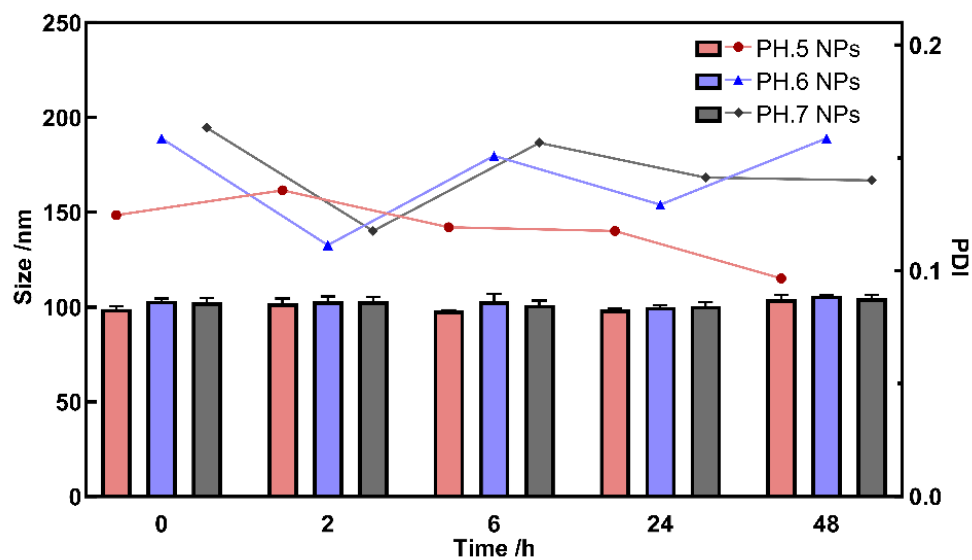


**Figure 4.8.** The stability profiles of prepared nanoparticles (non-targeted nanoparticles) under various pH conditions

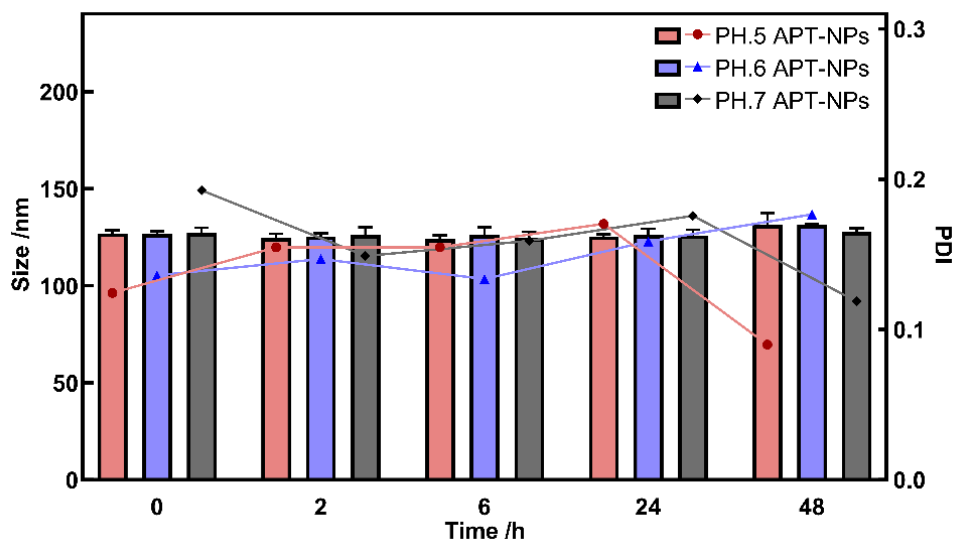


**Figure 4.9.** The stability profiles of prepared nanoparticles (APT-NPs) under various pH conditions

#### 4.2.2. Assessment of cellular uptake

Cellular uptake of nanoparticles (NPs) and AS1411-functionalized nanoparticles (APT-NPs) was evaluated at 1 hour (**Figure 4.10**), 2 hours (**Figure 4.11**), and 6 hours (**Figure 4.12**) incubation for HCT-116 using confocal laser scanning microscopy. Fluorescence imaging revealed evident intracellular accumulation at all time points, indicating efficient uptake by the cells. Notably, the fluorescence intensity observed at 2 hours was higher than that at 1 hour, suggesting a time-dependent enhancement in nanoparticle internalization. However, extended exposure to APT-NPs for 6 hours resulted in a visible reduction in cell viability, likely attributable to the intrinsic antiproliferative activity of AS1411. Based on these observations, a 2-hour incubation period was selected for subsequent experiments to balance uptake efficiency and cellular viability.

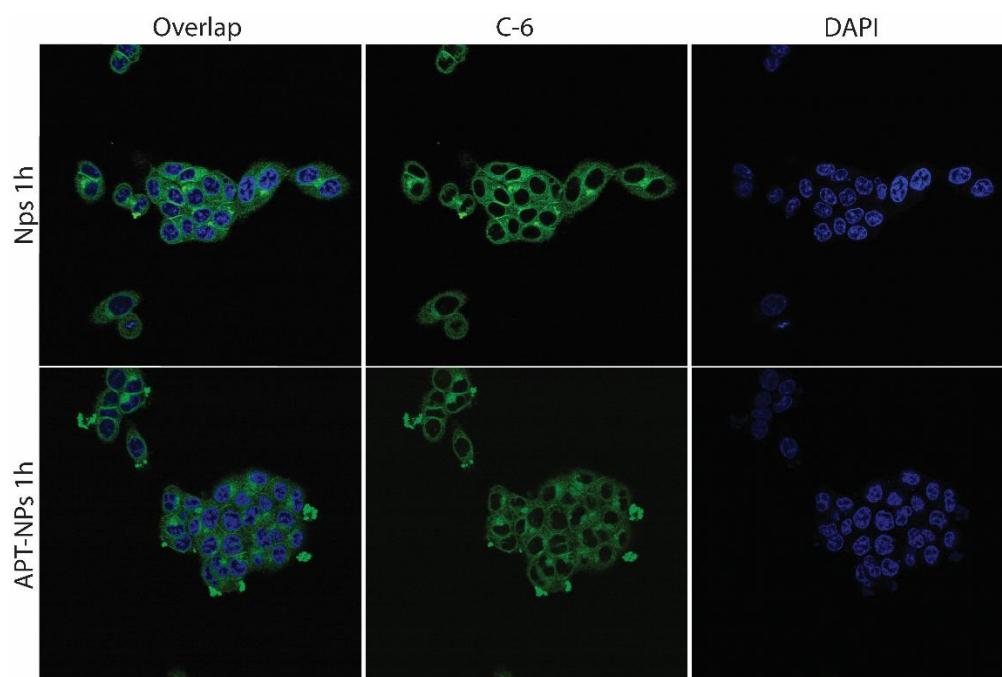


**Figure 4.10.** Confocal images of colocalization between the NPs loaded with C-6 (green channel) and cell nuclear stained by DAPI (blue channel) for 1 hour incubation. Scale bar, 20  $\mu\text{m}$ .

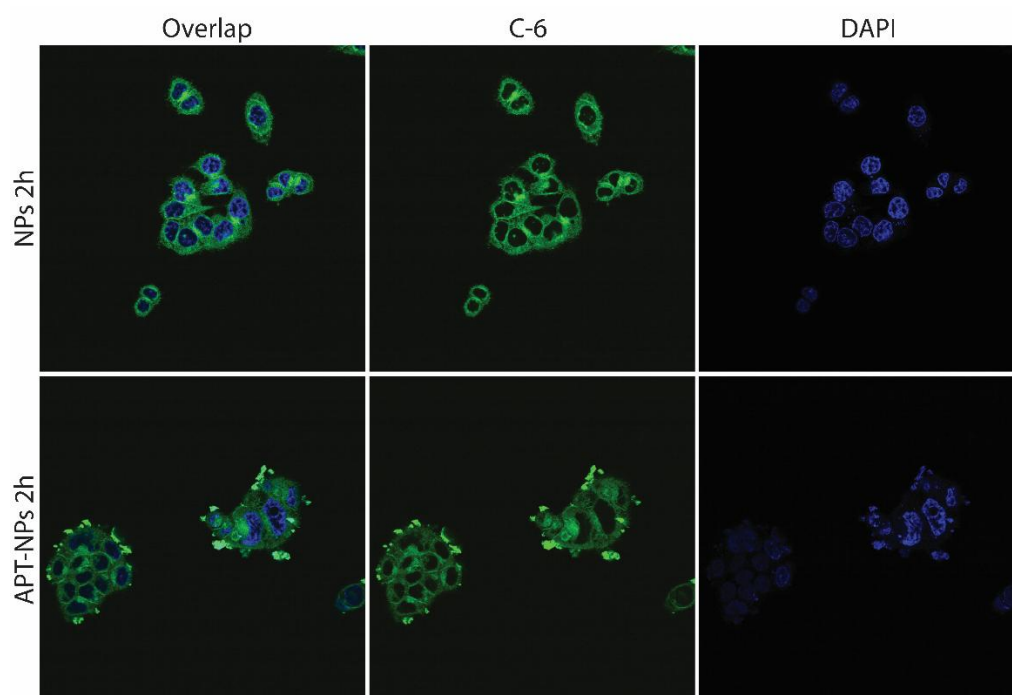


**Figure 4.11.** Confocal images of colocalization between the NPs loaded with C-6 (green channel) and cell nuclear stained by DAPI (blue channel) for 2 hours incubation. Scale bar, 20  $\mu\text{m}$ .

