INTRACELLULAR DELIVERY OF CRISPR/CAS9 PLASMID WITH ZIF-8 NANOCARRIER TO LIVER CANCER CELLS

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Hepatocellular carcinomas are the fifth most common cancers with poor prognosis, inefficient drug therapy, and severe side effects. C-Myc regulates up to 20 % of all human genes. C-Myc overexpression is involved in 70 % of liver cancers. Downregulating the c-Myc expression may provide a potential therapeutic alternative for future liver cancer treatments.

CRISPR/Cas9 method enables simple, stable, and inexpensive gene editing. Zif-8 biomineralization has been utilized to encapsulate CRISPR/Cas9 edited plasmid due to its stable structure, fast and easy assembling in room temperature aqueous solution, and flexibility in payload sizes since they can directly grow a nanolayer protection shell on top of the payload. Zif-8 enables pH-mediated cargo release, which can promote endosomal escape. The aims are to deliver the CRISPR/Cas9 system to knockout c-Myc genes in HCC cells with Zif-8 nanocarrier, monitor endosomal escape, analyze the downregulated c-Myc expression, and investigate the treatment effects on cancer cells.

As a result, the CRISPR/Cas9 plasmid has been successfully and efficiently delivered into HCC cells; high ratio endosomal escape after 4 hours of transfection, whereas nuclei targeting was observed at 6 hours. The transfected HCC cells with c-Myc knockout plasmid result in lower c-Myc expression than wild-type HCC cells. Results indicate the potential of intracellular c-Myc knockout with Zif-8-mediated CRISPR/Cas9 plasmid delivery in HCC cells.

Although the c-Myc expression downregulation utilization has not yet proceeded into clinical trials, it is a potential therapy for future liver cancers. The delivery method must become more accurate and suitable for further *in vivo* experiments.

KEYWORDS: CRISPR/Cas9, c-Myc, gene editing, hepatocellular carcinoma, Zif-8

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Table of abbreviations

Cas9	CRISPR-associated protein 9 nucleases
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
Cy5.5	Cyanine5.5 NHS ester
c-Myc	Cellular Myc
DMEM	Dulbecco´s Modified Eagle´s Medium
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescence Protein
FBS	Fetal Bovine Serum
HBSS	Hank`s Balanced Salt Solution
HCC	Hepatocellular carcinoma
HDR	Homologous directed repair
HepG2	Human liver cancer cells from hepatocellular carcinoma
HEPES	N-2-hydroxyethylpipeperazinr-N´-2-ethanesulfonic acid
HNEJ	Non-homologous end joining
Huh-7	Human cellular carcinoma cells
KO	Knock-out (modified by inactivating or removing specific gene)
MOFs	Metal-Organic frameworks
NEAA	MEM Non-Essential Amino Acids solution
PAM	Protospacer Adjacent Motifs
PBS	Phosphate Buffered Saline
sgRNA	single guide RNA
TALEN	Transcription-like effector nuclease
TEM	Transmission electron microscopy
tracrRNA	Trans-acting CRISPR RNA
Zif-8	Zeolitic imidazolate framework-8
ZNF	Zinc finger nuclease

1 REVIEW OF THE LITERATURE

1.1 LIVER CANCER

Liver cancers are ranked globally as the most frequent fatal malignancy with poor prognosis (Anwanwan et al. 2019). Malignant tumors in liver cancers are categorized as hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, or mixed types (Lv et al. 2021). Over 90 % of liver cancers are hepatocellular carcinomas, whose current treatment is based on chemotherapy and immunotherapy (Anwanwan et al. 2019). Hepatocellular carcinomas are ranked the fifth most common cancer (Asafo-Agyei & Samant 2023) and the fourth of the most common cancer-related deaths (Llovet et al. 2021). In HCC, hepatocytes form malignant changes leading to tumors (Asafo-Agyei & Samant 2023). HCCs are thrice more common among males than females. Simultaneously, the number of patients with liver cancer is increasing worldwide as the number of people with risk factors, such as obesity, diabetes mellitus, hepatitis virus B and hepatitis virus C. Risk of getting HCC increases during aging (Llovet et al. 2021). The prognosis is size-dependent on the tumor, differentiation, grade of the tumor, metastases presence, and tumor extension. Over 50 % of advanced-stage HCC patients have abdominal pain, weight loss, or liver dysfunction (Llovet et al. 2021).

The treatment alternatives are narrow for the advanced stages of HCC nowadays (Anwanwan et al. 2019). The more advanced stages of HCC have challenges with the current therapeutics (Lin et al. 2016). Most patients with advanced HCC are treated with palliative or local treatment. Although there have been developed multiple mono-targeted therapies, they have not managed to control HCC due to the heterogeneity of the tumor of complex cell signaling. For less advanced stages of HCC, the treatment alternatives include hepatic resection and liver transplantations (Llovet et al. 2021). The current therapy alternative for the intermediate stage of HCC is based on intra-arterial therapy (Roddy et al. 2022). The single-drug therapies for advanced-stage HCC are orally taken sorafenib and lenvatinib. However, more than two-thirds of HCC patients are unable to benefit from long-term

treatment due to toxicity, drug inefficacy, or resistance within half a year (Anwanwan et al. 2019). Drug resistance has become one of the major challenges in the therapy of cancers (Yao et al. 2020). The outcome of drug resistance may lead to cancer progression and poor prognosis. Notably inefficient therapy remains the major reason for poor prognosis and low survival rate (Lin et al. 2016). Monotargeted therapy provides low efficacy whereas multi-targeted agents have their limitations in long-period therapy. The current multi-targeted agents such as sunitinib, brivanib, and sorafenib have slight impacts on the advanced stages of HCC. These therapeutics share short-time tolerable effects before they show severe side-effects, or resistance in HCC patients. Chemotherapy is used as a treatment for HCC patients when other therapy alternatives are limited. However, chemotherapy provides only slight benefits for HCC and easily develops toxicity in patients without long-term efficiency. The five-year survival rate of HCC is low, barely 18 % after diagnosis (Asafo-Agyei & Samant 2023). Therefore, novel applications with fewer side effects and system toxicity for treatment are needed to achieve a better prognosis and decrease the death rate (Anwanwan et al. 2019).

Biomolecules may have difficulties passing through a cell membrane because of their bulky weight and size (Xing et al. 2020). A carrier for biomolecules with excellent biocompatibility and biodegradability must develop to enhance passing and release. Nanotechnology might enhance the therapy and therapeutics for cancer by improving targeting and creating more effective therapeutics (Anwanwan et al. 2019). Nanoparticle-mediated drug delivery has enhanced biocompatibility, optimal cancer cell targeting, and stability with reduced toxicity and drug resistance (Yao et al. 2020). Furthermore, nanoparticle-mediated delivery methods will become more attractive in future cancer therapy designs due to their diverse properties to overcome current challenges. By increasing the permeability, retention, and pharmacokinetics with fewer side effects, the nanotechnology-based treatment could achieve better outcomes in the malignancies and the survival percentages (Anwanwan et al. 2019).

1.1.1 PERSONALIZED MEDICINE FOR HEPATOCELLULAR CARCINOMA

Since HCC has become more common worldwide with increased mortality rates without preventative or efficient treatment, there is a need to develop novel therapy approaches (Min et al. 2021). Within recent years, personalized medicine has become an attractive research topic for HCC (Chan et al. 2022). Furthermore, most HCC patients receive their diagnosis at the advanced stage, which creates a necessity to enhance early diagnostics and focus on personalized medicine (Min et al. 2021). Personalized medicine aims to achieve more reliable information about the mechanisms of diseases, which have been utilized in drug development (Kallio & Pylkkänen 2019). Furthermore, personalized medicine will consider age, lifestyle, vital functions, and genetic features, which creates the necessity to research even more specific therapeutics and therapies. Typically, the development of a disease or cancer is fast, so the utilization of genomic information should be advanced more often to create better and more personalized therapies in the future. Pervasive genomic information will utilize the quality of treatment and safety while simultaneously increasing expenses.

Personalized medicine aims to promote the health and well-being of an individual by using novel technologies utilizing a wide range of research fields (Sosiaali- ja terveysministeriö 2023). In general, the main goal of personalized medicine is to improve diagnostics to achieve more effective and safer therapies. The number of cancer therapy alternatives has increased to treat cancer patients from a personalized medicine point of view (Yao et al. 2020). Although there are multiple novel therapeutics of targeted agents or immune inhibitors, only a minor amount of HCC patients benefit from them (Chan et al. 2022). Personalized medicine has become essential when considering which therapy would benefit the most for each individual. Gene editing for liver diseases novel therapy has become an absorbing and logical approach since most malignant forms of liver diseases are caused by mutations in a single gene (Aravalli & Steer 2017). Therefore, the monogenic nature of liver diseases could be repaired easily with optimal genome editing applications.

1.2 C-MYC IN HEPATOCELLULAR CARCINOMA

The family of Myc consists of proto-oncogenes (Ryan & Birnie 1996). They are associated with various malignant human cancers. The Myc family members are involved with transcription, cell cycle progression, and differentiation. The family has complexities, but mutations occur in human malignancies. Cellular Myc (c-Myc) oncogene belongs to the Myc family. C-Myc has a significant role in regulating various cellular and molecular effects in cells, such as transcription, proliferation metabolism, differentiation, and apoptosis of cells (Whitfield & Soucek 2011). The major impact of the c-Myc regulation remains to promote the cell cycle progression (Nevzorova et al. 2013).

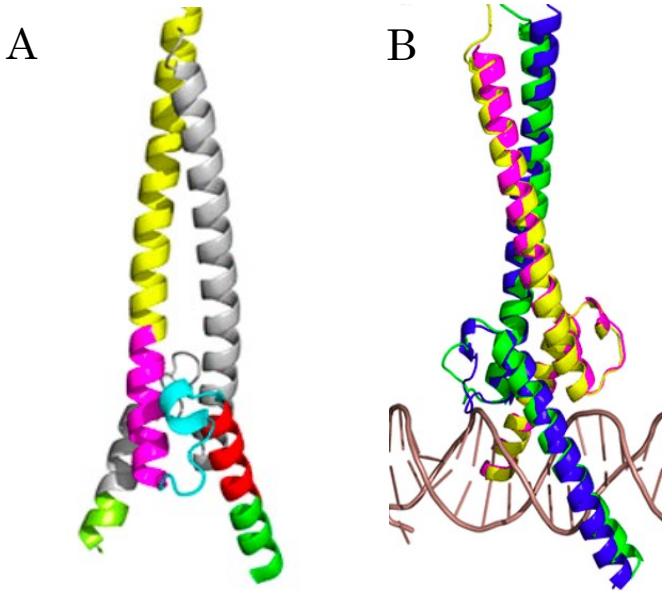


Figure 1. Image A is a cartoon representation of the structure of c-Myc. The zipper region is colored yellow, the helixes red and magenta, the loop cyan, and the basic regions of c-Myc green. MAX is colored gray (Sammak et al. 2019). Image B is a cartoon representation of the c-Myc-MAX complex binding to DNA. The complex controls the gene transcription. MAX is colored magenta, whereas c-Myc is blue.

The structure of c-Myc is consisted of helix-loop-helix-zipper (Abcam 2023). There is a N-terminal domain, a C-terminal domain, and a central region in c-Myc. The N-terminal domain is responsive to protein stability and transcription regulation (Sammak et al. 2019). MAX is in the C-terminal

domain. The domain is associated with cell proliferation and apoptosis. The central region composes nuclear localization signals and is associated with transforming activity, transcription, and apoptosis. Figure 1 represents the structure of c-Myc and the c-Myc-MAX complex binding to the sequence DNA. The c-Myc-MAX complex is responsible for controlling gene transcription. C-Myc consists of 493 residues. The molecular weight of c-Myc is around 62 kDa (Madden et al. 2021). The location of c-Myc is on chromosome 8q24.1 (Ji et al. 2018).

C-Myc is responsible for regulating 15 to 20 % of human genes. C-Myc controls the cell cycle, protein synthesis, cytoskeleton, and cell motility (Lin et al. 2010). How the genes interact together remains unknown. The overexpression of c-Myc is the highest of all oncogenes in human cancer types (Lin et al. 2010). The deregulation of c-Myc expression is common in various cancers (Nevzorova et al. 2013). The mutations of c-Myc are due to an altered signal transduction rather than self-caused (Whitfield & Soucek 2011). The cancer-promoting oncogene c-Myc remains one of the significant factors in developing HCC by the amplification and overexpression of c-Myc.

The lifetime of c-Myc in proliferating cells remains brief, approximately only 20 – 30 minutes (Lin et al. 2010). The activation level of c-Myc is highly regulated, and the response to different cellular effects depends on the activation. However, when the expression of c-Myc is imbalanced, the protein functionality may change, create resistance, or the transcription may lead to dysregulation. Distinct levels of activating c-Myc expression cause variations of functions in cells (Akita et al. 2014). C-Myc is associated with over 70 % of all cancers (Zheng et al. 2017). The overexpression of c-Myc leads to changes in controlling cell proliferation, growth, metabolism, DNA replication, cell cycle progression, cell adhesion, and differentiation. The cause of the overexpression is commonly genomic amplification, which correlates with high proliferative activity and levels of p53.

The high expression and the frequent amplification of c-Myc can be seen in hepatocellular carcinomas (Lin et al. 2010). C-Myc is over-expressed in most HCCs, which highlights the significant role of c-Myc in regulating cellular

processes. C-Myc overexpression develops hepatic cells, which leads to hepatocellular carcinoma. The activation level of c-Myc is the greatest for the pathogenesis of liver cancers if compared to the activities of other oncogenes (Shachaf et al. 2004). However, the overexpression of c-Myc is associated more with the advanced liver cancer stages (Zheng et al. 2017).

The interaction between c-Myc and hepatocytes in proliferation occurs during the regeneration of the liver while the hepatocytes enter cells for replication. Hence, c-Myc is considered one of the significant factors in the cell cycle activating hepatocytes from the G_0/G_1 phase into the S phase. The location of C-Myc is near the nucleus, with greater expression levels during proliferating fetal hepatocytes. Its localization has changed during the cell proliferation closer to the nucleus.

C-Myc has impacts up to thousands of target genes (Miller et al. 2012). It can either positively or negatively regulate the downstream pathway of the target genes. Overexpression increases malignant changes. As listed in Figure 2, c-Myc has a wide range of effects on cellular and molecular processes. Several pathways activate c-Myc, and c-Myc activates simultaneously multiple metabolic transitions leading to malignancy (Miller et al. 2012). In Figure, c-Myc in normal conditions increases cellular proliferation, metabolism, transcription, protein synthesis, glutaminolysis, and glycolysis while it decreases genes that inhibit growth. In the overexpression, these effects increase more.

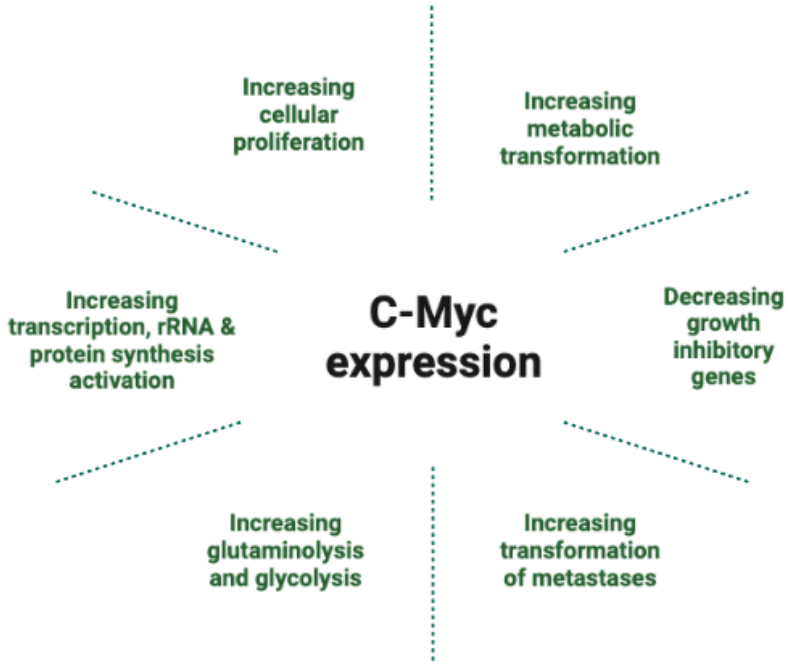


Figure 2. C-Myc has multiple effects on both molecular and cellular levels. Regular c-Myc expression affects maintaining the homeostasis of the cell. The image was created with BioRender.com.

C-Myc is able to induce hepatocellular carcinoma tumorigenesis, which has been studied in *in vitro studies* on human cancers (Zheng et al. 2017). Due to its crucial role in the regulation, it provides opportunities for future therapeutic development (Miller et al. 2012). That has led to increasing liver cancer research focusing on the role of c-Myc. The inactivation of c-Myc stabilizes the regular cellular balance with apoptosis, proliferative arrest, microenvironment remodeling, and suppression of vascular angiogenesis (Zheng et al. 2017). Whitfield & Soucek investigated that Myc inhibition did not cause resistance in the tumor lesions. That suggests possible therapeutic applications due to a non-adaptive link between the tumor and its microenvironment. Animal models have shown the potential of the inactivation of c-Myc as a treatment because it has decreased the neoplastic properties of carcinoma (Shachaf et al. 2004).

Since the current treatment is not efficient enough to raise the survival rate, there is a necessity to develop novel therapies and drugs (Lin et al. 2010). Towards more advanced stages of HCC, c-Myc increases its levels, which

might be a potential place to decrease the advancement of cancer since various activity levels of c-Myc activate the different target genes. Due to the importance of c-Myc in liver cancers, c-Myc has become an attractive target for future cancer treatment. Down-regulating the c-Myc expression tumorigenesis could potentially decrease and regress (Pelengaris & Khan 2003). Previous studies of the c-Myc downregulating have shown decreasing activity (Lin et al. 2010). The reduced activation causes the regression and differentiation of tumors. The small molecule inhibitors of c-Myc have fast metabolism with low concentration in tumors, which should be improved by different pharmacokinetic profiles such as a novel nanocarrier.

1.3 METAL-ORGANIC FRAMEWORKS

Metal-organic frameworks (MOF) have become one of the most attractive and valuable applications in the storage and delivery of cargo (Zhou et al. 2012). The structure of MOFs combines a metal ion or cluster and an organic ligand. Typically, the synthesis of MOFs is self-assembled of the metal and the organic ligand (Xu et al. 2021). Biomineralization through MOFs enable potential applications for the future due to its inexpensive and fast process (Liang et al. 2015). Biomineralization can be determined as a process where minerals are produced in living organisms (Yan et al. 2020). MOFs enable growing a nanolayer in distinct substrates under the biocompatible conditions for protection regardless the shape of the cargo (Liang et al. 2015). Biomineralization utilizing MOFs raise hopes in biotechnology field. MOFs provide protection and controlled release of its loaded cargo with encapsulation (Zou et al. 2020).