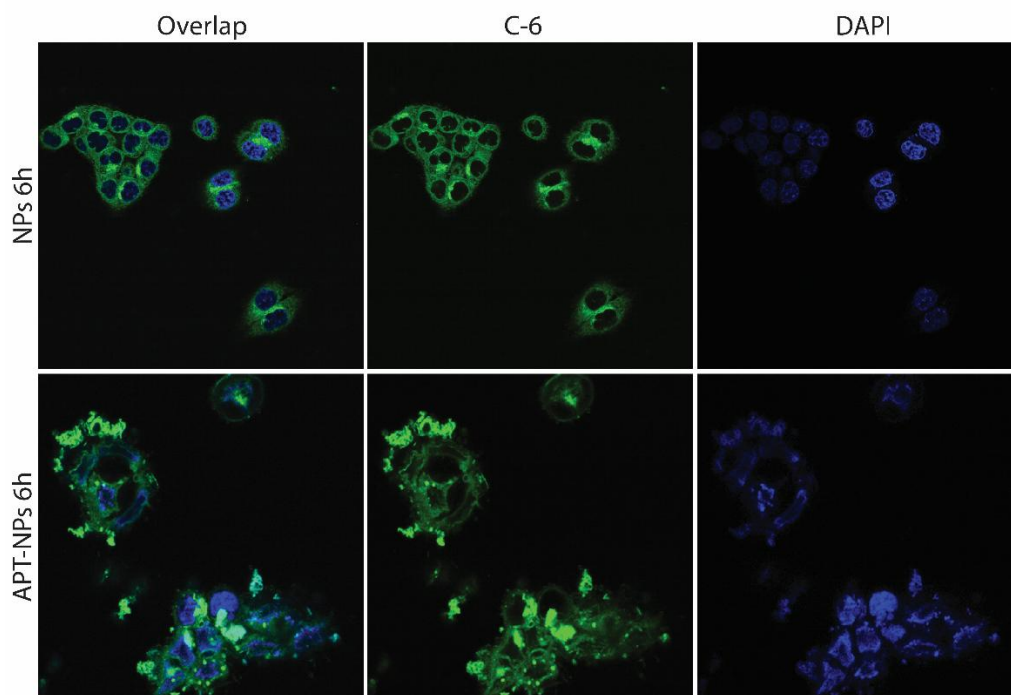


**Figure 4.12.** Confocal images of colocalization between the NPs loaded with C-6 (green channel) and cell nuclear stained by DAPI (blue channel) for 6 hours incubation. Scale bar, 20  $\mu\text{m}$ .

To evaluate the effect of AS1411 concentration on cellular uptake and viability, AS1411-functionalized nanoparticles were prepared using increasing volumes of aptamer solution (5, 10, 20, and 40  $\mu\text{L}$ ) and incubated with cancer cells for 2 hours. Confocal microscopy (**Figure 4.13**) revealed successful uptake in all groups, with intracellular fluorescence signals clearly observed. However, a progressive decline in cell viability was noted with increasing AS1411 content. Cells treated with nanoparticles modified using 5 or 10  $\mu\text{L}$  of AS1411 remained morphologically intact and exhibited no signs of cytotoxicity. In contrast, treatment with 20  $\mu\text{L}$  resulted in compromised cell morphology, while 40  $\mu\text{L}$  led to evident cell death. These observations suggest a concentration-dependent cytotoxic effect of AS1411. Consistent with these morphological changes, MTT assay results (**Figure 4.14**) showed that relative cell viability was approximately 90% for the 5  $\mu\text{L}$  group and 80% for the 10  $\mu\text{L}$  group but declined to below 70% and 50% with 20  $\mu\text{L}$  and 40  $\mu\text{L}$  of AS1411, respectively. Based on these findings, 10  $\mu\text{L}$  was selected as the optimal aptamer amount for subsequent experiments to balance uptake efficiency and cellular viability.

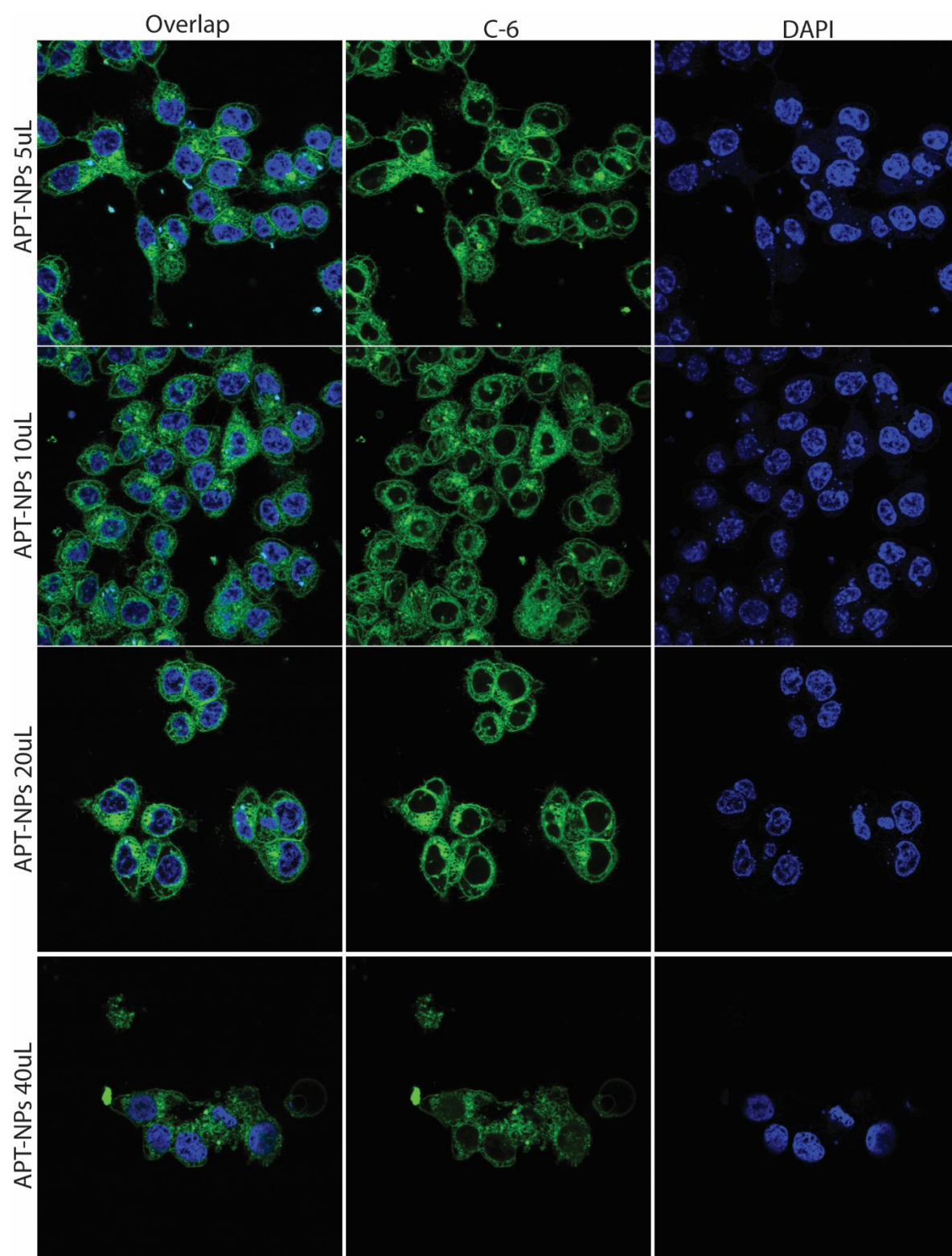


**Figure 4.13.** Confocal images of different Aptamer concentration (AS1411) NPs loaded with C-6 (green channel) and cell nuclear stained by DAPI (blue channel) for 2 hours incubation. Scale bar, 10  $\mu\text{m}$ .