MOFs share versatile features with effortless synthesis (Yang & Yang 2020). Non-toxic metals, such as Fe, Zn, Ca, and Mg, are used to form metal compounds (Xing et al. 2020). MOFs share large surface areas with adjustable internal surface properties and pore size. MOFs can be grown under biocompatible conditions and in diverse substrates, such as films, particles, and gels (Liang et al. 2015). The large surface area provides enhanced drug

adsorption. The large pore size enables protection for therapeutic by encapsulation of the cargo, such as therapeutics (Xu et al. 2021).

The surface of MOFs can be easily functionalized and modified due to their high surface areas and tunable biocompatibility (Yang & Yang 2020). The adjustable porosity enables utilization in diverse biomedical applications. The structures of MOFs have up to 90 % free volume, which leads to high porosity (Kaur et al. 2017). The pore windows vary between 5 to 25 Å. The surfaces of MOFs can be up to 6000 m^2/g. Drug deliveries MOF-mediated provide advantages for current drug delivery systems by enhancing the bioavailability and therapeutic effects of drugs (Yang & Yang 2020). The wide range of metal ions and clusters of MOFs provide diverse morphologies, compositions, adjusted sizes, and chemical properties for wanted cargos. High surface areas and large pores of MOFs allow high cargo loading capacity. For drug delivery, MOFs enable controllable release to enhance the safety and effectiveness of a drug. MOFs share weak coordination bonds, which leads to proper biodegradability. Notably, MOFs have low densities and thermal, and mechanical stabilities, which make them attractive subjects in sensing, drug delivery, and storage due to their extraordinary abilities. As a delivery method for therapeutics, MOFs provide a biocompatible and stable alternative without causing toxicity (Xing et al. 2020). MOFs have increased their usage in drug release due to their adjustable size, shape, chemical composition, easily modified structure, and surface functionalization (He et al. 2021). MOFs enable better cargo and drug loading efficiency, instability, and fewer side effects and toxicities when compared to other nanocarriers, such as liposomes, quantum dots, and polymers. The components consist of frail coordination bonds enhancing biodegradability. The usage of MOFs as carriers has increased drug loading, drug solubility, stability, targeting, and bioavailability. MOFs have enhanced drug accumulation in tumors. Despite the challenges, MOFs have become widely used in bioimaging, drug delivery, and electrocatalysis (Xing et al. 2020). MOFs have diverse applications in a wide range of research fields (Yang & Yang 2020). The biomedical applications of MOFs are shown in Figure 3. The applications of MOFs are versatile, which enables the utilization of MOFs in bioimaging, biosensing,

and functionalization. MOFs enable the delivery of cargo, such as therapeutics, nucleic acids, and proteins.

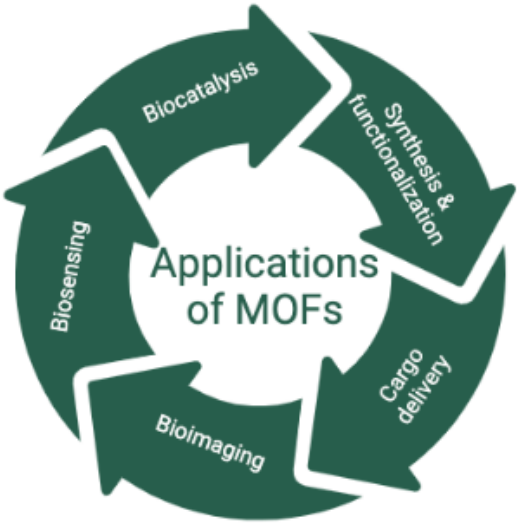


Figure 3. The biomedical applications of metal-organic frameworks. Due to the versatile applications of MOFs, researchers have recently become more attracted to MOFs and their unique properties.

Despite all the applications, there remain multiple challenges among MOFs (Xing et al. 2020). The covalent bonds and electrostatic interactions might change in the combination of MOF and biomolecule, which may lead to an unsuitable incorporation. Due to the biomolecular crystal structure that may vary, the stability of MOFs might vary. MOFs are most likely targeting correctly despite risks of off-target remaining during development to achieve a suitable biosafety level without causing toxicity. The most important part is to achieve optimal protection for the active biomolecule to achieve the benefit. However, biotoxicity, autophagy, and immunogenic response are common challenges among MOFs at this stage of research, which needs more research and development. Therefore, MOFs-based drug delivery requires further studies in nanoparticle uptake and cytotoxicity to prove great ability to work as a drug carrier (Spitsyna et al. 2022).

1.3.1 ZEOLITE IMIDAZOLATE FRAMEWORK–8

Zinc-based MOFs have a tetragonal shape (He et al. 2021). Zeolite imidazolate framework-8 (Zif-8) belongs to the MOFs family (Spitsyna et al. 2022). Zif-8 nanoparticles are prepared by combining a Zn^{2+} with a 2-methylimidazole (2-MIM). Zn^{2+} works as a metal central ion, and 2-Methylimidazole is an organic ligand (Wang et al. 2020). Zinc is an endogenous metal ion commonly used to construct MOFs without being a toxic metal. Due to the metal properties, Zif-8 provides high thermal and chemical stability (Hugenschmidt et al. 2020). Zif-8 has supplied excellent biocompatibility in several cell lines (Yan et al. 2020).

Zif-8s enable high surface area and adjustable sizing of pores with good biocompatibility (Spitsyna et al. 2022). Zif-8 provides a pH-mediated drug release with highly efficient nanoparticle-mediated therapeutics due to its ability to host nucleic acids and proteins. Zif-8 indicates promising results in gene editing and cancer cell therapy by delivering therapeutics. Because Zif-8 is pH-responsive, which leads to drug release and endosomal escape promotion, it enables excellent possibilities for future cancer therapies (He et al. 2021). Zif-8 provides controllable release due to its great biodegradability in an acidic environment. Zif-8 remains stable in a physiological environment, and only acts in an acidic environment, which enable payload release in cancerous conditions (Xing et al. 2020). Zif-8 enable great stability, protection and controlled release of its cargo (Zou et al. 2020).

The structure of Zif-8 is easily modified into the desired shape and size to enhance the combination of organic linker, penetration, and encapsulation. (Xing et al. 2020). Zif-8 is a functionalized drug carrier due to its capability to the high load, controlled release, and plasticity of pores. The release of the compound in the optimal environment provides biosafety and better benefits from the drug. Zif-8 is a great tool to store, protect, and carry several forms of organic molecules. Exceptional chemical bonding, surface modification, and internal encapsulation of the Zif-8 complex enhance stability, capacity, and affinity. Zif-8 has cell targeting abilities due to its ability to target movement and release in the correct location.

1.3.2 APPLICATIONS AND CHALLENGES WITH ZIF-8

Zif-8s have been in cancerous research as a nanocarrier because of their low toxicity and decent biocompatibility (Wang et al. 2020). Zif-8s in cancerous research have multiple other advantages. Due to their highly crystalline structure, they can be conductive while determining the spatial structure. Zif-8s have high porosity and specific surface area, which ease the loading of a compound. Zif-8 can be loaded with multiple distinct organic bridging ligands since their structures are simple to modify and design. Overall, the structure of Zif-8 enables effortless modification due to its framework because the frame and surface can be adjusted. Encapsulation or other coating methods could improve immune system escaping (Xing et al. 2020).

Zif-8 has shown great promise as an efficient delivery platform for CRISPR/Cas9 edited plasmid (Duan et al. 2021). A plasmid encoding Cas9 or sgRNA enables easy transfection through an efficient nanocarrier. However, there are still some challenges with Zif-8 due to its new appearance in the research. Since the diameter of pores in Zif-8 is small, only 11.6 Å, Zif-8 might not be suitable for large and bulky molecules, such as proteins and nucleic acids, with a high molecular weight that is unable to diffuse (He et al. 2021). Biostability, biosafety, and biopharmaceuticals must be considered while developing novel carriers. However, Zif-8 as a carrier still requires more research before proceeding to the clinical stage (Xing et al. 2020). The induction and other nucleic acids and proteins that impact growth must be studied. The protein interaction and possible changes in the morphological structure remain unknown.

1.4 NANOPARTICLE-MEDIATED DELIVERY

Gene editing has been under development since the 1980s (Duan et al. 2021). The most effective gene editing tools for eukaryotic cells are zinc finger nuclease (ZNF), transcription-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR) nowadays. ZFN uses a zinc-finger protein, TALEN uses an RVD tandem repeat region of

TALE protein, whereas CRISPR uses a single-guide RNA as its recognition site (Li et al. 2020). TALEN and ZFN use a Fok1 nuclease as a modification pattern, whereas CRISPR uses a Cas9 nuclease as its modification pattern.

ZNF cleavage a non-specific through DNA binding domain (Li et al. 2020). Hence, ZNF may have various interactions with the homologous sequences of DNA. TALEN enables more specific and efficient gene editing when compared to ZNF, although it lacks DNA cleavage. To edit the genome, TALEN and ZFN have remained in the shadows due to CRISPR/Cas9's ability to target various DNA by its multiplexing (Rabaan et al. 2023). CRISPR allows DNA to bind to RNA with Cas9 nuclease, whereas ZNF and TALEN use proteins to bind DNA with the FokI nuclease. CRISPR enables targeting multiple targets, whereas the abilities of several targeting for TALEN and ZNF are low. CRISPR provides a non-complicated design when compared to others. To continue the gene editing in a different DNA template, TALEN and ZNF require enzyme reengineering (Li et al. 2020). That will lead to separate synthetization, whereas Cas9 enables engineering to the new DNA template by altering the sequence of its sgRNA.

Gene editing based on TALEN has necessitated developing more effective and specific methods (Savić & Schwank 2016). The TALEN method is time-consuming and complicated, which is why researchers have discovered easier and less time-consuming gene editing methods. CRISPR/Cas9-based gene editing method has provided simple and time-effective gene editing, which enables the utilization of future genome editing and the development of novel therapeutics for diseases with poor prognoses. The CRISPR/Cas9 gene editing method has been ranked as the most successful method nowadays (Liu et al. 2021). Table 1 gathers the most used gene editing methods with their main characteristics. CRISPR has become the most used and effective method to edit genes and genomes due to its wide use in research and clinical investigation.

The delivery method must provide high efficiency in editing, low immunogenicity, and efficient delivery of both sgRNA and Cas9 nuclease into the wanted target (Yang et al. 2021). Several delivery methods have been

developed, but the previous methods have not provided the desired outcome due to their limited editing efficiency, lack of Cas9 protein activity, and immunogenic responses (Yang et al. 2021). There has become a necessity to develop better delivery methods to enhance the efficacy of Cas9/sgRNA delivery.

Table 1. Gene editing methods and their comparison (Li et al. 2020).

	CRISPR/Cas9	**TALEN**	**ZFN**
The pattern of modification	Cas9	Fok1	Fok1
Recognition site of DNA	sgRNA	RVD tandem repeat region of TALE protein	Zinc-finger protein
The size of targeting sequence	20 bp guide sequence + PAM sequence	14-20 bp for monomer, 28-40 for pair	9-18 bp for monomer, 18-38 bp for pair
Specificity of gene editing	Tolerance of multiple/positional consecutive mismatches	Tolerance of tiny number of positional mismatches	Tolerance of tiny number of positional mismatches

Table 2 represents the most common delivery methods. As can be observed, all the delivery methods have unique limitations and advantages. The delivery methods are used in distinct experiments depending on the experiment's aim. Viral vectors have raised concerns about *in vivo* delivery of CRISPR/Cas9 due to the inefficiency of targeting cells and safety (Sioson et al. 2021). The most common source of the Cas9 nuclease is from DNA plasmid due to its cost-efficiency, stable structure, and easy editing. Inorganic nanomaterials have become attractive because of their unique characteristics. Notably, inorganic nanomaterials provide non-toxic, biocompatible, and stable cargo delivery. Inorganic nanocarriers have given promising results for gene delivery of CRISPR/Cas9 for therapeutic applications (Duan et al. 2021).

Zif-8, as the CRISPR/Cas9 carrier, has provided promising results due to its efficient loading capacity and high binding affinity (Liu et al. 2021). The major advantage of the Zif-8 carrier is the pH-mediated cargo release. Nanoparticle-mediated delivery of CRISPR/Cas9 has attracted researchers because of its high loading capacity, scale-up capability, and low immunogenicity (Sioson et al. 2021). Regardless of the promising advances of nanoparticle-mediated delivery, low delivery efficiency remains a challenge.

Table 2. The most common delivery methods for CRISPR/Cas9 are represented in the table below. The advantages and limitations of the methods are listed. (Lv et al. 2021; Yang et al. 2021; Yip 2020).

Delivery method	Advantages	Limitations
Viral vectors (adenovirus, lentivirus & adeno-associated virus vector)	Efficient delivery, non-integrating (adenovirus, adeno-associated virus vector), can be used in *in vivo*, *in vitro* and *ex vivo*	Limited size of cargo, integration risk (lentivirus), immune responses, long expression time, off target effects
Lipid nanoparticles	Low cost, high compatibility, availability. Encapsulations provide successful delivery, causes no stress	Low editing efficiency, depending on the cell type, requires a lot of optimalization.
Microinjection	Provide efficient, direct delivery, the delivery amount and dosage easily manageable, utilized in *in vitro* and *ex vivo* experiments	*In vivo* delivery unavailable, challenging method to perform
Electroporation	Efficiency, simple operation, utilized in *in vitro* and *ex vivo* experiments	Viability of cells and *in vivo* experiments challenging
Extracellular vesicles	Non-integrating, multiplexable, utilized in *in vitro, in vivo* and *ex vivo* experiments	Low amount of quantification methods
Non-viral vectors (e.g., inorganic nanomaterials,	Enhanced tolerance, biocompatibility, non-immunogenicity, encapsulation for	Low gene delivery efficiency and transgene expression requires optimization.

gold nanoparticles)	protection, delivery method without risk of virus, utilized in *in vivo* and *in vitro*	

1.4.1 CRISPR/CAS9 MECHANISM

Clustered regularly interspaced short palindromic repeat (CRISPR) / CRISPR-associated protein-9 nuclease (Cas9) has become the most widely used gene editing tool because of its ability to guide Cas9-targeted RNA to the DNA locus with high accuracy and specificity (Savić & Schwank 2016). The CRISPR/Cas9 complex was originally been isolated from prokaryotic cells (Lv et al. 2021) since CRISPR is originally found from bacteria and archaea (Cheng et al. 2020).

Six putative CRISPR systems occur, which differ by leading DNA recognition and cleavage (Kim et al. 2017). There are two classes of CRISPR. Class 1 consists of types I, III, and IV of CRISPR. CRISPR types in class 1 use Cas protein complexes. Class 2 of CRISPR consists of types II and VI of CRISPR. The CRISPRs in class 2 use mainly single Cas protein. Class 2 are utilized for RNA-guided nucleases. The type II of CRISPR bases on Cas9 nuclease, which has become the most used type of all the CRISPR types.

Editing based on CRISPR requires a guide RNA and a non-specific CRISPR-associated nuclease (Sioson et al. 2021). The guide RNA consists of synthetic RNA. The RNA has a scaffold sequence for binding the Cas9 protein and a targeting sequence for editing. The sgRNA will lead the Cas9 protein to edit specific sequences by inducing double strands to break. The sgRNA consists of 20 nucleotide-specific sequences for a target (Sousa et al. 2022). The sgRNA is responsible for leading Cas9 into the target DNA site, where the DNA has been cleaved previously. Cas9 then can cleave the double-stranded DNA into double-stranded breakings (DSB).

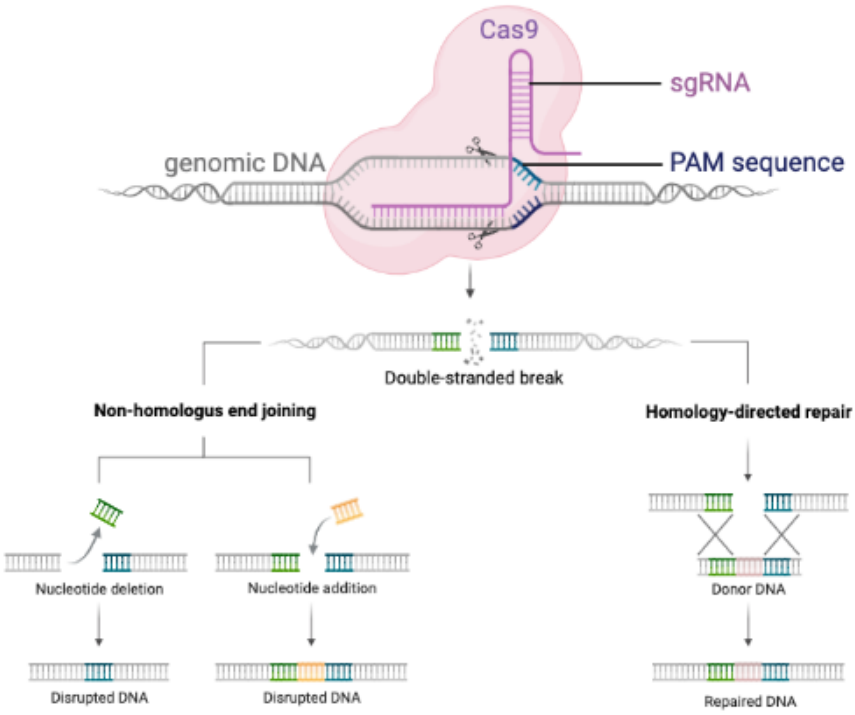


Figure 4. The mechanism of CRISPR/Cas9. Cas9 and single guide RNA form a complex. Cas9 cleaves a specific target sequence, which breaks double strands. There are two repair methods for cleaning. The non-homology end joining repairs through insertions or deletions. The homology-directed repair forms the donor template, which forms the modified DNA. The image was created with BioRender.com.

Figure 4 represents the basic principle of CRISPR/Cas9 editing. The gene editing begins with DNA cleaving into smaller pieces and attaching to the locus of CRISPR (Savić & Schwank 2016). There are two different RNA guides for Cas9 nuclease, called single guide RNA (Kim et al. 2017). The sgRNA is consisted of CRISPR RNA (crRNA) and trans-acting CRISPR RNA (tracRNA). The crRNA is composed of a complex with tracRNA (Savić & Schwank 2016). The complex composes single-guide RNA (sgRNA). The sgRNA leads the Cas9 nuclease into the target sequence DNA site. Protospacer adjacent motif (PAM) is located near the targeted sequence of crRNA to improve the DNA target selection (Rabaan et al. 2023). PAM transcribes crRNAs to help guide the Cas9 nuclease into the target DNA site (Uddin et al. 2020). Cas9 cleaves the DNA strands until the PAM sequence. CRISPR/Cas9 will target the 5' end of the PAM sequence (Yang et al. 2021).