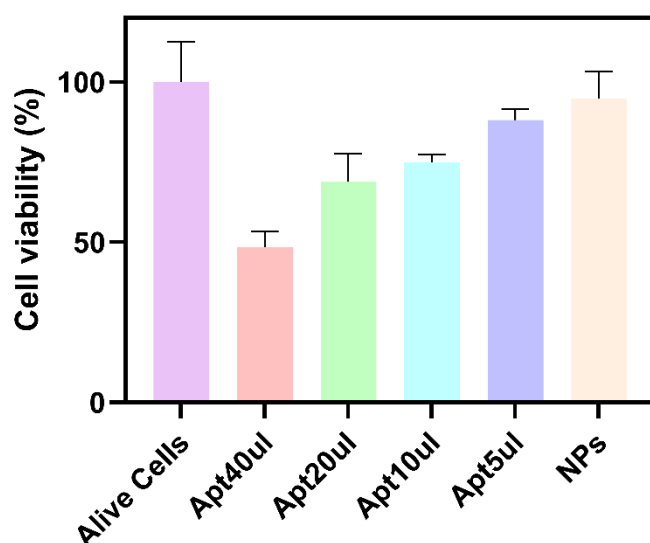


**Figure 4.14.** The viability of HCT-116 cells after the treatments with nanoparticles (NPs) and aptamer at different concentrations modified nanoparticles (Apt) at 24h by conducting MTT assay.

#### 4.2.3. Assessment of colocalization between nanoparticles and nucleus

The targeting capability of the nanoparticles was evaluated in two cancer cell lines. As shown in **Figure 4.15 a and b**, MDA-MB-231 cells treated with AS1411-NPs exhibited a significantly larger colocalization area (white regions) compared to those treated with non-targeted nanoparticles. Similarly, in the HCT-116 cell line (**Figure 4.15 c and d**), a notable increase in colocalization was observed with the use of targeted nanoparticles. To confirm that the nanoparticles were transported into the nucleus, colocalization images were further quantified using Pearson's correlation coefficient (PC) and Mander's split coefficient (MC) analysis. PC ranges from -1 to +1, where +1 indicates perfect correlation, 0 indicates no correlation, and -1 denotes perfect anti-correlation; noise can skew this value closer to 0 [350]. MC measure the fraction of intensity in each colour channel that colocalises with above-threshold intensity in the other channel, reflecting the proportion of colocalising pixels or voxels [351, 352]. A value of 0 indicates no colocalization, while a value of 1 indicates complete colocalization. A high MC1 coefficient suggests that most of the signal in channel 1 overlaps with channel 2, indicating that structures or molecules in channel 1 are largely colocalized with those in channel 2. Similarly, a high MC2 coefficient indicates that the signal in channel 2 largely overlaps with channel 1 [352, 353]. As shown in Fig.15e and 15f, the PC was higher for the cells treated with targeted nanoparticles compared to those treated with non-targeted ones (0.24 vs. 0.08) in both

cell lines, indicating improved colocalisation. Furthermore, MC1 and MC2 were also increased for the aptamer-modified nanoparticles, further supporting enhanced colocalisation compared to the unmodified nanocarriers.

To ensure the accuracy of our ImageJ-based data analysis, we also trained a CNN deep learning model utilising a U-Net architecture in a supervised manner, which has been primarily used for image segmentation tasks and obtained a high utility within the medical imaging community [354]. This approach was designed for segmenting medical images and identifying specific regions in microscopy images [355]. It employs libraries NumPy and OpenCV for image processing, and TensorFlow for defining and training the model [356, 357]. Images are pre-processed and loaded to the U-Net model by splitting them for training and validation, compiling with the Adam optimizer and binary cross-entropy loss and using callbacks to save the best model during training based on the validation loss. The process will predict masks for regions in C-6 fluorescence images (green channel), using DAPI masks representing nuclear regions (blue channel). According to the model's predictions, the PC for the cells treated with targeted nanoparticles was slightly lower than the values obtained using ImageJ. This discrepancy may be attributed to the impact of the AS1411 aptamer on cell activity, which may reduce cell viability [358]. This phenomenon, in turn, increases background noise from the affected cells, leading to errors in manual data analysis. By contrast, the model-based analysis mitigates these issues by removing noise from aggregated parts of the image and reducing interference between images, which improves accuracy and accounts for the slight coefficient differences.

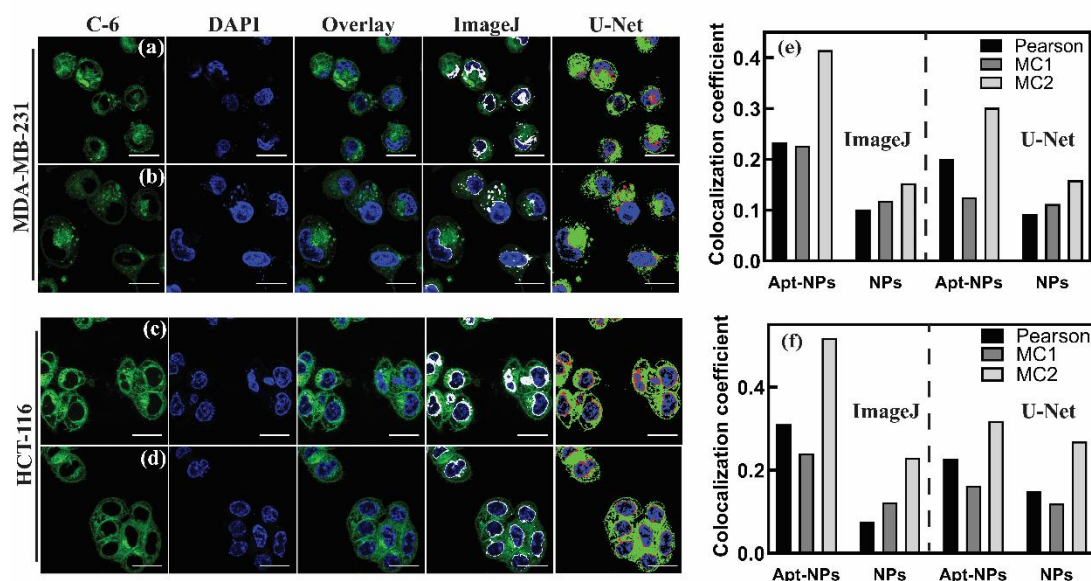


**Figure 4.15.** Confocal images of colocalisation between the nanoparticles and cell nuclear after 2 hours of incubation. (a) MDA-MB-231 cells treated with AS1411 modified nanoparticles, (b) MDA-MB-231 cells treated with pure nanoparticles, (c) HCT-116 cells treated with AS1411 modified nanoparticles, (d) HCT-116 cells treated with pure nanoparticles, Pearson and Manders coefficient analysed by using ImageJ and U-Net model for HCT-116 (e) and MDA-MB-231 (f). Scale bar, 20 $\mu$ m.

#### 4.2.4. Evaluation of Cas9 delivery to the nucleus by using nanoparticles

After confirmation of the nuclear-targeting capability of our nanoparticles, we further evaluated their ability to deliver Cas9 to the nucleus in the same cell lines. As shown in **Figure 4.16 a and b**, the colocalisation area (white region) between Cas9 and the nucleus was significantly increased in HCT-116 cells treated with targeted nanoparticles compared to those treated with non-targeted nanoparticles. ImageJ analysis indicated a notable increase in the PC (+0.36 vs. +0.24). A similar trend was observed in MDA-MB-231 cells (**Figure 4.16 c and d**), with an enhanced colocalisation area and PC (+0.031 vs. +0.021). Additionally, colocalisation analysis was performed using a CNN deep learning model. Similar to the results in **Figure 4.15**, the AS1411-modified nanoparticles caused increased noise in the cells, which resulted in a decrease in both the Pearson and Mander coefficients. Nevertheless, the computational analysis confirmed that the aptamer-modified nanoparticles exhibited more effective accumulation within the cell nucleus compared to the unmodified nanomaterials.

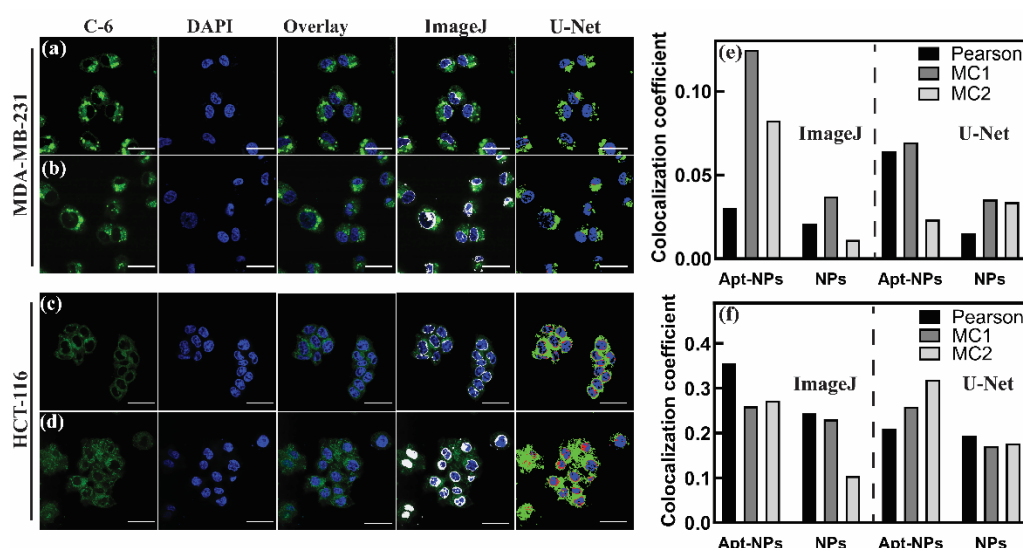


**Figure 4.16.** Confocal images of HCT-116 and MDA-MB-231 cells after the treatment with (a, c) pure nanoparticles and (b, d) aptamer-modified nanoparticles loaded Cas9-GFP; Pearson

*and Manders coefficient analysed by using ImageJ and U-Net model for HCT-116 (e) and MDA-MB-231 (f). Scale bar, 20 $\mu$ m.*

#### **4.2.5. Intracellular and Nuclear Uptake Assessed by 3D Confocal Imaging**

To assess the intracellular distribution and nuclear targeting capability of the functionalized nanoparticles, 3D confocal microscopy was performed following treatment of MDA-MB-231 cells with C-6 nanoparticles. Z-stack images were acquired using a Nikon A1R confocal microscope with a 100 $\times$  oil immersion objective and reconstructed using NIS-Elements AR software and ImageJ to visualize spatial localization.

In the non-targeted group (**Figure 4.17**), fluorescence signals corresponding to both C-6 (green) were primarily confined to the cytoplasm, indicating that nanoparticles were successfully internalized but remained largely excluded from the nucleus. In contrast, the nanoparticle modified with AS1411 aptamers demonstrated a markedly different distribution pattern (**Figure 4.18**). Reconstructed 3D images revealed co-localization of C-6 fluorescence with DAPI-stained nuclei, suggesting effective translocation of the payload across the nuclear envelope. Quantitative analysis of fluorescence intensity within defined nuclear volumes showed a significantly higher nuclear signal in the AS1411-targeted group compared to controls, confirming enhanced nuclear delivery. These results highlight the utility of aptamer-mediated targeting and support the functionality of the hybrid nanoparticle design in achieving subcellular-level delivery precision.

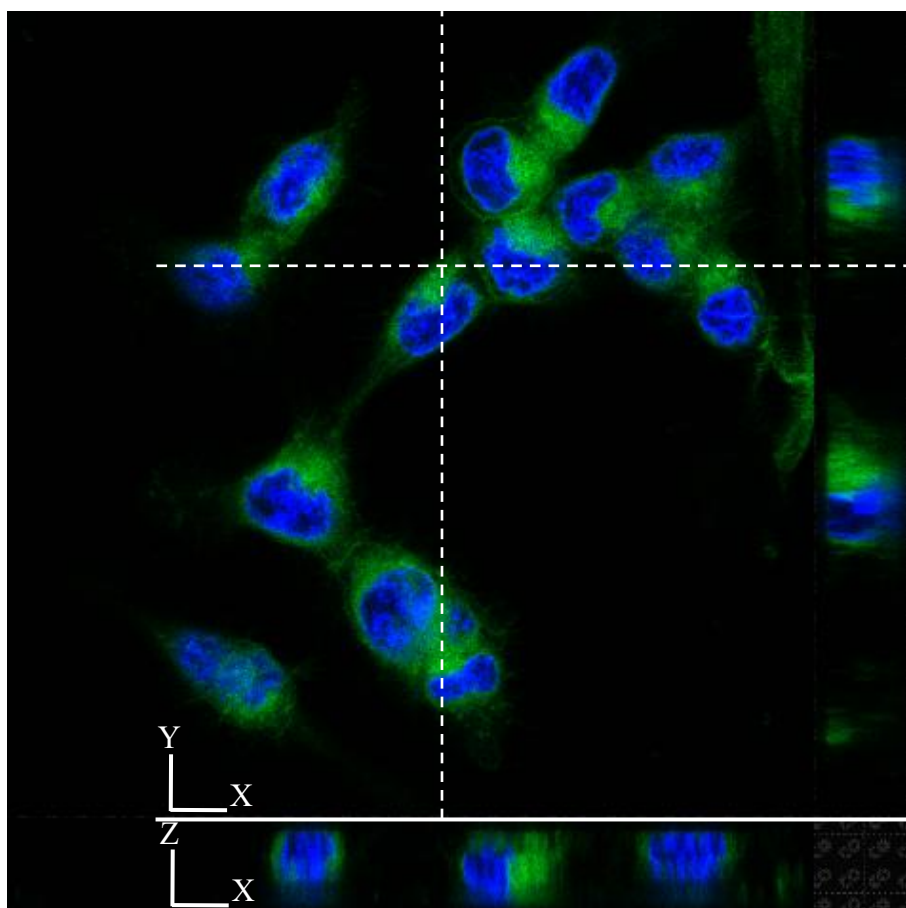


**Figure 4.17.** The 3D confocal images of colocalization between the nanoparticle loaded with C-6 (green channel) and cell nuclear stained by DAPI (blue channel) after 2 hours of incubation. Scale bar, 10  $\mu\text{m}$ .

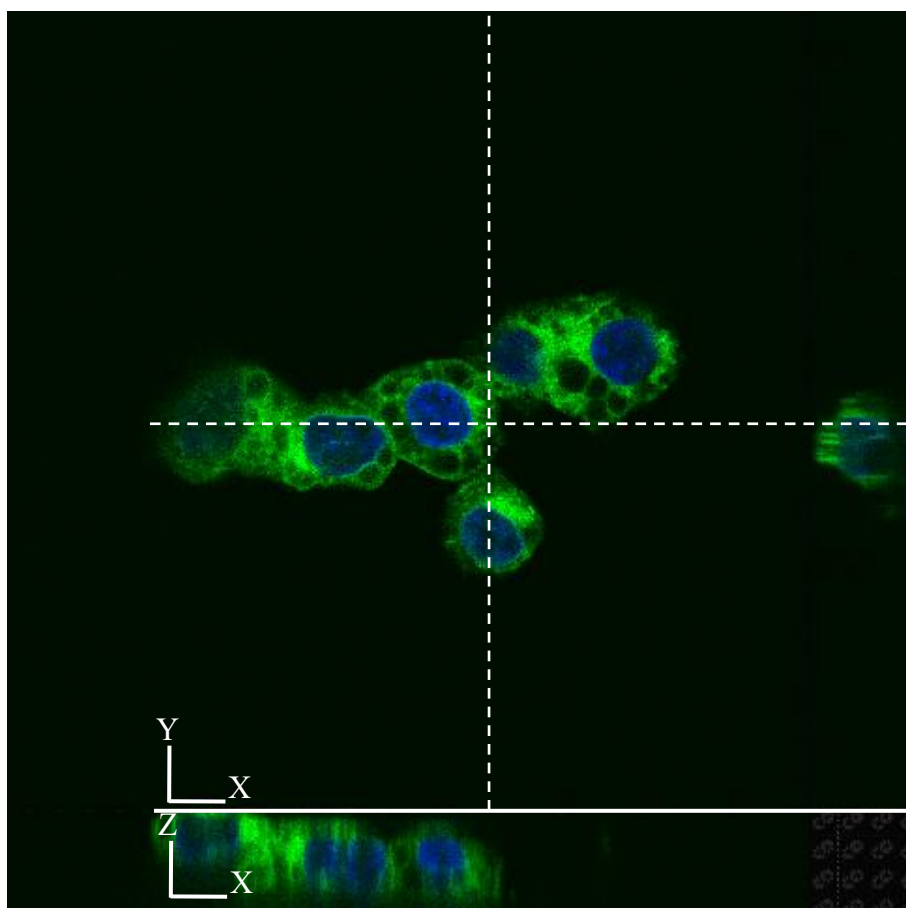


**Figure 4.18.** The 3D confocal images of colocalization between the AS1411 modified nanoparticle loaded with C-6 (green channel) and cell nuclear stained by DAPI (blue channel) after 2 hours of incubation. Scale bar, 10  $\mu\text{m}$ .