Cas9 pairs to the sequence through Watson-Crick base pairing, which will then induce the DSB of DNA (Savić & Schwank 2016). The previously cleaved DNA break is repaired through a repairing pathway. There are two different repair methods for DNA sites - non-homologous end joining (NHEJ) and homologous directed repair (HDR) (Kim et al. 2017). The repairing pathways are utilized in different gene editing regarding whether the will is to form knock-in or knock-out genes.

NHEJ forms indels in the targeted sequence through deletions or insertions. NHEJ is commonly used in most cell types when performing knockout experiments and frameshift mutations in mammalian cells (Uddin et al. 2020). NHEJ repairs the DSBs with indels without using a template sequence that leads to disruptions of the gene sequence (Sioson et al. 2021). NHEJ provides simple and effective repair but raises concerns about mutagenesis risks. The advantage of NHEJ as a repairing method is the lack of need for the homologous DNA template, enabling repairing with the staggered ends and the indels introduced at the repair DNA site, which will lead to the generation of missense or nonsense mutations in the coding sequence of proteins. NHEJ is used to repair non-homologous sequences (Rabaan et al. 2023). Hence, NHEJ can be used in future therapeutic development due to its ability to target gene disruption (Uddin et al. 2020).

The other repairing method based on HDR is to replace a mutated gene or insert genetic information between the cleaved DNA and similar sequences (Kim et al. 2017). HDR repairing utilizes a homologous donor template to repair the DBS site (Sioson et al. 2021). HDR repairing is utilized in gene knock-in editing. HDR will repair the damaged DNA template with a wild-type gene in the targeted cells, which can integrate into the homologous target location in the genome site (Aravalli & Steer 2017). However, the wild-type template can be integrated into an exogenous DNA leading to random insertions. The low efficiency of the editing remains the major challenge in the HDR-mediated DBS repairing method (Uddin et al. 2020).

When comparing the repairing methods, NHEJ repairing is more efficient, whereas HDR provides more accurate repairing when compared to NHEJ.

The activation of NHEJ during the cell cycle is longer when compared to the restricted S/G_2 phases of HDR (Aravalli & Steer 2017). NHEJ is used for disrupting the gene of interest, whereas HDR is used for correcting the gene of interest (Savić & Schwank 2016).

1.4.2 CRISPR/CAS9 APPLICATIONS

CRISPR/Cas9 gene editing technology provides more flexible, simpler, and more accurate outcomes compared to the other gene editing methods (Chen et al. 2019). The best advantage of CRISPR/Cas9-based gene editing is the capability of editing simultaneously several DNA sites. ZNF and TALEN are able to edit only one DNA site at a time (Lv et al. 2021). Simultaneous editing of multiple DNA loci decreases the time for gene editing, which provides time-efficient gene editing (Li et al. 2020). Cas9 can target a new DNA sequencing after modifying the sgRNA sequence without challenges (Kim et al. 2016).

CRISPR/Cas9-based gene editing can be utilized in epigenome editing, immunotherapy, anticancer drug development, cancer gene therapy, and tumor model development due to its versatility (Yang et al. 2021). CRISPR/Cas9 gene editing method has been utilized in manipulating human gene sequence and introducing the specific targeting of epigenetics. CRISPR/Cas9 enables a novel anticancer drug development (Chen et al. 2019). The method provides applications in drug development by identifying the resistance genes and finding the potential drug targets.

CRISPR/Cas9 gene editing enables simplification of a design correcting gene efficiently (Aravalli & Steer 2017). The specific targeting and cleaving of the target DNA site highly accurately are the major advantages of CRISPR/Cas9-mediated gene editing. The method provides versatility, which can be utilized in editing several types of cells through knock-in, knockout, gene activation, and repression editing (Sioson et al. 2021). The structure of Cas9 is stable and can be easily adjusted. CRISPR/Cas9 is more available for a wider range of researchers due to its lower expenses when compared to the other gene editing methods.

CRISPR/Cas9 has been utilized in detecting, imaging, manipulating, and annotating specific sequences of interest in various mammalian cells (Yang et al. 2021). CRISPR/Cas9-based gene editing provides a potential alternative to traditional cancer therapy through a permanent disruption of the genes of tumor survival (Sioson et al. 2021). CRISPR/Cas9 mechanism may enhance the recovery rates among patients in cancer therapy (Rabaan et al. 2023). For cancer therapy, the editing mechanism enables simple manipulation of the cancerous genome and its immunotherapy. The tumor suppressors activate oncogenes through the absence of oncogenes, mutations, and silencing genes, which may lead to tumorigenesis. For enhancing future cancer therapy, malignant tumorigenesis requires optimization and correcting mutations.

1.4.3 CHALLENGES OF CRISPR/CAS9 GENE EDITING METHOD

CRISPR/Cas9 has become an attractive method for the novel treatment of malignant cancers (Cheng et al. 2020). However, there are various challenges in human gene editing, such as off-target effects, editing efficiency, fitness of edited cells, immune response, and delivery methods. Despite the extraordinary advantages and applications, the CRISPR/Cas9 method provides, there are several issues and limitations regarding human gene editing (Yang et al. 2021). CRISPR/Cas9 gene editing has mostly raised concerns about efficacy and safety (Duan et al. 2021).

Protecting the CRISPR/Cas9 complex in the plasmid provides better stability, faster outcomes, and convenience (Lv et al. 2021). However, plasmid-mediated delivery may reduce gene editing efficiency, prolong the time for Cas9 cleaving, and increase the rate of off-target effects and the risks of gene integration. The DNA cleavage through CRISPR/Cas9 gene editing method may lead to the deletion of thousands of base pairs leading to novel genotypes (Wang et al. 2022). That may lead to pathogenic consequences in the cells.

Since the plasmid-mediated transfection with CRISPR/Cas9 encoding is easy, they provide easy loading to the nanocarriers (Duan et al. 2021). However, the size of CRISPR/Cas9 is large, which requires an optimal carrier to achieve the desired outcome. For future use, CRISPR/Cas9 gene editing requires

further experiments and development to obtain stable editing with high efficiency of targeting and enhanced loading and delivery efficiency (Duan et al. 2021). The development should proceed into safe and efficient therapy alternatives.

The correct delivery into the target cells or tissues remains challenging for the CRISPR/Cas9 components (Sioson et al. 2021). Hence, the delivery method for the CRISPR/Cas9 system remains one of the significant issues (Sousa et al. 2022). The delivery method should maintain its stable structure to protect the carried system from passing through the cell membrane and successfully release it in endosomal escape to the target cells (Duan et al. 2021). In CRISPR/Cas9, it is highly significant that the Cas9 nuclease is transported into the nucleus to achieve its functions in the target cell. The viral vectors provide high gene transfection efficiency but lack safety due to their long-term expression and immunogenicity (Sousa et al. 2022). Additionally, the viral vectors are more likely to have off-target effects when compared to the other delivery methods. The delivery method requires optimization to the wanted target. The accurate targeting of cells may create challenges if the delivery method is inefficient, or the delivery vehicle develops an immune response to the target cells (Duan et al. 2021). The delivery vectors may create problems due to their inaccurate migration into host cells through mutations or carcinogenesis (Duan et al. 2021).

The off-targets may generate effects with CRISPR/Cas9 technology since editing might generate indels at other genomic loci of the genome (Cheng et al. 2020). That might lead to undesired mutations and toxicity. The immune responses might occur due to the bacteria origin of the Cas9 protein. Since the bacteria-origin components can be used for eukaryotic cells, immunogenic responses are possible. Since CRISPR/Cas9 can bind to the target DNA, the DNA sequences with identical or homologous could be cleaved, which leads to off-target mutations (Li et al. 2020). The possible off-targets may cause genomic toxicity, instability, disruption in gene functions, and carcinogenesis. The off-targets may be the major factor to activate tumorigenesis (Wang et al. 2022). The CRISPR delivery to the target cells may cause immune

reactions (Kim et al. 2017). Hence, the delivery method should maintain its stable structure to protect the carried system from passing through the cell membrane and successfully release the carried system in an endosomal escape to the target cells (Duan et al. 2021). An optimized design should reduce the off-target effects (Uddin et al. 2020). In CRISPR/Cas9, it is significant that the Cas9 nuclease is transported into the nucleus to achieve its function in the target cell. Cytotoxicity remains challenging regarding the cancerous cells and their fast proliferation (Chen et al. 2019). The fast proliferation with enhanced survival may limit achieving the optimal and effective cell targeting and clearance without causing cytotoxicity. The off-target effects in the edited eukaryotic cells have become a major limitation (Yang et al. 2021). The possibility of the off-target effects depends on the cleaved sequences. The indels generated in other genomic loci might lead to undesired mutations and toxicity (Cheng et al. 2020). The toxicity caused by immunogenicity has become a crucial issue regarding gene editing (Yang et al. 2021). Due to the prokaryotic origin of Cas9 nuclease, the eukaryotic organisms may possess immunogenicity and antibodies.

Since gene editing leads to the permanent change of DNA, there are some problems with safety and ethicality (Li et al. 2020). The ethical challenges have raised concern among the population and researchers due to the lack of studies and the possibility of major risks (Yang et al. 2021). The ethical issues require further, more detailed experiments for developing novel therapeutics to gain more knowledge of the long-term effects and safety. Hence, the main challenge regarding human gene editing is the ethical question (Kim et al. 2017). There is no clear boundary on which human traits can be ethically modified. Therefore, the issues must be optimized when developing novel delivery methods for human gene editing to enhance the delivery and safety concerns (Sousa et al. 2022).

2 AIMS AND HYPOTHESIS

Cancer rates increase worldwide, which creates a necessity for more efficient and promising therapeutics more cost-efficiently. The gene editing technology provides a novel therapy approach for various gene-related diseases, such as cancers. Hepatocellular carcinoma has become more common worldwide without long-term treatment and without causing resistance or side effects. Hence, my proposal is that down-regulating c-Myc could be a potential therapeutic approach in liver cancer since there have been engaging results *in vitro* concerning how the downregulating of c-Myc inhibits or decreases the activation of cell cycle and growth. I will use two distinct hepatocellular carcinoma cell lines to achieve more reliable results and compare the cell lines to provide more accurate results in an environment of liver cancer *in vitro*. The thesis work will be a base for a clinical translational work for *in vivo* experiments.

Zif-8 indicates excellent biocompatibility in distinct cell lines with great protection and controlled release. Therefore, it was chosen as the nanocarrier for the CRISPR/Cas9 c-Myc knockout plasmid. Zif-8 nanocarrier provides great protection for plasmids. Due to its less complex nature when compared to other more complex carriers, such as virus delivery systems, Zif-8 maintains well the loaded plasmid providing protection and pH-mediated release due to its properties. Furthermore, its cytotoxicity is inconsequential when compared to other. The master's thesis aims to achieve successful delivery of Zif-8 CRISPR/Cas9 plasmids into liver cancer cells to study the release of nanoparticles in acidic pH and analyze the c-Myc protein expression through using various imaging techniques. After the preparation, Zif-8 will be transfected to a couple of cancerous cell lines to proceed with the study. After the cellular uptake, the edited gene will escape from lysosomes towards the nucleus. If this delivery method works as proposed, it could be utilized for other gene experiments due to the versatility of the CRISPR/Cas9 system. This delivery method is believed to raise hopes due to the potential of the system for future *in vivo* studies.

The objectives of this thesis are specified:

1. To successfully load Zif-8 with the CRISPR/Cas9 edited plasmid into different hepatocellular carcinoma cells and observe its release in acidic pH by using distinct imaging techniques.

2. Analyze the protein expression of c-Myc with different hepatocellular cell lines and perform the knockout experiments.

3. Investigate the endosomal escape properties and the location of nanoparticles in cells at different points in time.

3 MATERIALS & METHODS

3.1 SYNTHESIS AND ANALYSIS OF ZIF-8

Large pore sizes and variable sizes and shapes of nanoparticles provide a great benefit for enhanced cargo loading. The optimal particle size for the therapeutic carrier is approximately 100 nm since the nanoparticle should be the optimal size for cellular uptake (Mitchell et al. 2021). If the nanoparticle is larger than 200 nm, it may be eliminated or cause complement system activation. The pore size is ideally around 10 nm. If the nanoparticles share the properties, they should enable successful cellular uptake with a high loading capacity of plasmids and therapeutics.

Plasmids are ring-shaped eukaryotic organelles with their intact genes in the double-stranded DNA (Liu et al. 2021). Plasmids can be transformed into eukaryotic cells by transfection. Plasmids enable knockout experiments to identify and cleave the wanted DNA sequence. The plasmids are used in a wide range of research due to their properties and easy access.

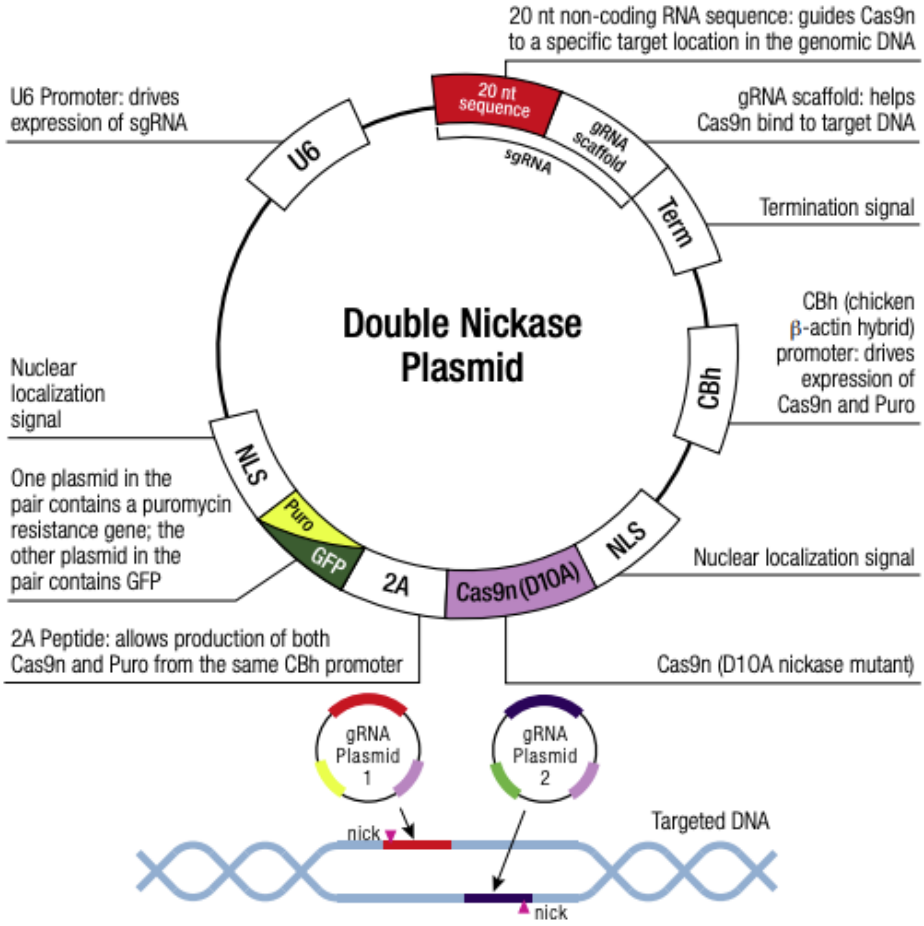


Figure 5. The main properties of Double Nickase Plasmid are presented. The plasmid contains a pair of plasmids, where one has a gene for puromycin resistance, and the other has GFP for monitoring the transfection (Santa Cruz Biotechnology 2023).

This project used a Myc Double Nickase Plasmid (h) (Santa Cruz Biotechnology) as a CRISPR/Cas9 edited plasmid. The structure and properties of the plasmid are shown in Figure 5. The plasmid is composed of a pair of plasmids with different effects. The plasmid system enables targeting with high specificity and a great level of KO efficiency. Both plasmids encode a D10A mutated Cas9 nuclease with specific targeted 20 nucleotide guide RNA. One plasmid was adjusted with a puromycin resistance gene for selecting the cells where the transfections were performed successfully, whereas the other plasmid was modified with a green fluorescent protein (GFP), which enabled the visualizing of the transfection.

3.1.1 Preparation of Cy 5.5 labeled Zif-8s

Zif-8 can be prepared through diverse synthesis methods (Lee et al. 2015). The most common methods are solvothermal, microwave-assisted, sonochemical, mechanochemical, and microfluidic methods. Different methods enable modification of the properties and the morphology of Zif-8, which can be utilized for distinct applications. The solvent solution, preparation temperature, and preparation time were the effects that played the most significant roles in achieving optimal nanoparticle size of Zif-8 preparation.

The one-pot method is mainly used for drugs with larger sizes than the pores of Zif-8 with enhanced drug loading capacity (Xie et al. 2022). The one-pot method enables drug loading into Zif-8s more conveniently and effectively than other preparation methods (He et al. 2021). The one-pot method, where all compounds are mixed, decreases the reaction time and produces less waste. Zif-8 is an ideal delivery method for the CRISPR/Cas9 edited plasmid. Figure 6 represents the basic principle of Zif-8 nanoparticle preparation. Zif-8 biomineralization to encapsulate the CRISPR/Cas9 edited knockout plasmid was performed in room temperature aqueous solution.

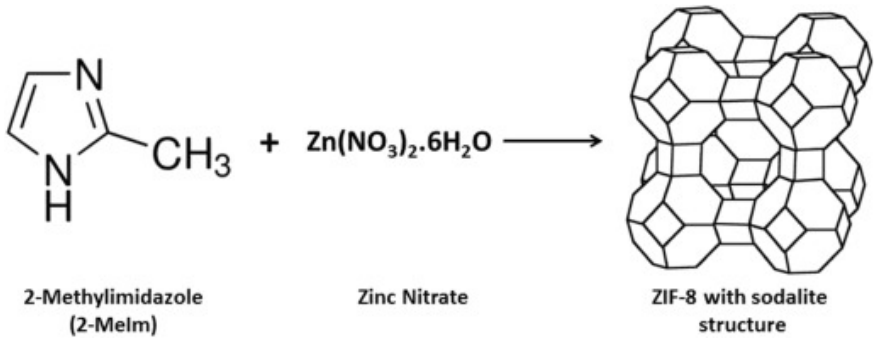


Figure 6. The basic principle of the preparation of Zif-8 from 2-methylimidazole and zinc nitrate hexahydrate (Kaur et al. 2017).

For synthesizing Cy5.5 labeled Zif-8s, both 6.8 mg of Zinc nitrate hexahydrate (Merck) and 46.7 mg of 2-Methylimidazole (Merck) were diluted into 100 µl of MqH_20 in separate 2 ml Eppendorf tubes. The nanoprecipitation method was later optimized to increase the solubility of 2-MIM in MqH_20 due to the low

solubility in water. The amount of MqH_20 doubled to increase the 2-MIM solubility. Although the solubility was considered in the further steps in preparation, the ratio of zinc nitrate hexahydrate and 2-MIM remained the same.