To further assess the selectivity of nuclear targeting, we evaluated nanoparticle uptake in non-cancerous human colon epithelial cells (CCD 841 CoN). As shown in **Figure 4.19 a and b**, while the nanoparticles were internalized by CCD 841 CoN cells, the fluorescence intensity was markedly reduced compared to that observed in cancer cells. Furthermore, no discernible nuclear localization was detected in the normal cell line, suggesting limited nuclear transport in non-malignant cells. This contrasted with the clear nuclear targeting effect observed in cancer cell lines. The lack of nuclear accumulation in normal cells is likely attributable to the low expression of membrane-associated nucleolin, the molecular target of AS1411. Unlike cancer cells, which often overexpress nucleolin on the cell surface to support their high proliferation rate, normal epithelial cells exhibit limited nucleolin availability, thereby reducing the efficacy of aptamer-mediated nuclear delivery.

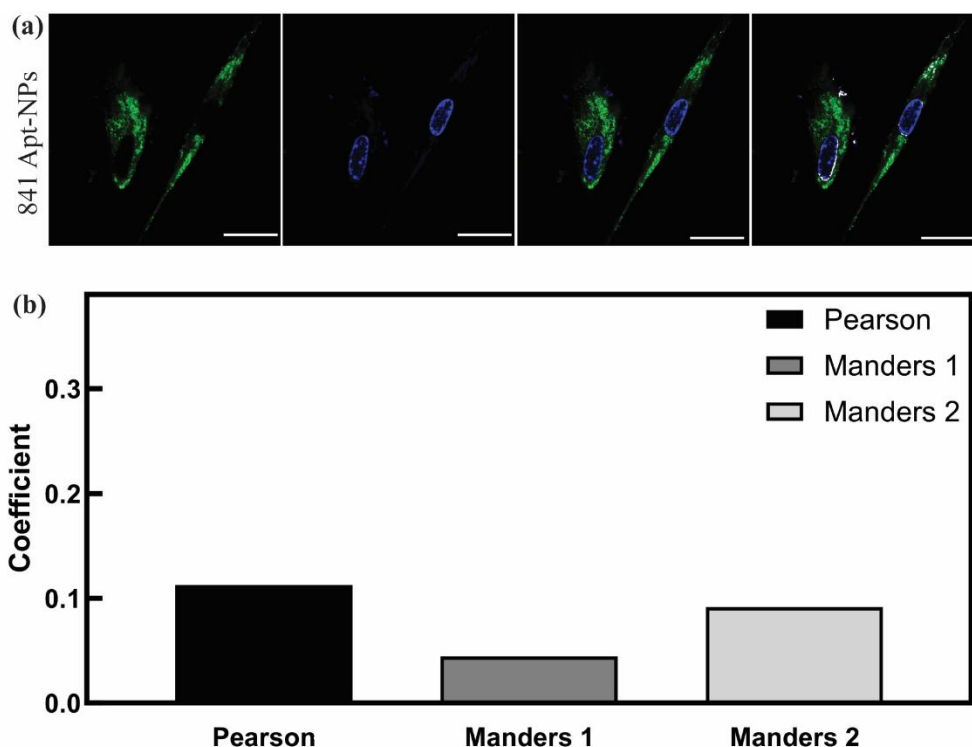


**Figure 4.19.** Colocalization analysis of CCD-841 CoN normal cells after the treatment. (a) Confocal images of CCD-841 CoN cells after the treatment with aptamer-modified nanoparticles loaded Cas9-GFP; (b) Pearson and Manders coefficient analysed by using ImageJ. Scale bar, 20μm.

### 4.3. Conclusions

In this chapter, the prepared nuclear-targeted nanoparticles were characterised at the cellular level. Systematic characterization, including dynamic light scattering and zeta potential analysis, confirmed the size, stability and successful surface modification. Cellular uptake studies demonstrated efficient internalization of the nanoparticles, with significantly enhanced uptake observed in the AS1411-functionalized group. Furthermore, both 2D and 3D confocal imaging revealed effective nuclear delivery of Cas9-GFP in targeted formulations, validating the role of aptamer-mediated delivery in achieving subcellular specificity. Collectively, these findings validate the use of nucleus-targeted nanoparticle systems and provide a solid foundation for subsequent functional genome editing studies discussed in the following chapter.

## Chapter 5: Nuclear-targeted delivery of CRISPR/Cas9

### 5.1. Introduction

Advancements in gene editing technologies have facilitated precise genetic modifications, offering significant therapeutic potential for a wide range of genetic diseases and cancers. Among these tools, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), which was awarded the 2020 Nobel Prize in Chemistry, has emerged as a groundbreaking innovation in revolutionising biotechnology and biomedical research through its unprecedented ability to control over gene activity in nearly any organism [1-3]. CRISPR machinery can be delivered as RNA, plasmid DNA and Cas protein [4]. CRISPR/Cas9 format relies on two critical components: a Cas endonuclease and a gene-targeting guide RNA (gRNA). It targets the genomic DNA of cells which is a linear double-helix molecule primarily stored in the nucleus of eukaryotic cells [5]. The CRISPR/Cas9 mechanism works by using the Cas nuclease to precisely cut DNA at specific sites, allowing the cell's natural DNA repair pathways to make the required modifications [6, 7]. Since this gene editing and the subsequent DNA repair processes primarily occur within the nucleus, the effective nuclear delivery of CRISPR/Cas9 is crucial for precise and efficient gene modification.

CRISPR/Cas9 delivery to the nucleus of live cells can be facilitated through viral vector systems [14, 84]. This is because viral vectors possess natural mechanisms for nuclear entry. For example, they exploit the host cell's transport proteins, such as importins and exportins, to facilitate their movement into the nucleus. These transport proteins recognise nuclear localization signals associated with viral components, enabling efficient nuclear delivery [16, 17]. However, the use of viral vectors raises significant concerns, including the risk of inducing immune responses, tissue inflammation and vector leakage [18, 359]. Nanoparticles have been explored as alternative carriers, providing a precise and safe tools of delivering CRISPR/Cas9 to target cells [20]. Various nanocarriers, including lipid nanoparticles, polymers and exosomes, have been explored for CRISPR/Cas9 delivery. Compared to viral vectors, nanocarriers present advantages such as lower immunogenicity, greater packaging capacity and an improved safety profile [21, 22]. However, nanoparticles often face challenges in achieving effective nuclear targeting. After being internalized, they must overcome the difficulty of escaping endo/lysosomes [360, 361]. Moreover, they generally lack the ability to interact with the

nuclear pore complex, which restricts the transport of large molecules or complexes into the nucleus [362-364].

To overcome this challenge, researchers have focused on active targeting strategies by surface modification of nanocarriers with nuclear targeting peptides and aptamers [365]. These targeting molecules have binding affinities for nuclear transport receptors that located in the cell nucleus. By attaching these molecules to the surface of nanocarriers, they can effectively deliver the gene editing components into the nucleus for precise therapy. A well-known nucleus-targeting aptamer is AS1411, a G-quadruplex DNA aptamer that binds to nucleolin, a nuclear protein that shuttles between the cell membrane, cytoplasm and nucleus [358]. Due to its high affinity for nucleolin, AS1411 facilitates the internalization of the nucleocytoplasmic shuttle, delivering it to the cell nucleus [358]. Furthermore, nucleolin expression is upregulated in cancer cells, making AS1411 particularly advantageous for cancer treatment [366]. This aptamer has been extensively studied and utilized in nuclear-targeted nanocarriers for the transport of various cargos, including chemodrugs [367, 368] and detection probes [369]. However, there were few studies exploring its potential for nuclear-targeted CRISPR/Cas9 delivery.

In this chapter, we mainly focus on the evaluation of gene editing efficiency after CRISPR/Cas9 delivery using AS1411-functionalized polymer–lipid hybrid nanoparticles in cancer cell lines, which were highly related to Aim 3. Specifically, we assess the *in vitro* gene editing performance and cellular consequences following the delivery of CRISPR/Cas9 complexes targeting two genes: green fluorescent protein (GFP) and lipocalin-2 (Lcn-2). The first part of this chapter presents the evaluation of GFP gene knockdown efficacy in GFP-expressing cell lines, serving as a model system to quantify the editing efficiency of the delivery platform through fluorescence microscopy and indel analysis. The second part focuses on the knockdown of Lcn-2, an oncogenic marker implicated in cancer progression and metastasis [370, 371]. CRISPR/Cas9 mediated knockdown of the Lcn-2 gene was performed in MDA-MB-231 cells, and editing efficiency was quantified using RT-qPCR and T7 endonuclease I (T7EI). Finally, we investigate the cell response and biological effects following Lcn-2 gene knockdown, including cell migration, proliferation, and wound healing, to explore the impact of Lcn-2 knockdown on cellular response.

## 5.2. Result and Discussion

### 5.2.1. Assessment of *in vitro* GFP gene knockdown efficacy of CRISPR/Cas9 delivered by targeted nanoparticles

To determine the optimal incubation period for assessing gene knockout efficiency, Cas9/gRNA-loaded nanoparticles were applied to GFP-expressing cells and incubated for either 48- or 72-hours post-treatment. As shown in **Figure 5.1**, GFP fluorescence intensity declined in both nanoparticle (NPs) and aptamer-functionalized nanoparticle (Apt-NPs) groups. Quantitative results are summarised in **Table 5.1**. At 48 hours, fluorescence intensity decreased from 17.70 (control) to 15.60 in the NPs group and 10.39 in the Apt-NPs group, corresponding to knockout efficiencies of 11.86% and 41.29%, respectively. At 72 hours, the difference became more pronounced, with GFP intensity declining from 27.85 (control) to 12.19 and 4.22 in the NPs and Apt-NPs groups, respectively, yielding knockout efficiencies of 56.24% and 84.84%. These results indicate that extended incubation enhances editing outcomes and that aptamer-mediated delivery further improves gene disruption. Based on these findings, a 72-hour post-treatment incubation period was selected for all subsequent knockout analyses.

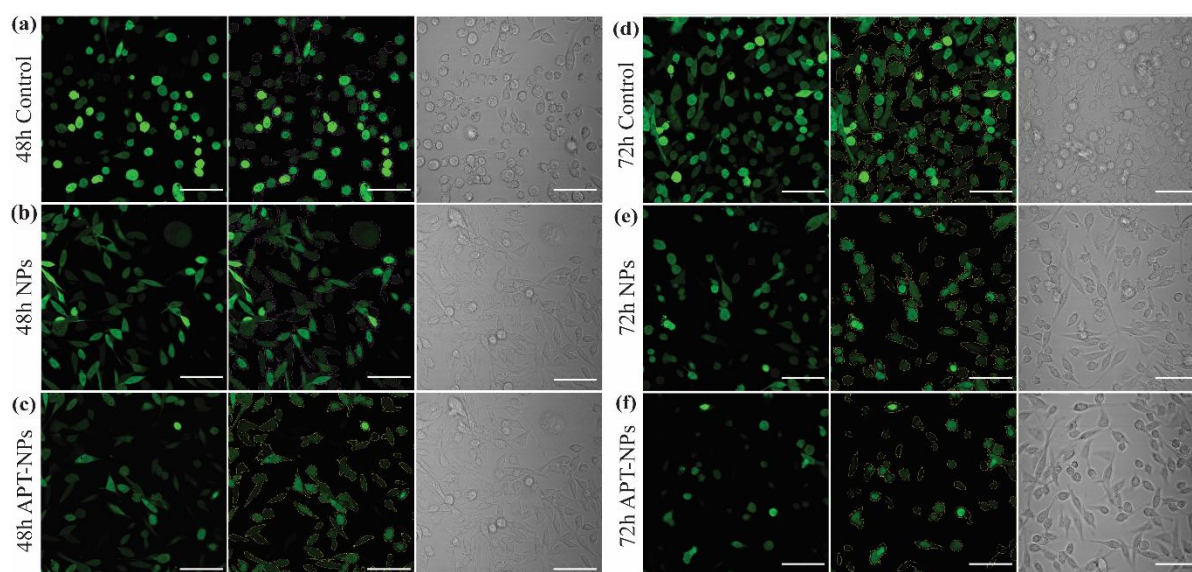


**Figure 5.1.** GFP fluorescence knockout analysis by confocal microscopy following nanoparticle treatment of MDA-MB-231-GFP cells for 48 and 72 hours of incubation. Scale bar, 50  $\mu$ m.

**Table 5.1.** GFP fluorescence intensity analysis following nanoparticle treatment of MDA-MB-231-GFP cells for 48- and 72-hour incubation.

|     | Control | NPs    | Apt-NPs | NPs knockout percentage | APT knockout percentage |
|-----|---------|--------|---------|-------------------------|-------------------------|
| 48h | 17.695  | 15.596 | 10.388  | 11.86%                  | 41.29%                  |
| 72h | 27.854  | 12.189 | 4.223   | 56.24%                  | 84.84%                  |

We first assessed the transfection efficiency of our nanoparticles targeting GFP expressed by MDA-MB-231 cells (MDA-MB-231-GFP). As shown in **Figure 5.2a**, there was a noticeable decrease in GFP fluorescence intensity in cells treated with 100  $\mu\text{g/ml}$  of nanoparticles loaded with CRISPR/Cas9 editing agents for 72 hours, compared to the control group. Flow cytometry data further confirmed these reductions. The GFP expression profile showed that 70.8% of the cells were GFP positive in the control group. In contrast, GFP expression decreased to 40.8% in cells treated with non-targeted nanoparticles and 30% in cells treated with aptamer-modified nanoparticles at 72 hours post-transfection (**Figure 5.2b**), indicating that the nuclear-targeted nanocarriers further enhance the GFP knockdown effect of CRISPR/Cas9 in cells.

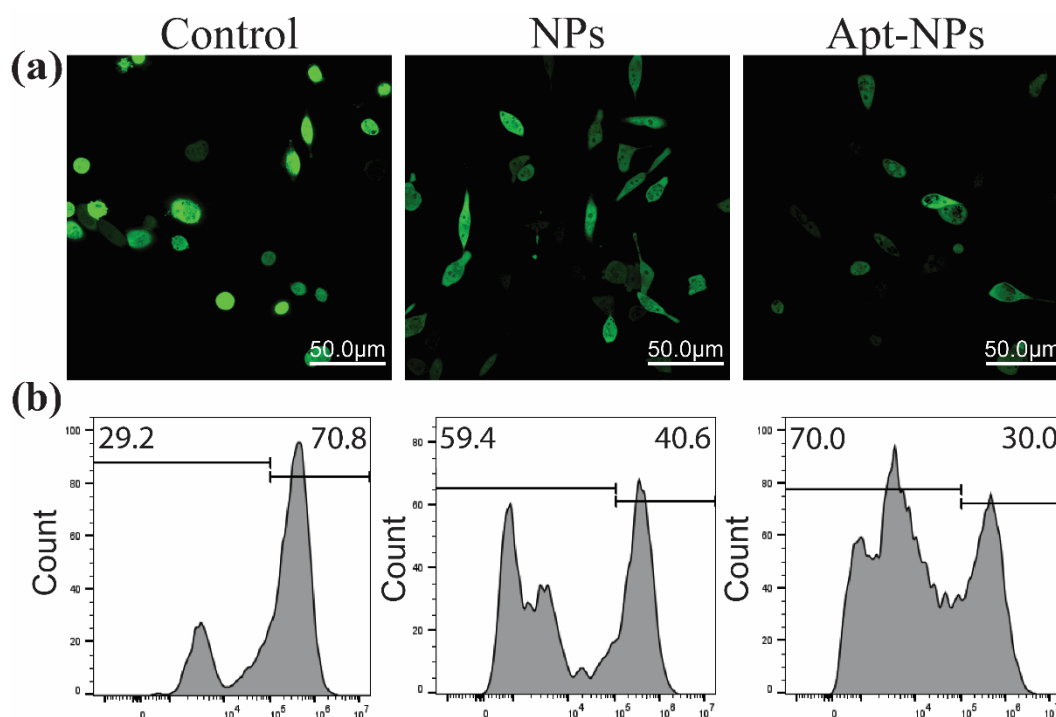


**Figure 5.2.** GFP fluorescence intensity analysis following nanoparticle treatment of MDA-MB-231-GFP cells. (a) Representative confocal images of cells after different treatment conditions indicated in the figure, (b) Percentage of GFP-positive cells quantified by flow cytometry. The X- and Y-axes indicate GFP fluorescence intensity and cell counts, respectively.