First, 4 µl of the previously prepared zinc solution and 1 µl of the Myc Double Nickase plasmid were mixed for 3 to 5 minutes by pipetting gently to avoid air bubbles in an Eppendorf tube. Then, in the Eppendorf tube were 40 µl of 2-MIM, and 1 µl of Cyanine 5.5 NHS ester (5 µg/µl) (Lumiprobe) added to the solution and briefly mixed a couple of times. The prepared solution was then covered from light by aluminum foil and incubated for 45 – 60 minutes by placing the Eppendorf tube on ice.

After the incubation, the Eppendorf tube was placed inside a centrifuge, and the Eppendorf tube was centrifuged at 17115 rcf (13500 rpm) for 9.5 minutes at 10 °C to wash the solution. After the centrifuging, the supernatant was collected carefully and 1 ml of fresh MqH_20 was added to break the previously formed pellet with sonicating and Vortex. These washing steps were repeated two more times to ensure that any residuals from nanoprecipitation were removed. The Cy5.5 labeled Zif-8 nanoparticles were then stored in a refrigerator for further experiments, such as transfection or characterization.

3.1.2 CHARACTERIZATION OF ZIF-8

There are several techniques to characterize Zif-8 to observe the morphology, structure, and stability of Zif-8 nanoparticles (Kaur et al. 2017). To investigate the structure, morphology, and interaction between loaded plasmid and Zif-8s, transmission electron microscopy (TEM), UV-Visible spectroscopy, and Zetasizer NanoZS were used.

TEM provides essential information on the shape, morphology, and structure of MOFs (Hugenschmidt et al. 2020). Approximately 10 µl of the prepared Cy5.5 labeled Zif-8 solution was added to the carbon-coated copper TEM grid (Ted Pella, Inc.). After preparing the grids for imaging, the grids were let to dry appropriately before imaging. A Jeol JEM-1400 Plus Electron Microscope,

with an 11 Mpix bottom-mounted digital camera, was used to capture the TEM images.

An AMG EVOS FL fluoresce Imaging Microscope was used to investigate the optical properties, plasmid loading, and release of Zif-8. The imaging enabled the observation of the transfected HepG2 and Huh-7 cells due to the GFP of the plasmid. The GFP plasmid provided information about the location of the plasmid and the effects within the different points in time. A GFP filter, with 470 nm excitation and 525 nm emission, was used to observe the locations of the plasmid.

Zetasizer NanoZS (Malvern Instruments) and Malverns Zetasizer Software V.7.02 were used to observe the average sizes and surface changes of Zif-8s. Zetasizer was used for characterizing the plasmid inside of Zif-8. The received results were compared to empty Zif-8 nanoparticles.

3.2 TRANSFECTION OF ZIF-8 LOADED CRISPR/CAS9 PLASMID SYSTEM INTO HEPG2 AND HUH-7

In vitro transfection enables genetic material into targeted cells to be delivered successfully (Fus-Kujawa et al. 2021). Transfection success relies on the quantity and the quality of DNA, the transfected cells, the incubation time, and the cell culture medium.

The main challenge with transfection is nucleic acid degradation through cell nucleases during endocytosis (Fus-Kujawa et al. 2021). The transfection is most likely to fail when nucleic acids are transfected alone without a delivery vehicle. To avoid failure, nucleic acids require an optimal delivery method for gene transfection. The optimal delivery method should be ideal for cancerous cells with high transfection efficiency and low cell toxicity without affecting the regular cell environment.

3.2.1 Cell lines and culture conditions of HepG2 and Huh-7

Human hepatocellular carcinoma epithelial-like cell lines, HepG2, and human cellular carcinoma cell lines, Huh-7, were used to deliver the CRISPR/Cas9 edited system using Zif- 8s nanoparticles. The cells were grown in Dulbeccos Modified Eagle Medium (DMEM High Glucose, EuroClone) where 50 ml of 10 % of heat-inactivated Fetal Bovine Serum (FBS, Gibco), 2 ml of penicillin 100 units/ml / streptomycin 100 U/ml (Lonza), 5 ml of 2 mM L-glutamine (Lonza), 5 ml of HEPES (Gibco) and 5 ml of 100X MEM Non-Essential Amino Acids Solution (NEAA, Gibco) added to propagate. The cells were grown in an incubator at 37 °C in 5 % CO_2. The cells were treated on 10-centimeter diameter culture dishes (Greiner Bio-One) and divided into a new dish when the dish had 75 – 90 % confluence.

Dividing cells into a new dish or preparing them for further experiments, the growth media was aspirated from the dish. The cells were rinsed with Dulbeccos Phosphate Buffered Saline (PBS, EuroClone) several times to discard the dead cells, prevent cells from rupturing, and maintain the pH. Approximately 2 ml of trypsin-EDTA solution (0.25 % trypsin, 1 mM EDTA, Gibco) was added before incubation at 37 °C for 2 - 4 minutes until the cells were detached from the bottom of the culture dish. Then 3 ml of fresh culture medium was added before placing the solution in a Falcon tube into a centrifuge and the cells were centrifuged at 200 rcf for 3.5 minutes. The trypsin-EDTA-growth medium was then aspirated, and cells were re-suspended with a fresh growth medium and transferred into a new culture dish to maintain the cell growth for further experiments. Cells were transferred into 96-well plates for cytotoxicity tests. Transfections were performed in 24-well plates. Confocal imaging was performed by using samples in glass bottom confocal dishes.

3.2.2 Zif-8 mediated transfection

The Zif-8 mediated transfection began with normal Huh-7 and HepG2 cell culturing. The old DMEM was aspirated from the culture dishes. The cells were then rinsed with 2 ml of PBS. Approximately 2 ml of trypsin-EDTA

solution was added, and the dish was left to incubate at 37 °C in 5 % of CO_2 for 2 - 4 minutes until most of the cells were detached. Approximately 3 ml of fresh DMEM was added to the dish for dilution. The solution was then divided into a couple of 2 ml Eppendorf tubes placed into a centrifuge. The cells were centrifuged at 200 rcf for 3.5 minutes. The supernatant was aspirated. The cell pellet was resuspended into Opti-MEM Reduced Serum Medium (Gibco) to achieve an ideal transfection medium. After the pellet was resuspended into Opti-MEM, the cells were counted and transferred into a 24-well plate (Greiner Bio-One). The number of cells was 2 x 10^5 which was transferred for each well. After the cells had been attached, the previously prepared Cy5.5 labeled Zif-8 nanoparticles were slowly pipetted into cell-Opti-MEM solution. For the transfection, the Zif-8 amounts of 10 μg, 25 μg, or 50 μg were used to be able to compare the efficacy and cytotoxicity caused by Zif-8 transfection, but also to be able to compare the c-Myc protein expression in different lysates in a protein expression analysis.

The well plate was incubated for approximately 6 hours at 37 °C in 5 % of CO_2. Opti-MEM was then discarded and replaced with fresh DMEM. Cells were left to incubate for a few days before analyzing and observing the transfection efficacy. With successful transfection, the cells were cultured and transferred into 6-well plates for obtaining the living of transfected cells for further experiments.

3.2.3 Lipofectamine transfection

As a control method for delivering the plasmid into liver cancer cells, Lipofectamine™ 3000 Transfection Reagent (Invitrogen™) was used. Lipofectamine 3000 Transfection Reagent enables high efficiency for cells to transfection and enhanced cell viability for most the cell lines with low costs (ThermoScientific 2023). Lipofectamine 3000 is used as a control due to its gentle transfection to obtain a reliable comparison between the Zif-8 and Lipofectamine 3000-mediated transfections.

Approximately 2 x 10^5 HepG2 or Huh-7 cells were transferred into a 24-well plate (Greiner Bio-One). The well plate was incubated until 70-90% confluences were achieved. Volumes of 0.75 μl and 1.5 μl of Lipofectamine

3000 Reagent were diluted into 25 µl of Opti-MEM into two separate Eppendorf tubes and then vortexed for a couple of seconds. A DNA master mix was then prepared, by diluting DNA (0.5 – 5 µg/µl) 1 µg into 50 µl of Opti-MEM medium. To the diluted solution, 2 µl of P3000 Reagent (2 µl/µg DNA) was added. The solution was mixed properly, with Vortex. Then, 25 µl of the diluted DNA was mixed with each tube of 25 µl of Diluted Lipofectamine 3000 Reagent in a ratio of 1:1. The Eppendorf tubes were left to incubate at room temperature for 10 – 15 minutes. For each well, the steps above were performed. Therefore, the total volumes of each reagent required scaling proportionally at the beginning.

After the incubation, 50 µl of DNA-lipid complex was diluted into each well. Then, the 24-well plate was incubated at 37 °C in 5 % CO_2 for 4 – 6 hours before discarding the solution and replacing it with the fresh DMEM. After the transfection, the cells were observed and analyzed after incubating them at 37 °C in 5 % CO_2 for 2 – 4 days.

3.2.4 ZIF-8 ASSESSMENT OF CELL TOXICITY

The plasmid had a puromycin resistance gene, which required a puromycin test to discover which concentration was the lowest, where all the transfected cells could not grow after 48 hours of incubation. Puromycin Dihydrochloride, sc-108071 (Santa Cruz Biotechnology), was used to assess the cell toxicity. Puromycin could kill 99 % of cells in an accurate environment within two days. First, the puromycin with 50 mg/ml concentration was diluted into MgH_20 to create stocks of distinct concentrations. The stocks were sterilized by transferring the puromycin through a pre-wet 0.22 µm syringe filter into a new Falcon tube to achieve clearer and more reliable results. The prepared stocks were stored at -20 °C.

The transfected HepG2 cells were plated into a 96-well plate (Greiner Bio-One) with seven distinct concentrations. The concentrations of 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 10 mg/ml were tested and compared to the negative control of HepG2 cells. To be able to compare the results in Huh-7 wild type and Huh-7 Zif-8 transfected cells, the concentrations of 0, 2, 3, 4, 5, and 10 mg/ml were

tested. Into each well of the 96-well plate, approximately 4,000 cells were added and left to incubate for 24 hours at +37 °C in 5 % of CO_2.

The dilution series of puromycin was performed to obtain the correct concentrations by mixing the puromycin with the DMEM. After completing the dilution series, the old DMEM was discarded, and 100 ml of new puromycin-DMEM solution was added, into each well with the wanted concentration. Then, the well plate was left to incubate for 2 days at +37 °C in 5 % of CO_2.

After the two-day incubation, the cells were observed. The concentration, where most of the cells died after incubation was determined under a microscope first. As a control, the cells were then observed with a VarioSkan Flash spectral scanning multimode reader (Thermo Scientific) after adding the WST-1 Assay Reagent. Firstly, the old solution, from the 96-well plate, was discarded. Then, 100 µl of fresh DMEM and 10 µl of WST-1 Assay Reagent (Abcam) were added to the wells. There were a couple of control wells without puromycin treatment to have a comparison of blank control. The 96-well plate was left to incubate for 2 hours at +37 °C in 5 % of CO_2. The well-plate was then placed in VarioSkan and measured the plate with a 440 nm absorbance to achieve data for cytotoxicity assay.

3.3 ANALYSIS OF THE C-MYC EXPRESSION

Western blot allows for separating and identifying the proteins by size, molecular weight, and marking the wanted proteins using specific primary and secondary antibodies (Mahmood & Yang, 2012). Correct validation of both primary and secondary antibodies is essential to achieve accurate antibody and antigen interaction and determine the dilution factor properly (Taylor & Posh, 2014). Western blot analysis was performed to detect the protein target, c-Myc, in our samples.

The concentrations for liver cancer lysates were set to 20 µg to have an equal amount of each lysate to obtain better and more reliable results. Each lysate

was moved to a 2 ml Eppendorf tube with 4x Laemmli Sample Buffer (BioRad) in a volume ratio of 3:1. Dithiothreitol (DTT) was added to Laemmli in a ratio of 1:9 to stabilize enzymes and proteins of the lysate while reducing disulfide bonds. Therefore, the prepared lysates will provide cleaner and more reliable results when reducing the disturbing disulfide bonds. The lysate samples were placed in a +95 °C heat block for 5 minutes to reduce and denature the disulfide bonds, which enables better loading into the electrophoresis gel.

The samples were loaded into Mini-Protean TGX Gel (4-15 %) (BioRad), which had the ideal protein size variation for the protein expression of c-Myc. The gel was placed in a 1X Tris/Glycine/SDS Buffer (BioRad) running buffer. First, a 5 µl of PageRuler Prestained Protein Ladder (Thermo Scientific) was added to the gel, and then 20 ng of the other samples were loaded for protein analysis. The gel electrophoresis running started at 80 voltages. After 20 minutes of running, the voltages were increased to 120, and the electrophoresis ran for 60 minutes.

After running, the proteins transferred into a nitrocellulose membrane during transfer running. Firstly, the membrane, filters, and sponges were soaked for 10 minutes in a 1X Tris/Glycine/Methanol transfer buffer to assemble a transfer "sandwich". After placing the electrophoresis gel and the membrane correctly inside the "sandwich", the "sandwich" was then rolled a couple of times with a roller to remove air bubbles to achieve enhanced results, leading to more efficient transfer and more reliable analysis of results. The transfer cassette with the "sandwich" was then ready for wet transfer. The transfer running took 80 minutes at 90 voltages and was performed with an ice block and ice around the rack to prevent heating and make the transfer neater.

Blocking after the transfer run was performed with Everyblot Blocking Buffer (BioRad), which enabled performing it in 5 minutes. The primary antibody of c-Myc Monoclonal Antibody (9E10) (Invitrogen) consisted of two primary antibodies, where the ratio of c-Myc monoclonal antibody was 1:500, and the ratio of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was 1:2000. GAPDH was used as a control for visualizing the equal amounts of loaded

lysates. The membrane was incubated in the primary antibody Everyblot Blocking Buffer solution overnight at +4 °C with gentle rocking. After the incubation, the membrane was washed three times with 1X Tris Buffered Saline Tween (BioRad) (TBST) for 10 minutes each with gentle rocking.

A Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (Invitrogen), was added to the solution in a ratio of 1:2000. The membrane was incubated in the secondary antibody in Everyblot Blocking Buffer solution for 1 hour at room temperature while gently rocking. After the incubation, the membrane was washed thrice with TBST for 5 minutes with gentle rocking. After completing the washes, the membrane was covered with 2 ml of Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) for a couple of minutes. Supersignal activates the HRP conjugate of the secondary antibody, which creates light and enables imaging with a clear signal and better visualization. The images were captured with iBright CL1500, and the image analysis was with both iBright CL1500 and ImageJ.

3.4 ANALYSIS OF THE ZIF-8 MEDIATED DELIVERY

Cellular uptake and endosomal escape experiments are essential to studying the features of nanoparticles (Liu et al. 2021). The preparation for the experiments began with transferring HepG2 and Huh-7 cells into 35 mm glass bottom confocal dishes (Greiner Bio-one) with fresh DMEM. The cells were incubated overnight at 37 °C in 5 % CO_2 to achieve 60 - 70 % confluence in the dish. The old DMEM was discarded and replaced with an Opti-MEM solution. Into the Opti-MEM solution, approximately 40 µl of Cy5.5 labeled Zif-8 nanoparticles was added to each confocal dish. The HCC cells were transfected with Zif-8 and incubated at 37 °C in 5 % of CO_2 for 1, 3, 5, or 19 hours to create samples with different transfected points in time.

After achieving the desired time point, the old solution was discarded from the dish. The cells were washed twice with 700 µl of Hank`s Balanced Salt Solution (HBSS) - HEPES pH 7.4 medium by pipetting the HBSS-Heps medium against the wall of the confocal dish slowly and discarding the

medium. The lysosomes were then stained with 300 µl of LysoTracker DND-26 Green (10 mM, Invitrogen) and incubated for 60 minutes at 37 °C in 5 % CO_2. After the incubation, the cells were fixed, which ended the cell activity. The fixation led to samples with 2, 4, 6, and 20 hours after the transfection, which began with adding approximately 500 µl of 4 % paraformaldehyde and incubated for 15 minutes at room temperature. The samples were then washed twice with the HBSS-HEPES medium. For nuclei staining, 9 µl of 4,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (5 mg/ml), was added to the cells. DAPI was incubated for 6 minutes at 37 °C in 5 % CO_2 and washed thrice with the HBSS-HEPES medium. 700 µl of HBSS-HEPES medium was added to the fixed confocal dishes, which were covered from the light, with aluminum foil. The fixed samples were observed with a Zeiss LSM 880 confocal microscope with 63x/1.20 oil immersion objective with 405nm Diode, 488nm Argon, and 633 nm HeNe lasers.

3.5 DNA EXTRACTION AND PURIFICATION

Santa Cruz Biotechnology does not share information about the sequence of the knockout of the sgRNA. Sequencing was performed to achieve the information on c-Myc knockout cleavage and sequence. The protocol of Monarch Genomic DNA Purification Kit (New England BioLabs Inc.) was used for DNA extraction and purification.

The extraction began with sample disruption and homogenization by the regular cell culturing of HepG2 and Huh-7. The cell culturing was performed by following the protocol of 3.4.1. After the cells were formed in the pellet in the centrifuge, the supernatant was discarded and replaced with 100 µl of ice-cold PBS into each Eppendorf. After resuspending the cell pellet into PBS, 1 µl of Proteinase K, and 3 µl of Monarch RNase A were added straight into the solution and vortexed briefly. Then, 100 ml of Monarch gDNA Cell Lysis Buffer was added and vortexed thoroughly until the solution altered more viscose. The solution was incubated at 56 °C in a thermal mixer with agitation.

DNA binding and elution began with adding 400 µl of Monarch gDNA binding buffer to the sample and mixing well with vortex for 10 seconds. The buffer mix was then carefully pipetted into the Monarch gDNA purification column inside a collection tube. The tube was centrifuged for 3 minutes at 1000 g to bind the gDNA to the sample. The tube was centrifuged at full speed for 1 minute to achieve a clear membrane. The flow solution was then discarded. The column was then transferred into a new collection tube. 500 µl of Monarch gDNA Wash Buffer was added. The tube was centrifuged at full speed for 1 minute, and the flow solution was discarded after the centrifuging. The column was then placed into a 1.5 ml Eppendorf tube and 50 µl of 60 °C Monarch gDNA Elution Buffer was added and incubated at room temperature for 1 minute. Lastly, the Eppendorf was centrifuged at full speed for 1 minute to achieve purified and eluted DNA for sequencing. The purification and concentration of DNA samples were measured with the UV-Vis Spectrophotometer (Nanodrop 2000c, ThermoFisher). The samples were then diluted with nuclease-free water to achieve the wanted amount, 10 ng/µl, of the purified DNA for sequencing. The DNA samples were then sent to Eurofins Genomics for sequencing by the Sanger Sequencing method.