### 5.2.2. Assessment on *in vitro* Lcn2 gene knockdown efficacy

The Lcn2 gene plays a critical role in tumour development. It encodes a secreted glycoprotein that regulates intracellular iron homeostasis, supporting metabolic activity and enhancing the survival of cancer cells [370]. Furthermore, Lcn2 is closely linked to epithelial-mesenchymal transition (EMT) in cancer cells, with elevated expression promoting the cellular migration and invasion [370]. Therefore, targeting Lcn2 using CRISPR technology may offer a promising therapeutic strategy for the treatment of certain cancers. To evaluate the gene knockdown efficacy of our targeted nanoparticles in MDA-MB-231 cells, we first performed the T7EI assay to confirm CRISPR/Cas9 mediated gene editing at the Lcn2 locus. As shown in **Figure 5.3a**, the presence of cleavage fragments confirmed successful disruption at the Lcn2 gene locus. Quantitative analysis revealed a mutation efficiency (indel%) of 25.5% achieved with Cas9/sgRNA delivered by non-targeted nanoparticles. In contrast aptamer-functionalised nanoparticles produced a more prominent mismatch-induced cleavage band, indicating a mutation efficiency of 31.1%.

We further assessed Lcn2 mRNA expression levels under different treatment conditions. As shown in **Figure 5.3b** and **Table 5.2**, both non-targeted and targeted nanoparticle formulations led to a significant reduction in mRNA expression levels compared to the untreated control. Particularly the aptamer-functionalised nanoparticles exhibited higher knockdown efficiency, with approximately a 60% reduction in Lcn2 expression, compared to 40% observed with the non-targeted formulation. Taken together, these findings suggest that nuclear-targeted nanoparticles facilitate more efficient delivery of the CRISPR/Cas9 system into the cell nucleus, thereby enhancing gene knockdown efficiency and effectively reducing Lcn2 expression.

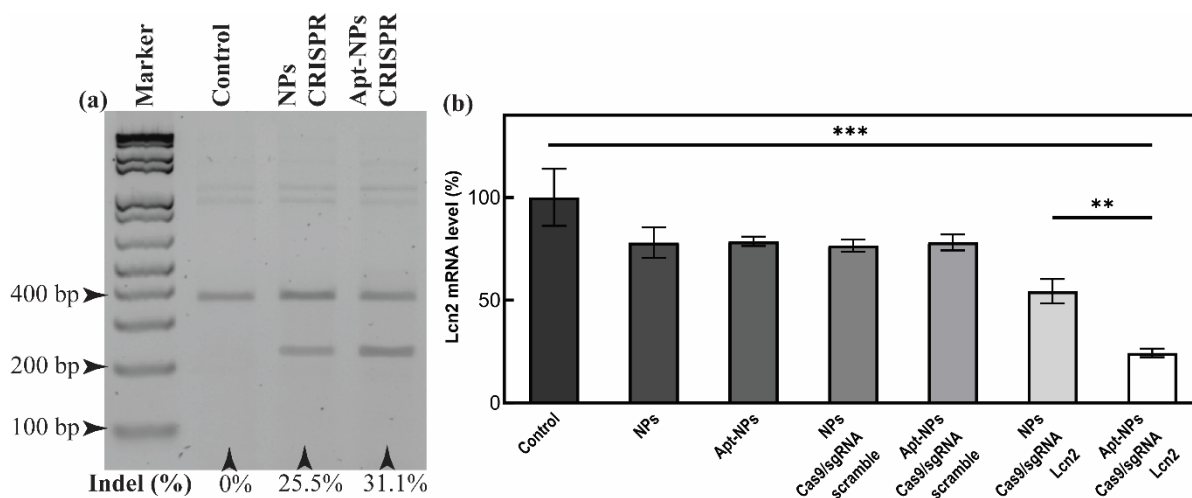


**Figure 5.3.** *In vitro* Lcn2 gene knockdown via the CRISPR/Cas9 system. (a) Agarose gel following T7EI assay. Expected PCR products and T7EI cleaved products are indicated. (b) Lcn2 mRNA expression level by RT-qPCR. All assays were independently repeated three times. Data represent mean  $\pm$  SD, \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

**Table 5.2.** The RNA expression by RT-qPCR.

|                           | Lcn2   | GAPDH       | dCt           | ddCT   | RQ           |       | Fold change        |
|---------------------------|--------|-------------|---------------|--------|--------------|-------|--------------------|
| <b>MDA-MB-231 control</b> | 27.001 | 12.680      | 14.321        | 0.180  | 0.883        | 0.877 |                    |
|                           | 26.814 | 12.642      | 14.172        | 0.031  | 0.979        | 0.973 |                    |
|                           | 26.531 | 12.601      | 13.930        | -0.211 | 1.157        | 1.150 |                    |
|                           |        | <b>Mean</b> | <b>14.141</b> |        | <b>1.006</b> |       | <b>1</b>           |
|                           |        |             |               |        |              |       |                    |
| <b>NPs treat</b>          | 27.349 | 12.424      | 14.925        | 0.784  | 0.581        | 0.577 |                    |
|                           | 27.661 | 12.455      | 15.206        | 1.065  | 0.478        | 0.475 |                    |
|                           | 27.405 | 12.481      | 14.923        | 0.782  | 0.581        | 0.578 |                    |
|                           |        |             |               |        |              |       | <b>0.543278402</b> |
|                           |        |             |               |        |              |       |                    |
| <b>NPs control</b>        | 27.532 | 12.961      | 14.572        | 0.431  | 0.742        | 0.737 |                    |
|                           | 27.559 | 12.987      | 14.572        | 0.431  | 0.742        | 0.737 |                    |
|                           | 27.329 | 12.989      | 14.341        | 0.200  | 0.871        | 0.865 |                    |
|                           |        |             |               |        |              |       | <b>0.779933766</b> |
|                           |        |             |               |        |              |       |                    |
| <b>APT-NPs treat</b>      | 29.087 | 12.918      | 16.168        | 2.027  | 0.245        | 0.244 |                    |
|                           | 28.936 | 12.878      | 16.058        | 1.917  | 0.265        | 0.263 |                    |
|                           | 29.374 | 13.068      | 16.305        | 2.164  | 0.223        | 0.222 |                    |
|                           |        |             |               |        |              |       | <b>0.24286836</b>  |

### 5.2.3. Investigation of cellular response after the Lcn2 gene knockdown

To investigate the cellular response following Lcn2 gene knockdown, wound healing, proliferation and transwell assays were conducted. As shown in **Figure 5.4a**, the cells treated with aptamer-modified nanoparticles exhibited the delayed wound closure compared to the non-targeted and control groups, indicating a strong inhibition of cell migration. Quantitative analysis (**Figure 5.4b**) confirmed that cells treated with aptamer-modified CRISPR/Cas9

nanoparticles achieved only 35.5% wound closure within 48 hours, whereas non-modified CRISPR/Cas9 nanoparticles resulted in approximately 60% closure. Similarly, the proliferation assay showed that aptamer-modified nanoparticles significantly reduced cell proliferation compared to the control groups (**Figure 5.4c**). In addition, Lcn2 knockdown impaired cell migration, with a relative migration rate of 64.3% in the group treated with targeted nanoparticles-substantially lower than the 98.5% observed in the control group (**Figure 5.4d**). These findings indicate that CRISPR/Cas9 delivered via our targeted nanoparticles achieved effective in vitro gene knockdown, highlighting their potential for improved in vivo gene editing and better tumour response.