4 RESULTS

4.1 CHARACTERIZATION AND ANALYSIS OF ZIF-8

TEM provides an observation method for Zif-8 nanoparticle characterization due to its ability to determine particle size, morphology, and uniformity (Shahsavari et al. 2022). Zif-8 nanoparticles were observed without a plasmid load and with CRISPR/Cas9 edited plasmid. Figure 7 represents Zif-8 nanoparticles. The size of only Zif-8 is smaller when there is no cargo inside. In Figure 8, Zif-8 nanoparticle sizes remained distinct. However, they share similar shapes, and the diameter of the nanoparticles is approximately between 100 and 200 nm, which enables cargo loading. The size of the prepared Zif-8 nanoparticles is optimal for cellular uptake.

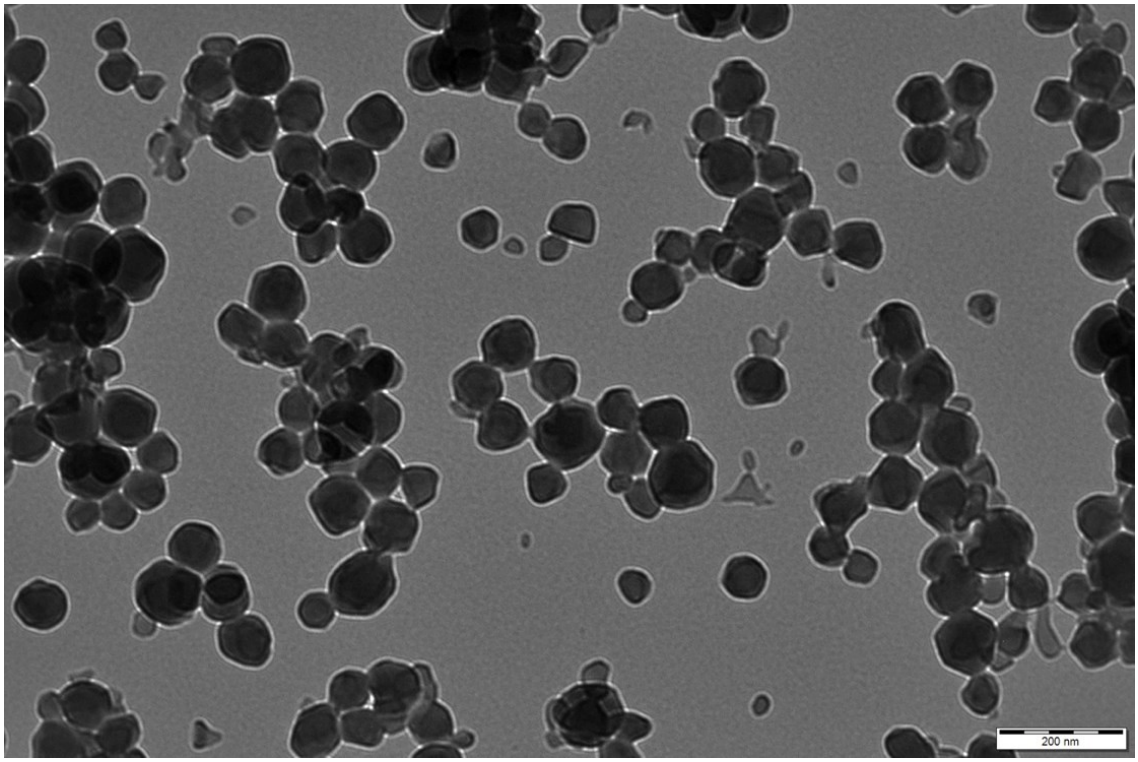


Figure 7. Empty Zif-8 nanoparticle characterization observed with TEM. The scalebar is set to 200 nm.

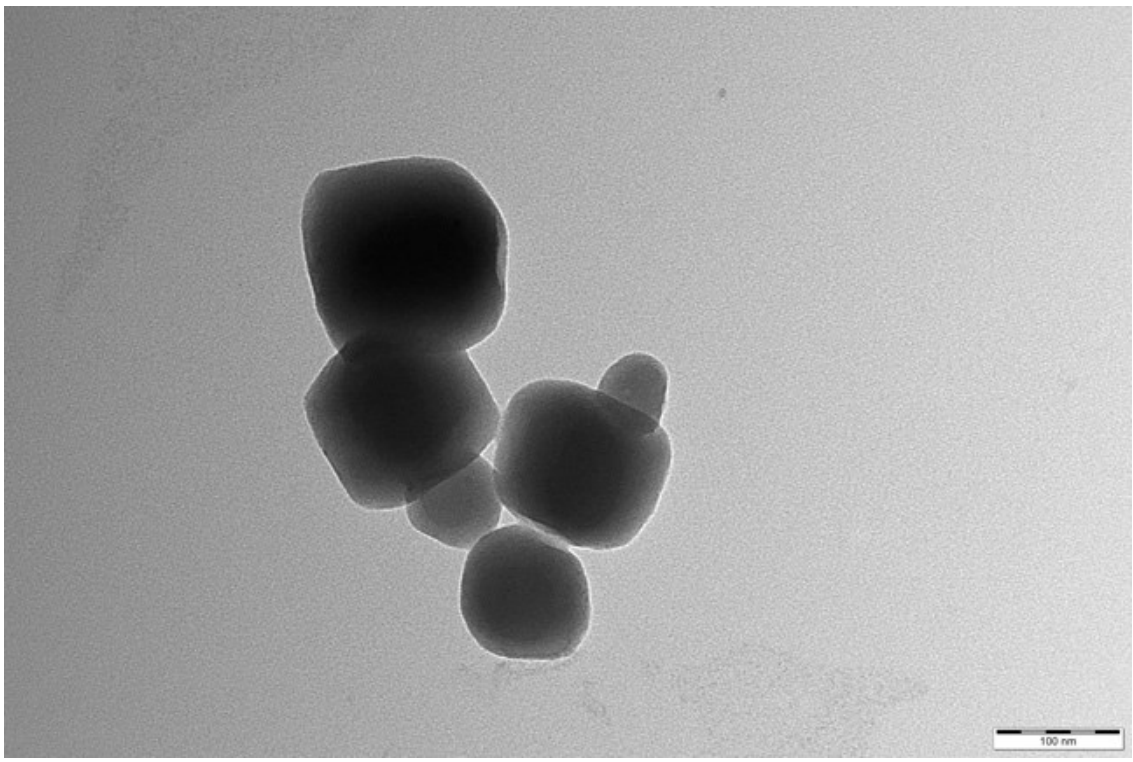


Figure 8. TEM images of Zif-8 nanoparticles containing the plasmid. The scalebar is set to 100 nm.

In Figure 8, plasmid-loaded Zif-8 nanoparticles are represented. The nanoparticles share a similar shape as the empty Zif-8 nanoparticles. Notably, the loaded nanoparticles have larger diameters when compared to the regular Zif-8 nanoparticles that do not have cargo loaded.

Table 3. Comparison between loaded and load-free Zif-8 nanoparticles to observe the plasmid intake and the effects of the plasmid on nanoparticle size and potential.

	Average sizes	Average zeta potentials
Zif-8 nanoparticles without loading	99.78 ±2.24 nm	22.8 ± 4.39 mV
Zif-8 nanoparticles loaded with plasmid	117.5 ±5.11 nm	-29.6 ± 2.82 mV

When investigating how cargo loading affects the Zif-8 properties, the average size and zeta potential should be measured. Table 3 gathers the most noteworthy results of comparing Zif-8 nanoparticles without plasmid and Zif-8 with the edited plasmid. The loaded Zif-8 nanoparticles had a larger

average size, which indicates that the loaded plasmid is inside the nanocarrier. Despite the loading increasing the average size, the Zif-8 is in the ideal size range in both ways. Since DNA contains negatively charged phosphate groups in its nucleotides, the overall potential is negative in DNA plasmids. Hence, the Zif-8 with loaded plasmids should have negatively charged zeta potential. In Table 3, the Zif-8 without plasmids has positive zeta potential, approximately +22.8 mV. In contrast, in the Zif-8 containing the plasmid, the average zeta potential alters into negative is approximately -29.6 mV. The zeta potential change indicates that the plasmid has successfully been taken inside Zif-8 nanoparticles. Polydispersity index (PDI) of the nanoparticles was approximately 0.15, which indicates relatively homologous structure of the nanoparticles.

4.2 DELIVERY AND TRANSFECTION OF PLASMID

The plasmid contains puromycin resistance, which enables selection. The gene was used to determine which cells have successful transfection and can survive after the puromycin treatment. Puromycin for Huh-7 and HepG2 cells was performed. The cells were seeded into 96 well plates and incubated for 48 hours after adding puromycin in distinct concentrations. The cells were observed under microscopy to determine which concentration was crucial to the cells. As a control, WST-1 stain was added into the wells, which will provide the determination of the survived cells and highlight which cells have the resistance towards puromycin antibiotic after imaging with VarioSkan. The results are more reliable since WST-1 staining highlights the number of living cells, whereas observing with a microscope provides information on the environment without providing information on how many cells can live. The determination of living cells after WST-1 staining was performed regarding the protocol of Abcam WST-1. The cell cytotoxicity assay started by subtracting the background of DMEM wells from the wells in the assay to have corrected absorbance. Then, the cytotoxicity percentage was calculated by the following formula, where control is the negative control concentration (0 μg/ml) and the sample other concentration.

$$Cytotoxicity\ (\%) = \frac{100 * (Control - Sample)}{Control}$$

The cytotoxicity percentage was then adjusted into cell viability by the formula below. The results are listed in Table 4 and Table 5.

$$Cell\ viability\ (\%) = 100\ \% - Cytotoxicity\ \%$$

HepG2 wild-type cells were observed under the microscope. Below the concentration of 1.5 mg/ml, most cells survived. HepG2 cells were dead at 3 μg/ml or higher concentration. When observing the Zif-8 mediated transfected cells, they survived at 4 mg/ml concentration under a microscope. The wild-type HepG2 cells could resist the puromycin antibiotic surprisingly well without the puromycin resistance gene when observed under a microscope.

Then, the cytotoxicity experiment was repeated, and the concentrations were observed with WST-1 staining. The results are shown in Table 4, where it can be visualized that the transfected HepG2 cells were alive at the concentration of 3 μg/ml, but they could not survive at the concentration of 5 μg/ml. The HepG2 wild-type cells did not survive after puromycin treatment. The puromycin resistance gene in the transfected HepG2 cells enhances the resistance.

Table 4. The HepG2 cell viability after 48 hours of incubation of distinct concentrations of puromycin. Grey represents Zif-8-mediated transfected HepG2 cells, whereas green represents HepG2 wild-type cells.

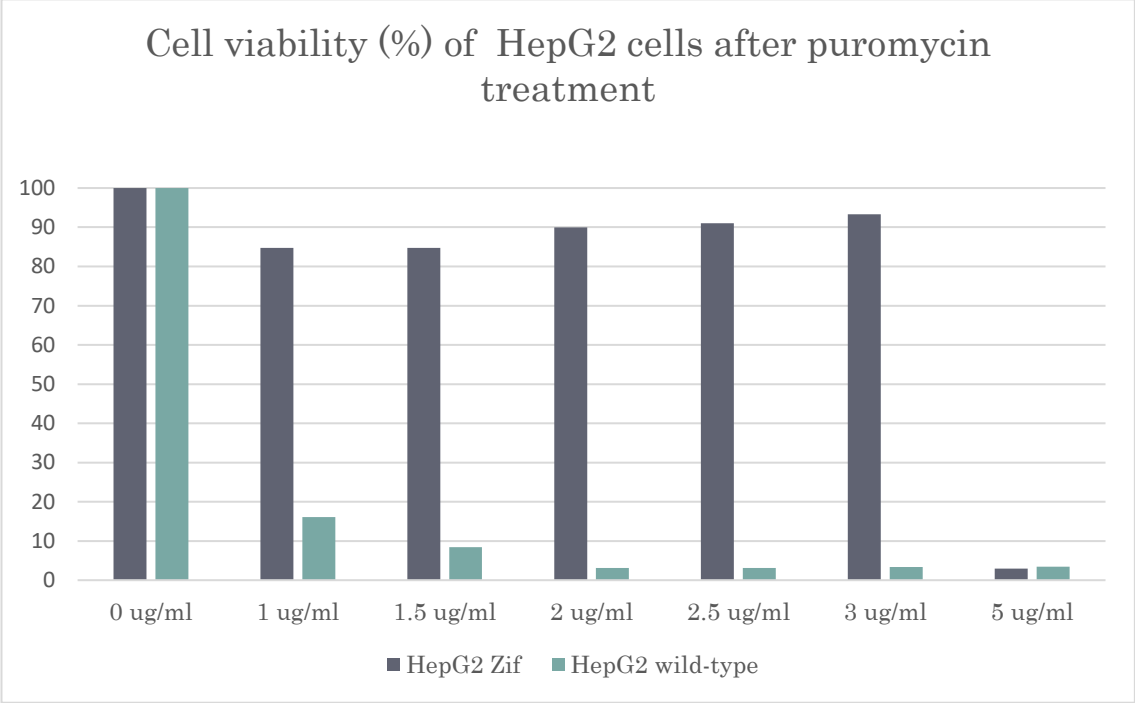
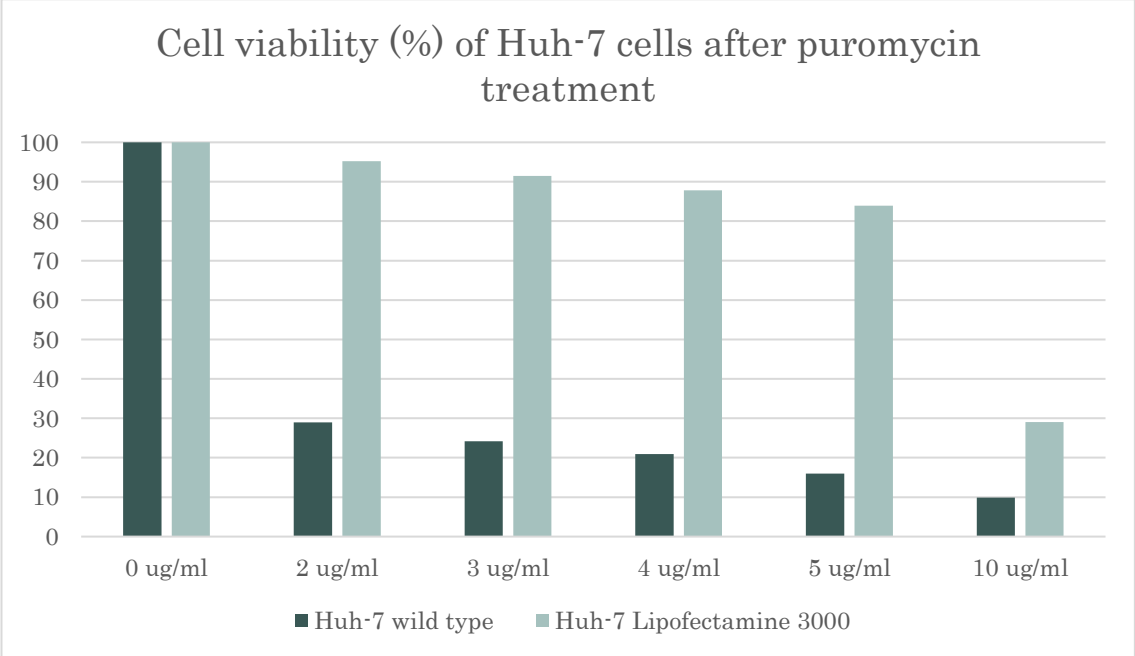


Table 5. Cell viability after 48 hours of incubation of distinct concentrations of puromycin. Darker green represents the results of Huh-7 wild-type cells, whereas lighter green represents Huh-7 cells with Lipofectamine 3000-mediated transfection.



In Table 5 represents results from both Huh-7 wild-type and Lipofectamine 3000-mediated transfected Huh-7 cells. The wild type Huh-7 cells could not

maintain living after adding puromycin significantly well. Notably, there occur alive cells in the highest concentration. The transfected Huh-7 cells resist puromycin better overall when compared to the HepG2 cells. Tables 4 and 5 indicate that the cells continued living in both cell lines similarly until the concentration of 3 µg/ml. Table 5 demonstrates that Huh-7 provided a better outcome in this cell viability assay after transfecting the CRISPR/Cas9 edited plasmid with the resistance gene, despite the HepG2 cells lack of information at 4 µg/ml concentration.

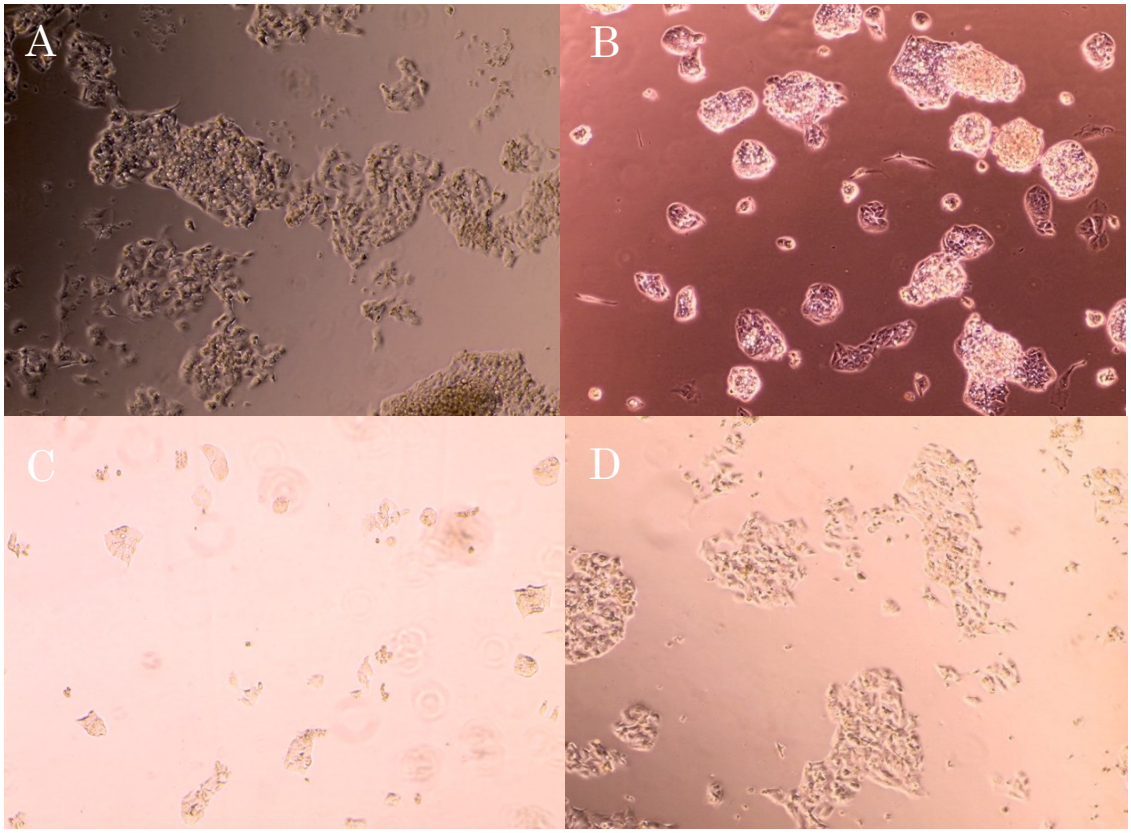


Figure 9. A image represents the CRISPR/Cas9 edited sgDNA plasmid transfected Zif-8 mediated into HepG2 cells. B image represents the Zif-8 mediated transfection of the GFP plasmid into HepG2 cells. C image represents HepG2 cells with Lipofectamine 3000-mediated transfection of the CRISPR/Cas9 edited sgDNA. D image represents wild-type HepG2 cells as a control. All the images are captured with EVOS digital microscopy.

Figure 9 enables visualization of HepG2 cells after successful transfections with different plasmids and methods. The images are taken with EVOS digital microscopy to highlight the similarities and differentiations after successful plasmid transfections. In image A, HepG2 cells with CRISPR/Cas9

edited sgDNA plasmid through Zif-8 nanoparticle transfection. In image B, the HepG2 cells are transfected with GFP plasmid through Zif-8 nanoparticle-mediated transfection. In image C, the HepG2 cells are transfected with sgDNA plasmid through Lipofectamine 3000-mediated transfection. The D image represents control of HepG2 wild-type cells to enable comparing the cell morphology. Figure 9 highlights that lipofectamine-mediated transfection altered the cell morphology the greatest. The wild-type HepG2 and sgDNA plasmid transfection with Zif-8 share similar morphology.

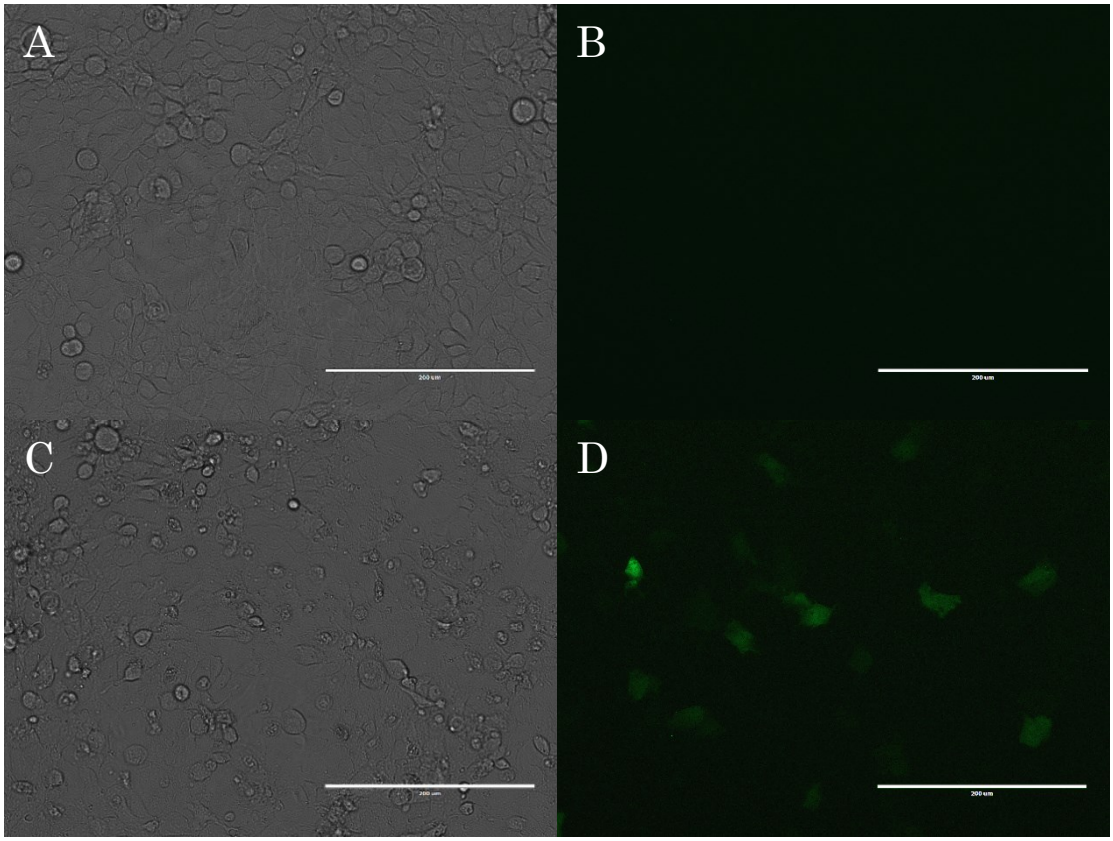


Figure 10. Control image of Huh-7 cells, A with light field and B with GFP filter. Control image of Lipofectamine™ 3000-mediated transfected Huh-7 cells 24 hours after transfection, C with light field and B with GFP filter. Images are captured with an EVOS fluorescent microscope (AMG), and scalebars are set to 200 μm.

Figure 10 represents the result from Huh-7 wildtype cells compared to Lipofectamine™ 3000-mediated transfected Huh-7 cells. Images A and B are captured from the same position with the light field and GFP filter. with EVOS fluorescent microscope. In Images A and B, there are wild-type Huh-7 cells as a control to enable visualization of the lack of fluorescent plasmids.

Images C and D are captured from the same position with the light field and GFP filter. In images C and D, Lipofectamine™ 3000-mediated transfected Huh-7 cells were captured 24 hours after transfection.

The imaging was performed to enable visualization if transfection was successful, and the transfected plasmids activate under fluorescence with the GFP filter. The results from Figure 10 indicates the delivery of the plasmid was performed successfully. The plasmids confirm the delivery into Huh-7 cells since the GFP markers of the plasmid remain activate when visualizing with the GFP filter.

4.3 PROTEIN EXPRESSION ANALYSIS OF C-MYC

Western blot analysis was performed to gain information on how transfected HepG2 cells differ when compared to wild-type HepG2 cells. The main goal for western blot protein analysis was to investigate how c-Myc was expressed in the HepG2 cells after different transfection methods and compare the results to the wild-type, unedited HepG2 cells. Proteins were loaded in equal amounts to achieve reliable results. GADPH was used as a control to highlight the same concentrations of the loaded lysates.

The c-Myc protein expression comparisons are represented in Figure 11 below. The greatest expression of c-Myc was in the wild-type HepG2 cells, and the least amount of c-Myc protein expression was observed in the Lipofectamine 3000-mediated transfected HepG2 cells. When comparing the results of c-Myc expression in Zif-8 mediated transfection, the expression is lower when the amount of Zif-8 is greater. That can indicate that transfection leads to the downregulation of c-Myc expression.

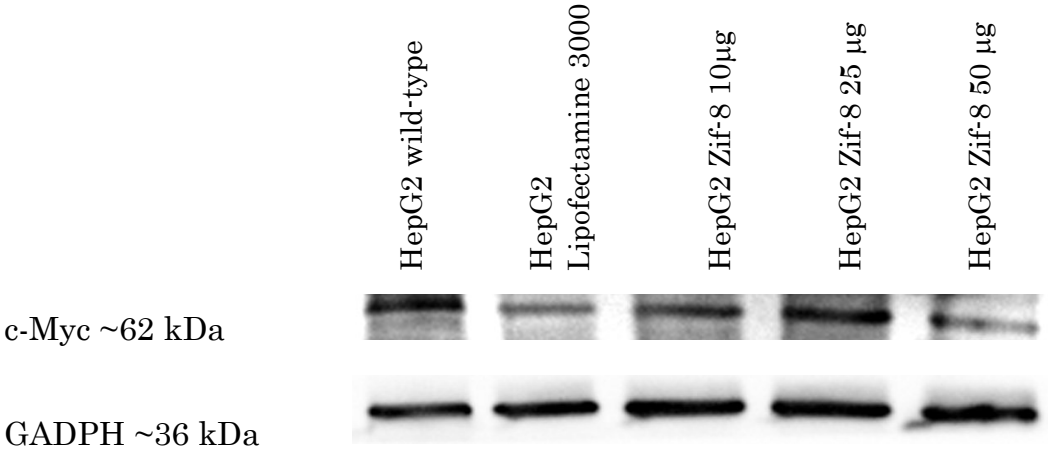


Figure 11. The protein expression of c-Myc was observed in chemiluminescent western blot analysis. The results were captured with the iBright CL1500 imaging system. Image normalization and densitometric analysis were performed with iBright CL500 and Fiji ImageJ software.

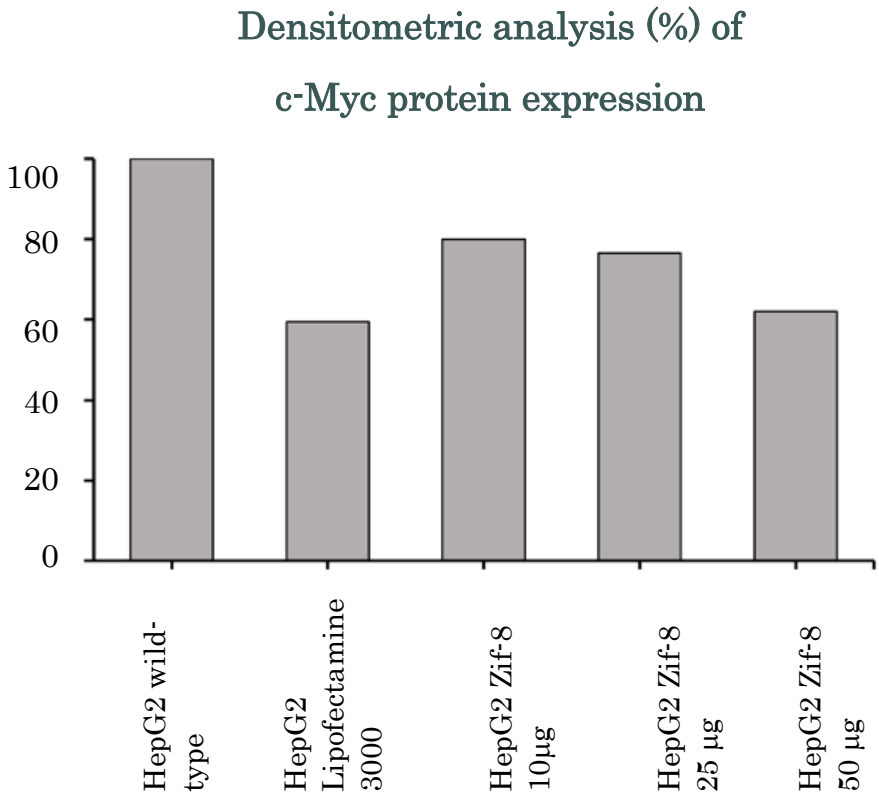


Figure 12. Densitometric analysis of c-Myc protein expression against the control GADPH. The results are normalized against wild-type HepG2 cells. The densitometric analysis, quantitation, and normalization were performed using the iBright CL500 imaging system and ImageJ software.

The densitometric analysis of c-Myc protein expression represented in Figure 12 provides information about the quantities of expression in percentages. The results demonstrate that the lowest amounts of c-Myc protein expression occur after Lipofectamine 3000-mediated transfection, whereas the highest expression level occurs in wild-type, unedited HepG2 cells. The comparison of the plasmid containing Zif-8 nanoparticles with distinct amounts indicates that the greater the amount of Zif-8, the lower the expression of c-Myc occurs. The densitometric analysis provides significant information about the successful transfection and downregulation of c-Myc. Lipofectamine 3000-mediated transfection was used as a control method to achieve successful transfection, and it was used to compare the results to the Zif-8-mediated transfection method and its efficacy in downregulating.

4.4 CELLULAR UPTAKE, ENDOSOMAL ESCAPING & NUCLEUS TARGETING OF THE PLASMID

For achieving the effective outcome of CRISPR/Cas9, edited plasmid, and its nucleus targeting, the cellular uptake and endosomal escaping may require optimization. The cellular uptake, endosomal escape, and nucleus targeting were observed by Zeiss LSM780 confocal laser scanning microscopy. Confocal imaging was based on fixed samples at the distinct fixation points in time. The fixed samples of HepG2 Zif-8-mediated transfected cells are at 2, 4, 6, and 20 hours. The fixed samples of Huh-7 Zif-8 mediated transfected cells are at 4 and 20 hours.

The fixation enables observing the results of the time points and how the nanoparticles, endosomes, and nucleus are represented in distinct colors. The co-localization of two objects alters the color as a combination due to overlapping locations, which enables observing distinct colors from the used dyes. There are three dyes to color the nucleus, endosomes and lysosomes of the cells, and the Zif-8 nanoparticles. LysoTracker™ Green DND-26 colored endosomes and lysosomes green. Cyanine5.5 NHS ester (Cy5.5), seen as red

color, was used to highlight the nanoparticles and their components at 684 nm excitation maximum and emission maximum at 710 nm. DAPI (4,6-Diamidino-2-phenylindole, dihydrochloride) highlighted the nucleus of the cells. DAPI colored the nuclei blue fluorescence.

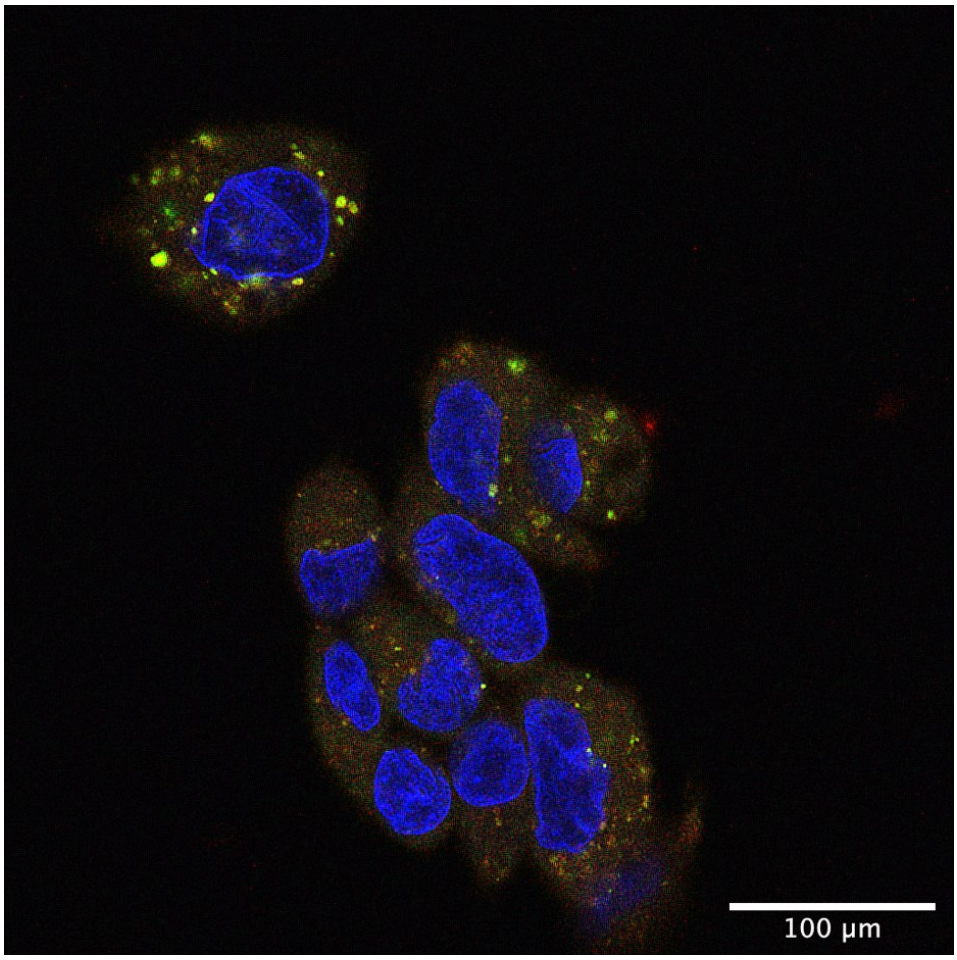


Figure 13. The fixed sample of 2 hours represented after Zif-8 mediated transfection of HepG2 cells. Nanoparticles are colored red, nuclei blue, and endosomes and lysosomes green. The scale bar is set to 100 µm.

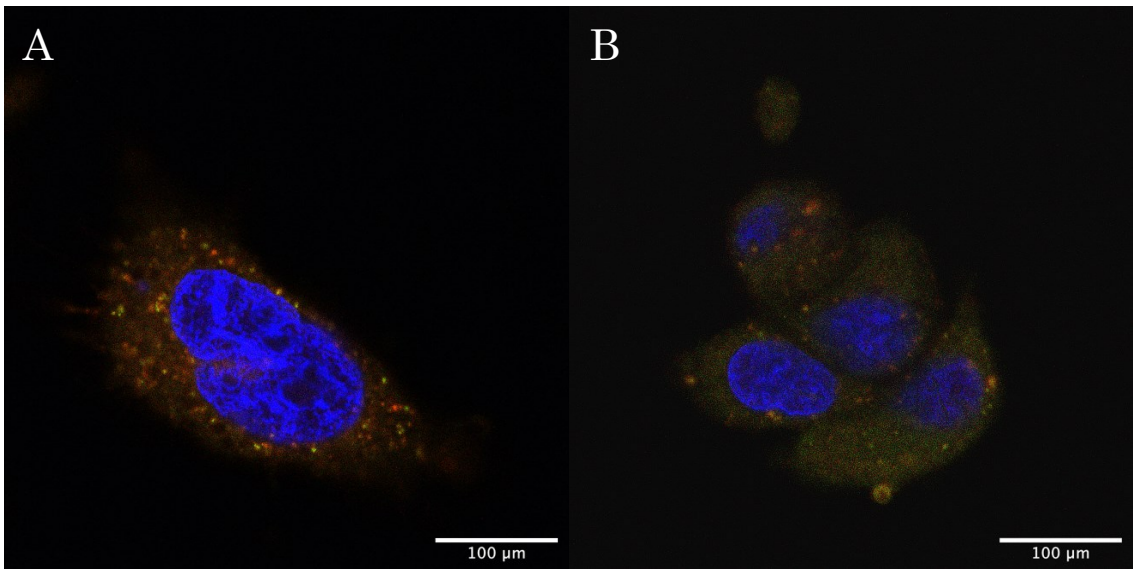


Figure 14. The fixed samples of 4 hours represented after Zif-8 transfection. The A image represents the Huh-7 fixed sample, whereas the B image represents the fixed HepG2 sample. The scale bars are set to 100 µm.