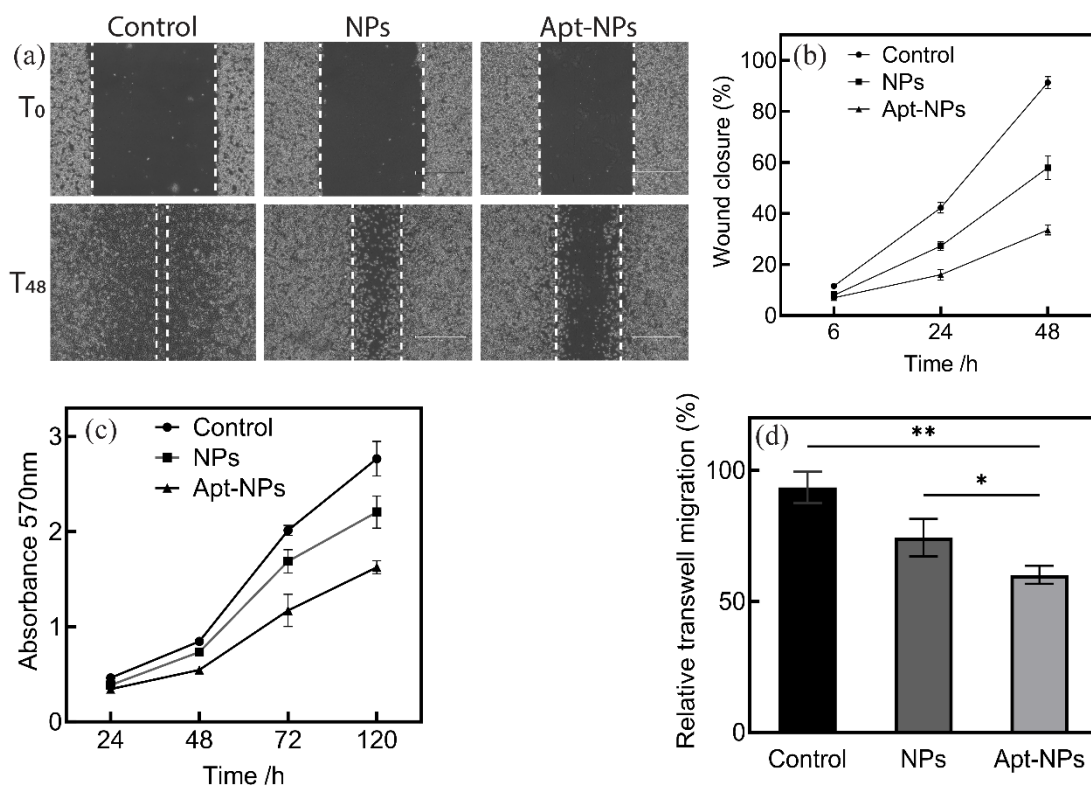


**Figure 5.4.** Evaluation of cellular responses after Lcn2 gene knockdown in MDA-MB-231 cells. (a) Representative cell images at 0 and 48 hours after wound creation. (b) Quantification of wound closure rate over time, expressed as the percentage of wound area covered by cells at each indicated time point. (c) Cell proliferation analysis at 24, 48, 72 and 120 hours. (d) Cell migration at 24 hours after the treatment of non-targeted and targeted nanoparticles loaded with CRISPR. All assays were independently repeated three times. Data represent mean  $\pm$  SD, \* $p$  < 0.05; \*\* $p$  < 0.01. Scale bar, 500  $\mu$ m.

### **5.3. Conclusion**

In summary, this study demonstrates that polymer–lipid hybrid nanoparticles functionalised with the nucleolin-targeting aptamer AS1411 enable efficient nuclear delivery of CRISPR/Cas9, resulting in enhanced gene editing efficiency compared to non-targeted systems. The targeted nanocarriers effectively knocked down both GFP and Lcn2 genes in vitro and achieved improved inhibition of breast cancer cell proliferation and migration compared to non-targeted counterparts. These findings indicate that aptamer-mediated nuclear targeting has the potential to enhance CRISPR knockdown efficacy by facilitating its delivery into the nucleus, thereby improving gene editing outcomes and providing a strong foundation for advancing nuclear targeting strategies for in vivo gene therapy.

## Chapter 6: Conclusions

My thesis has developed a nucleus-targeted CRISPR/Cas9 delivery platform based on polymer–lipid hybrid nanoparticles functionalised with the aptamer AS1411. The nanoparticle carrier (PLGA–lecithin–DSPE-PEG-COOH) was rationally engineered to combine a biodegradable polymer core for structural integrity with a lipid-PEG shell for biocompatibility and functionalization. Through carbodiimide chemistry, AS1411 aptamers were conjugated onto the particle surface, enabling nuclear targeting capability. Comprehensive physicochemical characterisation confirmed that the formulated nanoparticles exhibited a narrow size distribution indicative of favourable stability under a cell environment, and a slightly negative surface charge can reduce nonspecific protein adsorption and enhance colloidal dispersion. Moreover, UV-visible spectroscopy revealed a distinct shift in the characteristic absorption peak following aptamer conjugation, providing strong evidence for successful surface functionalization. These findings collectively demonstrate that the nanoparticles possess well-defined physical properties and stable surface chemistry, which are essential for ensuring reproducible behaviour in biological environments and effective targeted delivery.

We developed an AS1411 aptamer-conjugated polymer-lipid hybrid nanoparticle system, with physicochemical characterisation confirming the integrity of the targeted nanocarriers. Aptamer modification led to an initial increase in particle size; subsequent loading of the Cas9/sgRNA ribonucleoprotein further expanded the hydrodynamic diameter to over 200 nm and shifted the surface zeta potential toward neutrality (Table 4.1). These findings are consistent with previous studies of AS1411-functionalised nanocarriers [367, 368]. Because the targeted and non-targeted formulations showed comparable cargo loading efficiency (Table 4.1), the higher nuclear accumulation observed in the confocal images suggests that AS1411 primarily facilitates the intracellular trafficking and nuclear localization of the nanoparticles (Figure 4.15 and Figure 4.16). These findings indicate that the enhanced performance of the system is driven by improved subcellular transport rather than differences in initial cargo encapsulation. In both MDA-MB-231 and HCT-116 cells, AS1411-modified nanoparticles showed greater nucleus-associated colocalization than the non-targeted nanoparticles, as reflected by increased Pearson's and Mander's coefficients (Figure 4.15 and Figure 4.16). To validate the accuracy of our ImageJ-based analysis, we implemented a deep learning model utilising a U-Net architecture [354]. This supervised approach is widely recognized for its high utility in medical image segmentation and its ability to precisely identify specific regions within

complex microscopy datasets [355]. It employs libraries NumPy and OpenCV for image processing, and TensorFlow for defining and training the model [356, 357]. Although the coefficients generated by the deep learning model were slightly lower than those obtained via ImageJ, the overall experimental trend remained consistent across both methods (Figure 4.15 and Figure 4.16). This cross-validation indicates that increase in nuclear localization is a direct result of aptamer-mediated targeting, independent of the specific image-analysis method.

Crucially, improved nuclear accumulation correlated with enhanced gene knockout efficacy. In the GFP reporter cell model, the targeted nanoparticles reduced the proportion of GFP-positive cells more effectively than the non-targeted formulation (Figure 5.2). This trend was further validated at the endogenous *Lcn2* locus, where AS1411-functionalized nanoparticles yielded higher indel formation and a more pronounced reduction in *Lcn2* mRNA expression (Figure 5.3). These findings align with the nuclear-targeting capabilities observed previously (Figures 4.15 and 4.16). The biological relevance of this improvement is further evidenced by the potent *in vitro* therapeutic effects following *Lcn2* gene knockout. Specifically, the enhanced suppression of the *Lcn2* gene led to a significant inhibition of cellular proliferation, wound closure and transwell migration in MDA-MB-231 cells (Figure 5.4).

These results collectively demonstrate that AS1411-mediated nuclear targeting improves intracellular trafficking, intranuclear accumulation, and functional CRISPR/Cas9 activity in cancer cells, thereby translating enhanced subcellular delivery into measurable anti-tumour effects *in vitro*. The preferential interaction of AS1411 with nucleolin-overexpressing cancer cells also supports the selectivity of this strategy and highlights its potential value for targeted gene editing in precision oncology.

Despite the promising results demonstrated in this study, several limitations remain that warrant further investigation. Although the *in vitro* experiments demonstrated enhanced nuclear accumulation and gene editing efficiency, the applicability of this platform *in vivo* has not yet been assessed. Factors such as systemic circulation, immune clearance, and the complexity of the tumour microenvironment may significantly influence the pharmacokinetics and targeting specificity of the nanoparticles [105]. Additionally, this study employed a single nuclear-targeting aptamer, AS1411, which binds nucleolin. Although most tumours have been reported to exhibit elevated nucleolin expression, comprehensive profiling across different tumour subtypes remains incomplete [372]. Consequently, the universality of AS1411-mediated nuclear targeting requires further validation by testing it on various cancer cells, therefore the

targeting strategy may require adaptation or expansion to ensure broader applicability. Additionally, endosomal escape remains a critical barrier for efficient intracellular delivery, and while our carrier system showed promising internalization and nuclear localization, we did not quantitatively assess endo/lysosomal escape efficiency, which could decrease the efficiency of targeting and editing.

Looking ahead, several critical research directions will advance this nucleus-targeted CRISPR delivery platform toward clinical application. First, in vivo studies using tumour-bearing animal models are essential to validate nuclear localization, gene editing efficacy and biosafety within a complex biological environment. Second, enhancing tumour selectivity through dual-ligand strategies, such as combining AS1411 with peptides or antibodies targeting tumour-specific receptors, could further improve therapeutic precision. Third, optimising on-target editing efficiency remains a priority, such as analysis incorporating endosomal escape mechanisms to improve intracellular trafficking. Together, these efforts will ensure that this targeted nanoparticle system can evolve from a promising in vitro tool into a viable gene therapy platform for precision oncology.

## Reference

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