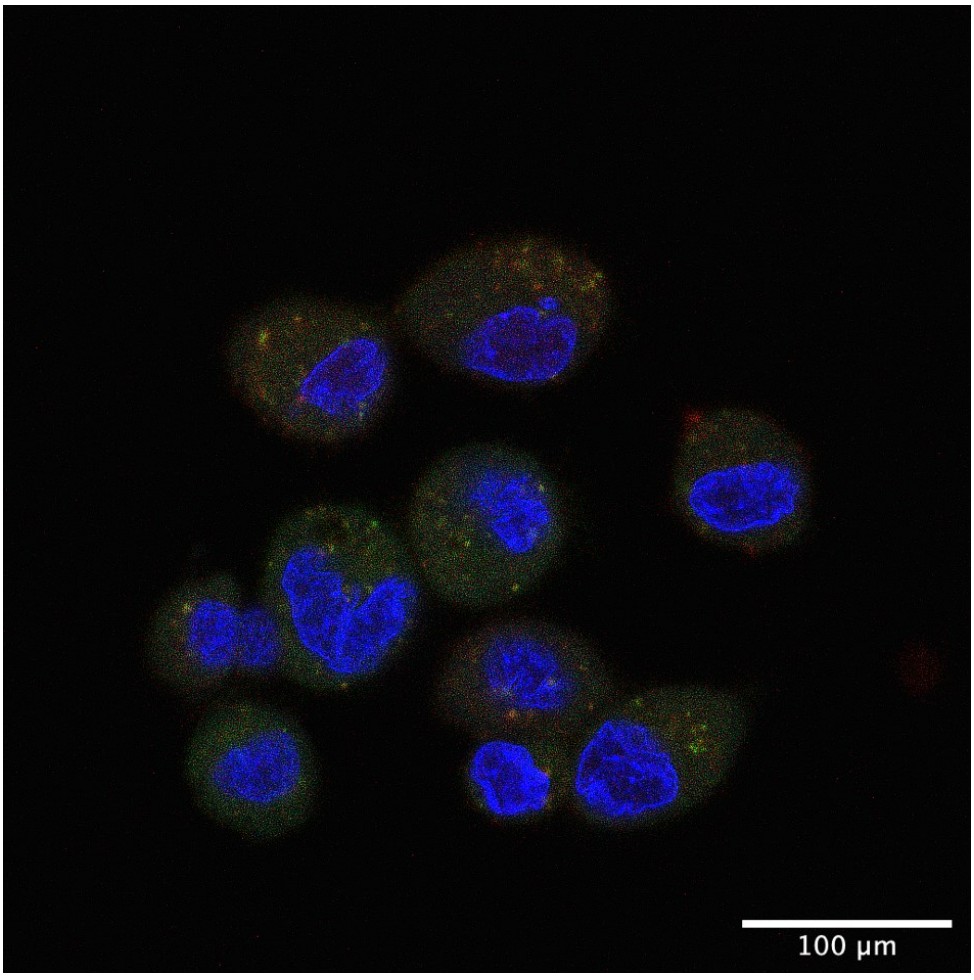


Figure 15. The fixed sample of 6 hours was represented after Zif-8 transfection into HepG2 cells. The scale bar is set to 100 µm.

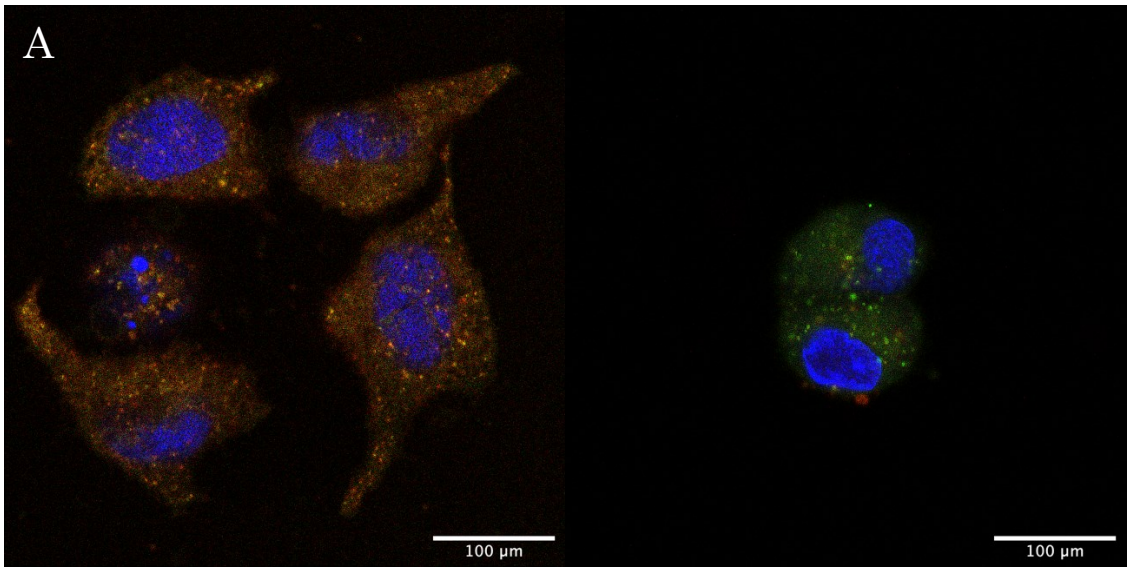


Figure 16. The fixed samples of 20 hours represented after Zif-8 transfection of Huh-7 cells in image A and Zif-8 transfection of HepG2 in image B. Scale bars are set to 100 µm.

After the delivery of the edited plasmid into the cell, the plasmid should migrate into the nucleus for further transcription of Cas9 mRNA and gRNA (Sioson et al. 2021). The delivered Cas9 nuclease is translated to mRNA. The delivered Cas9/gRNA complex then cleaves the DNA into a specific DNA site after the translocation of the nucleus.

In the sample of 2 hours after transfection in Figure 13, HepG2 cells had taken the Zif-8 complex into the cell. The colocalization of endosomes and Zif-8 nanoparticles had begun. However, the amount of colocalization is not high yet because the colors remain green and red in large areas inside the cell. Additionally, several nanoparticles occur in cytosol, and some endosomes remain empty. The colocalization creates a yellow color when combining red and green, which provides highlighted information about the locations of nanoparticles. At the 2 hours after transfection, the results highlighted that the cellular uptake has been successful since outside of the HepG2 cells, the Zif-8 nanoparticles do not occur at this filter setup. The nanoparticles had migrated into cells, and some Zif-8 nanoparticles had already been taken into endosomes.

Observing Figure 14 of the 4 hours after transfection of Huh-7 cells and HepG2 cells highlights the peak of the colocalization of endosomes and nanoparticles. I would state that 4 hours after the transfection is when the endosomal escape begins since most of the nanoparticles have been in the endosomes of the cells. Notably, Huh-7 cells have more red and yellow, which may be caused by better fitness of the cells and a higher number of Zif-8 nanoparticles compared to the HepG2 sample. The nuclei remain blue in both confocal images.

Figure 15 provides information on 6 hours after transfection into HepG2 cells. The nanoparticles have escaped from the endosomes since there is not much colocalization. The colors have brightened, and colocalization has its lowest point since all the colors remain mostly green, red, and blue. However, the nanoparticles have migrated to a more centered part of the HepG2 cell near the nuclei. Some have been taken into the nuclei for further transcription of the DNA inside of the plasmid. Although the endosomal escape may occur between 4 and 6 hours after transfection, nuclei targeting occurs approximately 6 hours after transfection.

Figure 16 represents the final sample of 20 hours after transfection of Huh-7 and HepG2 cells. The nanoparticles have returned to the cytosol in the Huh-7 cells. After 20 hours of transfection, the number of nanoparticles in HepG2 cells has decreased significantly. The nuclei remain blue, and the endosomes remain green, which may indicate the nanoparticle absence. In HepG2 cells, the lysosome degradation of Zif-8 nanoparticles may have begun earlier, whereas the degradation will begin due to the significant number of nanoparticles remaining in the cytosol in the Huh-7 cells.

The samples of 2, 4, 6, and 20 hours of Huh-7 and HepG2 provided valuable information about the migration and locations of nanoparticles within distinct points in time. These results indicate the possibility of using Zif-8 as a nanocarrier due to the protected release, endosomal escape, and migration into nuclei. After nuclei release of DNA and its genes loaded into a plasmid, the nanoparticles returned to the cytosol, where lysosomes should degrade the structures of Zif-8 nanoparticles.

Table 6. Merge of the nanoparticles, endosomes, and nuclei compared to freely located nanoparticles in HepG2 cells. ImageJ software provided the data of merged percentages from the HepG2 images above.

Percentage (%) of merged nanoparticles within different time points	The merged amount (%) of nanoparticles and endosomes	The merged amount (%) of nanoparticles and nuclei
2 hours after transfection into HepG2 cells	22.22 %	9.62 %
4 hours after transfection into HepG2 cells	13.19 %	11.62 %
6 hours after transfection into HepG2 cells	8.07 %	17.87 %
20 hours after transfection into HepG2 cells	19.50 %	3.7 %

The merged amounts as percentages in Table 6 highlight the migration and locations of nanoparticles at different points in time in HepG2 cells. Table 6 shares comparable results of the nanoparticle locations from Figures 12-15. Huh-7 samples provided data from 4 and 20 hours after Zif-8-mediated transfection. Therefore, the results from Huh-7 transfections were left out of Table 6. Table 6 supports the data from Figures 12-15. The colocation peaks 2 hours after the transfection, and some nanoparticles appear in nuclei. At time points 4 and 6 hours after the transfection, the number of nanoparticles decreases, whereas the number of nanoparticles increases in nuclei, which states the endosomal escape and migration towards nuclei. At 20 hours after transfection, the number of nanoparticles increases in endosomes. However, the same dye is used to stain lysosomes, so the nanoparticles have become dissipated. The nanoparticles release the edited plasmid into nuclei, where the transcription may begin.

5 DISCUSSION

Hepatocellular carcinoma has become more common yearly worldwide without efficient and great long-term therapeutics and therapies. Up to 90 % of all liver cancers and dysfunctions are caused by hepatocellular carcinomas, which lead to an urge to develop novel therapeutic alternatives without causing toxicity, severe side effects, or resistance. Since c-Myc is the major regulator of human genes and responsible for up to 70 % of human malignancies due to its overexpression, it could be utilized in novel therapeutic development by downregulating its expression in the cells. To downregulate c-Myc, gene editing and methods to deliver the edited genes into nuclei of the cells are required.

The thesis aimed to achieve a successful delivery of CRISPR/Cas9 edited plasmid of the c-Myc downregulation into hepatocellular carcinoma cells through Zif-8 nanocarrier. The aim was to observe how HCC cells will uptake the Zif-8 complex, and how the plasmid inside Zif-8 is migrating at different points in time. The thesis aimed to investigate if c-Myc downregulation through gene editing could be transferred into HCC cells and how that affects cell proliferation and cycle through KO experiments.

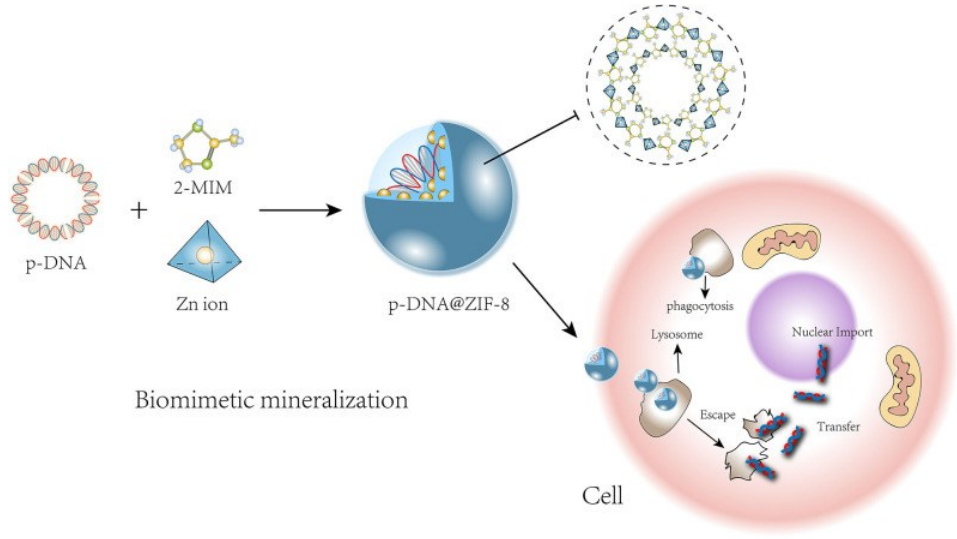


Figure 18. The CRISPR/Cas9 edited DNA plasmid combines Zif-8 for cell uptake (Xing et al. 2020). In the HCC cell, the edited plasmid will escape from

the Zif-8 complex in the endosome, and the plasmid will migrate towards the nucleus.

The schematic of this thesis can be visualized in Figure 18. The plasmid is combined with Zif-8, creating protection of cellular uptake and early release. After the cellular uptake in the acidic malignant environment, the endosomal escape has begun which promote release the plasmid and its edited genes, which will then migrate toward the nucleus of the HCC cell.

5.1 HCC AND ITS FUTURE ALTERNATIVES OF NANOPARTICLE-MEDIATED THERAPEUTICS

Hepatocellular carcinoma is commonly known for its high morbidity and mortality rates among HCC patients (Ji et al. 2018). The research of the genetic variation within various diseases has become easier and more archivable due to the enhanced technology to study the cellular processes throughout animal models (Kim et al. 2017). The technology developed for research has enabled us to widen the therapy alternatives for rare genetic diseases. CRISPR/Cas9 has enabled researchers to achieve the maximum benefit of identifying and targeting the sequence of interest for altering the sequence for the editing sequence-specific genes.

Although vaccinations and anti-viral therapies have reduced the rate of HCC, the number of patients increases worldwide simultaneously as the common risk factors (Llovet et al. 2021). Fortunately, the survival percentage has improved over the past years by enhanced prevention of the main risk factor, HBV (Chan et al. 2022). The nanoparticle-mediated novel cancer therapy has shown significant advantages due to their specific targeting, reduced side effects and lack of drug resistance (Yao et al. 2020). The ideal nanoparticle size and characteristics should choose the delivery method of nanoparticles for each cancer to achieve the most optimal delivery method for the therapeutic. Precise targeting will prevent normal cells from cytotoxicity and enhance the efficacy of the drug. The aim for future cancer treatment will be focused on targeting the active cancerous cells rather than previous methods

that include killing the normal cells among cancerous cells. The precise targeting of cancerous cells has shown promising results.

5.2 ZIF-8 AS A DELIVERY METHOD FOR CRISPR/CAS9 GENE EDITING PLASMID

The advantages of Zif-8 as a nanocarrier provide the controlled release for editing the human genes in cancers (Xu et al. 2021). Zif-8 is categorized as stimuli-responsive nanocarriers, which enable release in the accurate and optimal environment to achieve greater therapeutic performance and outcome. Zif-8 is pH-responsive due to the protonation capacity of imidazolate, which can be utilized in the release of therapeutics in the accurate cancerous environment, which decreases the off-targeting risks. The cancerous cells and tumors' environment has a low pH, about 4-6.5, whereas in the normal environment of the tissues, cytosol, and blood of a human or cells, the pH is about 7.4. In general, the longer the nanocarrier remains in the blood circulation, the better outcome of the cargo loading, and the release can be achieved (Xu et al. 2021). The loaded cargo will circulate longer in the blood circulation, which leads to reaching the optimal sites of the organism more accurately.

One of the most important impacts a nanocarrier should have is its stability and stable structure, which is important to reduce therapeutic degradation during the transport into the target cell. Under the physiological environment, Zif-8 remains stable until Zif-8 has reached its target cell or tissue to release its cargo. The nanocarrier-mediated delivery with the optimal size and surface characteristics might increase the half-time of the therapeutic and induce the accumulation into the target tumor cells or tissues (Yao et al. 2020). The characteristics of Zif-8, such as high biosafety, decomposition in acidic solutions, induction of cellular autophagy and large specific surface area, enable its applications in cancer therapies (Xie et al. 2022). The release of the therapeutic and its loading depends on the carrier's structure and the loaded therapeutic properties (Feng et al. 2022). The

surface should be adjusted to match the properties of the loaded therapeutic and the used carrier to enhance the loading, release, and dissolution.

Zif-8 has been widely utilized as a nanocarrier in drug delivery. It has been carrying chemical-based drugs, photothermal-, photodynamic-, and chemo dynamic-based agents, proteins and nucleic acid-based therapeutics and complexes, such as CRISPR/Cas9. Despite all the significant advantages of Zif-8 in drug delivery, there occur issues regarding the toxicity involved of Zif-8 nanoparticles (Feng et al. 2021). Some studies show that surface adjusting with PEG molecules decreases the toxicity Zif-8 nanoparticles may cause. The study of Mete et al. showed the potential of Zif-8 nanocarrier for the most commonly used orally taken sorafenib. The results showed that the nanocarrier mediated delivery enhances the cytotoxicity when compared to the drug effects of sorafenib itself. They suggested the importance of the Zif-8-mediated drug delivery, and showed how it is an effective therapy for HCC in the future. They determined the time difference of release in the physiological pH and compared the release to the acidic tumor environment, which provided the results of optimized and faster release in an acidic environment, as suggested.

Several issues require optimization and consideration when Zif-8 is used as a nanocarrier. One of the most important things to evaluate is the efficiency and specificity of the delivered CRISPR/Cas9 complex throughout Zif-8 nanoparticles into the targeted HCC cells. The delivery vehicle may require some modification of its surface chemistry, size, and shape to achieve better cellular uptake outcomes and the delivered genes' stability. However, the results will not be as accurate *in vitro* experiments when compared to *in vivo* experiments since *in vitro* experiments provide less information on- and off-targets than *in vivo* experiments. Furthermore, the amount of the delivered genes requires evaluation before determining enough of the complex into HCC cells.

Despite the lack of identical *in vitro* studies of Zif-8 nanocarrier for edited genes into HCC, numerous studies for Zif-8 carry different therapeutics and components into HCC cells. Feng et al. discussed widely different studies of

Zif-8 versatile usages to carry components into HCC cells with great loading capabilities, low cytotoxicity, and accurate pH-mediated release. However, since the studies of Feng et al. discussed were not performed using the CRISPR/Cas9 edited genes loaded into Zif-8, HepG2 cells were used as HCC cell lines and Zif-8 showed promising results with the numerous studies as carriers with the aimed impacts. Therefore, the results are not entirely in line with the previously published data because there has yet to be a similar experiment. Multiple studies have shown promising results of Zif-8 being an excellent cargo carrier for cancerous cells due to its versatile and unique properties and characteristics. As mentioned previously, the large surface area, biocompatibility and non-toxicity makes Zif-8 a great candidate for the future therapeutic carrier, as well as the pH-mediated release of the loaded cargo. The large surface ensures the therapeutics' high loading capacity, which provides more loaded cargo to increase therapeutic delivery efficiency. Feng et al. showed results of the successful delivery of CRISPR/Cas9 with Zif-8 with great loading capacity and controlled delivery of both sgDNA and Cas9 nuclease. In this thesis, the Zif-8 nanocarrier provided great protection for the charged sgDNA and Cas9. The nanocarrier released plasmid in an acidic environment providing excellent protection. However, the toxicity of CRIPSR/Cas9 requires further studies due to the concerns related to safety in the therapy of genetic diseases.

Despite all the significant applications and benefits MOFs have provided, there are several issues regarding the use of MOFs as cargo carriers (Linnane 2022). The most limiting issues are the stability of MOFs, the possibility of aggregation in a solution, the biodistribution and the specificity of targeting tissues. The functionalization of the surface of MOFs may enhance the issues, such as PEGylation, and encapsulation with biological derived coatings. However, multiple other challenges occur regarding a nanocarrier entering the tumor tissue (Xu et al. 2021). The nanocarrier may need help delivering cargo *in vivo* due to the lack of lymphatic drainage, dense extracellular stroma, and high tumor interstitial pressure. Furthermore, the tumor is challenging due to the acidic and hypoxic environment. The delivery of CRISPR/Cas9 complex may create issues to the cellular uptake due to the

strongly negatively charged RNA and the large size of Cas9 nuclease (Feng et al. 2021).

Although Zif-8 has shown promising results in cancer therapy as the carrier, further experiments are required to overcome the safety and efficiency levels to the aimed level (Xie et al. 2022). The lack of long-term *in vivo* safety and preclinical studies challenges the researchers to proceed. After overcoming the challenges with the needed experiments, Zif-8 could provide a safe and novel delivery method for future liver cancer therapies. After achieving the required results, this could be utilized for diverse cancer therapies due to the easy modification.

5.3 CRISPR/CAS9 AS THE GENE EDITING METHOD

An efficient gene editing method provides future therapeutic aspects and possibilities to create novel therapeutics for diseases with genetic etiology (Sousa et al. 2022). The CRISPR/Cas9-based gene editing raises hopes for future genome editing for novel therapeutics (Savić & Schwank 2016). The aim for the future is to replace the malignant mutations in the DNA sequences with the normal sequence to enhance cancer therapy with CRISPR/Cas9 method (Li et al. 2020). Although there are various gene editing methods currently, there is not yet a method that provides rapid, not complicated, and cost-efficient gene editing technology. Every current gene editing method has its challenges and advantages. However, the excitement meets with the challenges of social and ethical consequences due to human gene editing.

When preparing the efficient delivery method for the CRISPR/Cas9 complex, there are multiple issues to consider. Firstly, the transfection method should be efficient to achieve less harm for the transfected cells. The delivery vehicle should be optimized for the cells to accomplish the wanted transfection as efficiently as possible. The CRISPR/Cas9 complex should be investigated sufficiently to ensure the specificity and efficiency of targeting the target cells

rather than the off-target cells or tissues. Furthermore, the complex should be able to bind specifically to the target site of DNA to avoid off-target effects or possible mutations. The stability of the delivery method and the edited plasmid should be optimized to prevent the early release and achieve the desired effect. Lastly, the characteristics of HCC cells require investigation to perform the optimization to achieve the most significant outcome overall. In this thesis, two distinct transfection methods were performed for the used HCC cell lines with desirable outcomes. Lipofectamine™ 3000 was used as the positive control, which enabled observing the transfected plasmids were working. Therefore, it provided great comparison for Zif-8 transfection, which enabled trustworthy results.

CRISPR/Cas9 must be studied more widely in safety and efficacy before moving to clinical applications (Kim et al. 2016). To be able to enhance the specificity and efficiency of CRISPR/Cas9, the double strand breaks repair pathways, modifying nucleases and changing the delivery pathway could be potential alternatives to consider. HDR provides better gene correction and insertion but lacks in the activity of the cell cycle since it only appears in the S/G_2 phase, which limits its usage to the dividing cells. In contrast, HNEJ induces loss of functions by forming indels but provides longer activation during the cell cycle without needing the repair template.

Since the gene editing experiments were only performed *in vitro*, the results may not mimic the environment of *in vivo* experiments. Although this thesis showed promising results, the methods should be optimized for the safest and most efficient delivery of gene-edited plasmid into the wanted tissues or organs. Hence, the *in vivo* experiment requires more experiments in the different cell lines and environments before proceeding with further experiments to avoid challenges such as off-target mutations.

Despite the CRISPR/Cas9-mediated gene editing providing promising results regarding cancer therapy alternatives in the future, there are multiple issues and limitations to optimize before achieving the effective and safe gene editing and delivery enough to beneficiate cancer patients (Li et al. 2019). The major concerns include the efficacy of the transfection, the vehicle for

delivery, gene editing specificity and efficiency, the plasmid stability, and the possibilities of off-target effects. Some issues require consideration regarding the CRIPSR/Cas9 complex. The targeting optimization should be performed to ensure the edited genes will target the specific cancerous cells rather than the healthy cells. The optimized targeting will decrease the risks of off targets. The targeting remains an issue when it becomes possible for a mutation to be introduced due to gene editing. The mutations may lead to crucial outcomes and create significant health problems in *in vivo* experiments and among patients. Therefore, it is essential to carefully investigate and analyze the impacts to ensure safe and effective use. Despite the great possibilities of CRISPR/Cas9, proceeding from pre-clinical studies into clinical studies is challenging because of the lack of the safeness, versatility, and effectiveness of delivery methods (Sousa et al. 2022). Gene editing with CRISPR/Cas9 provides novel possibilities to knockout or repair genes.

Despite the multiple delivery methods for CRISPR/Cas9 edited genes showing potential results *in vitro*, the delivery efficiency remains low, which leads to limiting the potential applications in *in vivo* studies (Li et al. 2020). For future use, CRISPR/Cas9 gene editing requires further experiments and development to achieve stable editing with high efficiency of targeting and enhanced loading and delivery efficiency (Duan et al. 2021). The development should proceed into safe and efficient therapy alternatives. Furthermore, the *in vivo* experiments require more optimization to overcome the issues before considering as the therapeutic aspect (Xu et al. 2021).

5.4 THE HCC CELL LINES

The HepG2 cell line is commonly used for hepatocellular cell line experiments (Meex et al. 2011). However, the Huh-7 cell line has been used as an alternative cell line to the HepG2 cells. Despite the more native characteristics that the Huh-7 cell line provides using Huh-7 cells, there are no significant advantages compared to HepG2 cells. The HCC experiments

mainly focused on HepG2 cells in this thesis due to time limitations. During the experiments, other hepatocellular carcinoma cell lines were taken as a part of the study since HepG2 cells were growing slowly, especially after the transfection. This might be due to the fact that after reducing the activity of MYC gene cells grow slower. However, in these experiments, the Huh-7 cell line provided more detailed results and was more suitable for the experiments. The time for doubling is approximately 24 hours for Huh-7, whereas it is approximately 48 hours for HepG2 cells. Therefore, all the results may have been received earlier, which could have left more time for further experiments and their repeats to achieve greater and broader results from both HCC cell lines. Furthermore, all the results are not a comparison of the two HCC cell lines but rather HepG2 experiments. I will continue HCC-related experiments but mainly focus on the Huh-7 cell line. Huh-7 cell line demonstrates better outcome in further experiments than HepG2 cell line. HepG2 cells prone to aggregate more easily than Huh-7 cells. Therefore, the cell membrane passing will be complicated for nanoparticles.

The transfection into HCC cells decreased the doubling time and the general growth rate significantly in both the Huh-7 and HepG2 cells. This may indicate the successful transfection since c-Myc highly regulates cell proliferation and the plasmid aimed to downregulate the expression of c-Myc. This result was significant since the aim was to target cancerous cells with downregulating c-Myc expression. Cancerous cell lines typically grow and proliferate fast; the comparison of the growth rate before and after the transfection provides reliable results. Therefore, the plasmid transfection should reduce the expression of c-Myc by its downregulating, it should have an impact on the growth rates of the cells. However, there is a problem with this, since not all of the cells are edited and maybe do not die after puromycin selection either. The change detection will be more complicated since wild-type cells will gradually expand and make up the majority of the population.

The growth rate experiment would enable us to determine the number of cells at different time points by estimating the cell density (Hall et al. 2014). The rate of growth experiment provides essential information on the

characteristics and cell behavior (Pereira et al. 2020). Since the growth can be influenced by several factors, it is necessary to gain knowledge for further experiments (Lindström & Friedman 2020). However, the growth estimation after performing the growth rate experiment may not provide the information for additional steps if the factors of environment or toxins have been changed. Furthermore, further studies for comparing the cell viability percentage and the number of cells in the wild-type and the transfected HCC cells might be informatic. Other cell viability assays could include methods such as metabolic activity assays, and ATP concentration assays. This could provide significant informatics on how the downregulation of c-Myc affects the cells and their proliferation and doubling *in vitro*.

5.5 THE PROTEIN EXPRESSION OF C-MYC

The densitometric analysis of the c-Myc protein expression in HepG2 cells provided significant information on the wild-type c-Myc protein expression compared to the distinct amounts of the loaded gene to downregulate the expression throughout different transfection methods. The higher amounts of the delivered gene into HepG2 cell downregulated the expression the most. Most likely due to the fact that highest editing efficiency was achieved this way. This indicates the importance of the efficient delivery method and a great amount of the loaded gene to achieve the expression difference. Every transfection decreased the amount of expression, which indicates that the CRISPR/Cas9 edited plasmid works, and it has been successfully delivered and targeted the nuclei. However, the results of c-Myc delivery do not provide data for the *in vivo* experiments, although the delivery was successful with the controlled release in the acidic environment in *in vitro* experiments. The other thing to consider when comparing *in vitro* and *in vivo* results is the other cell lines or the lack of them since in vitro provides fewer possibilities for differentiation, which makes it impossible to observe the effects towards other cell lines that are attributed with c-Myc (Pelengaris et al. 2002).

To achieve the received results of the expression of c-Myc, multiple optimizations needed to be done while performing Western blot in every step of the analysis. The commercialized protein loading gels were optimized as well. At first, 4-20 % Mini-PROTEAN TGX Precast Protein Gels (BioRad) were used, which had the separation range of 2-400 kDa for polypeptides, which was replaced with lower concentrated gel of 4-15 % Mini-PROTEAN TGX Precast Protein Gel, which had the separation range of 5-200 kDa. This provided better results since the target proteins were located between 30 to 65 kDas. The running of proteins was enhanced by increasing the time and using lower voltage, which provided more detailed results with great separation. For transferring, the PVDF membrane was replaced with a nitrocellulose membrane, which provided more accurate results for the lower protein weights. The transfer in the colder environment enhanced the results, which led to performing the transfer with an ice block in the buffer tank, which had ice cubes around. For blocking, several different blocking buffers where used, but the best was achieved by EveryBlot, which decreased the time for blotting and provided greater results when compared to 5% BSA because the blocking step was crucial to reduce the non-specific antibodies from binding.

For future therapeutic development, c-Myc has attracted researchers due to its importance in the cell cycle, apoptosis, and protein synthesis (Chen et al. 2014). Therefore, c-Myc has been more widely investigated to achieve the most efficient route to develop a novel therapeutic for malignant cancer therapy. Zhao et al. 2013 highlighted the potential of downregulation by inhibiting c-Myc as a therapeutic alternative for treating HCC in the future. The western blotting required several months to succeed with plenty of optimizations. Since other western blot attempts did not succeed to illustrate the c-Myc expression due to the used antibody. The antibody did not provide results even when performing western blot with positive c-Myc control lysate. This indicates the difficulties to achieve results of c-Myc expression although the western blots were performed correctly since it provided results of equally loaded protein lysates through positive control protein, GAPDH. That is why there is no other western blot results represented in the master´s thesis

project. Due to the lack of time, there was no comparison of c-Myc protein expression between Huh-7 cells and HepG2 cells, which could have provided more information on the HCC cell lines, and where the expression is higher when using the same amounts and concentrations. Despite this, the c-Myc protein expression in HepG2 cells provided adequate information on the effects after transfection. The proliferation decelerated significantly after the transfection. The results received are in line with the published study data. The study of Zhao et al. 2013 is in line with this study of the decreased level of HepG2 cells after transfection. The reduced amount of proliferation of HCC cells may indicate that the c-Myc has a significant role for the proliferation of HCC. Additionally, the aim is to work on this topic in the future and publish the received data after accomplishing the western blots and gathering more data on c-Myc downregulation.

5.6 THE CELLULAR UPTAKE, ENDOSOMAL ESCAPE AND NUCLEI TARGETING IN HCC CELLS

CRISPR/Cas9 edited plasmid must be delivered effectively regardless of the multiple distinct barriers in *in vivo* (Xu et al. 2021). The delivery method provides a better outcome by encapsulating the CRISPR/Ca9 components with nanoparticles. However, firstly, the blood barrier and degradation of CRISPR/Cas9 must be overcome. The nanocarriers should have been cleared, which leads to nanoparticle aggregation and filtration. Secondly, the cargo should overcome the blood circulation barrier to be able to accumulate in the target tumor tissue. The last issue to overcoming the barrier for the delivery is passing the extracellular matrix due to the potential charges – both CRISPR/Cas9 edited plasmid and extracellular matrix are charged negatively, which may lead to challenges to passing the barrier and being trapped into the interstitial area due to similarity of potential charges. By encapsulating the cargo with nanoparticles, the uptake should be enhanced because the nanocarrier becomes positively charged due to electrostatic attraction. Nanocarrier could be also modified further with targeting motifs

to increase the delivery efficiency, and endocytosis. The advantage of what Zif-8 provides as the therapeutic carrier is related to the imidazolate ligand that works as a proton sponge in an acidic environment with pH stimuli responsive (Linnane et al. 2022). Several studies of Zif-8 in drug delivery advocate the pH-mediated release in the cancerous environment.

The particle size and shape of the carrier are crucial determinators of the interaction between the nanoparticle and the cell (Linnane et al. 2022). Those properties play significant roles in biodistribution, the capabilities of the interface of cell membranes, and the uptake of the target cell. Furthermore, MOFs have their limitations regarding cellular penetration and interactions with cells due to their lack of biological properties. Due to the properties of nanoparticles, endocytosis will be needed for cargo penetration into cells. There are issues related to MOFs with targeting the accurate component of the cell, avoiding lysosomal degradation and exocytosis. Optimal endocytosis is crucial due to cell signaling, motility and uptake. Endocytosis is possible when the particle size is between 50 to 150 nm, so cargo may be taken into the cell for further processing. However, the endocytosis pathway endocytosis is crucial due to the tight regulation and complexity.

The nanoparticles were taken into cells after 2 hours of the transfection, but the colocalization did not mainly occur since the green and red colors remained green and red rather than yellow. The colocalization of endosomes and nanoparticles was highest at the point of time at 4 hours after the transfection. After 6 hours of transfection, some nanoparticles migrated toward the nuclei and were already targeting the nuclei inside. As mentioned earlier, the Huh-7 cell line is more viable, but the results from confocal imaging were better and more accurate in HepG2 cells. Huh-7 cell line may have taken too much of the Zif-8 nanoparticles, which made it more challenging to visualize the exact localization of the nanoparticles at different points in time. This may indicate the enhanced efficiency of delivery and uptake of Huh-7 cells, which has been a better alternative for further experiments when compared to HepG2 cells.

Linnane et al. highlighted the enhanced endosomal escape of Zif-8-mediated CRISPR/Cas9 delivery. The delivery led to a 4-day gene expression of Cas9 with the fluorescent marker. The fluorescence imaging in CHO cells resulted in Cas9 location inside endosomes after 1 hour of transfection, whereas after 6 hours, the Ca9 was not located inside endosomes with LysoTracker green dye. These results are similar to the results of the thesis, although they had information at the time of 1 hour after transfection and this thesis lacked it. Although, the confocal images do not indicate the exact point of time after transfection when the nanoparticles have been taken into the cell, when endosomal escape occurs, and when the nucleus targeting begins. However, the results provide vast knowledge overall when the functions and effects in the cell can be predicted in general. More detailed and accurate results would be achieved with longer, live cell imaging.

5.7 FUTURE APPLICATIONS OF UTILIZING NANOPARTICLE MEDIATED DELIVERY AND DOWNREGULATION OF C-MYC

The current therapeutics are facing issues due to sensitivity towards the environment inside of the human body (He et al. 2021). The acidic environment, moisture, higher level of oxygen and temperature may lead to oxidation, degradation and crystallizing of the therapeutic, which creates a need for an enhanced carrier to achieve a better therapeutic outcome. MOFs could be utilized as a carrier of therapeutic since they can be altered to enhance the outcome of poorly soluble and hydrophobic therapeutic due to the stimuli-responsive release in the ideal environment. Oncogenes will be commonly used as therapeutic targets in various cancers in the future (Cheng et al. 2020). The formation and development of the tumor could be controlled with the powerful gene editing method.

Targeted therapy with molecularly targeted agents has provided alternatives for advanced cancer therapies (Lin et al. 2016). A combination of targeted therapy with chemotherapy might overcome more promise and be effective

for future cancer patients. Multiple studies have shown remarkable results by combining different treatments for HCC. This could be the future aspect of combining c-Myc inhibition with the current therapies to achieve greater results in the survival rates among HCC patients.

Due to the effective gene editing method of CRISPR/Cas9, it has already been utilized in human cell line cancers and animal models (Kim et al. 2017). It provides versatility in the applications due to its amazing capability to locate mutations, which can be further utilized in cancer therapy applications. Targeting of CRISPR/Cas9 remains challenging (Duan et al. 2021). To enhance the delivery efficiency, gene editing of ribonucleoprotein provides a more straightforward and efficient delivery method (Sioson et al. 2021). The ribonucleoproteins do not require additional expression and only require localization of nuclei. Due to its applications, multiple studies have been ongoing focusing on the ribonucleoprotein complex. The complex provides protection for sgRNA from enzymes which can cause degradation. Furthermore, the risks of off-target effects and mutations have decreased due to the short lifetime of the complex. Despite the great applications and advantages, the negatively charged sgRNA and relatively large-sized Cas9 nuclease require optimized encapsulation, which may lead to limiting the usage of ribonucleoproteins. However, the ribonucleoproteins could be utilized as carriers due to the delivery efficiency and fewer potential risks with the shorter duration of action. More studies will be required to achieve reliable results in comparing ribonucleoproteins to plasmids as carriers. Since CRISPR/Cas9 has provided better outcomes when using ribonucleoproteins instead of plasmids, the further studies will focus on the intracellular delivery of CRISPR/Cas9 ribonucleoproteins with ZIF-8 to HCC cells.

The overexpression of c-Myc is involved in a considerable number of cancers of advanced stages with a poor prognosis (Pelengaris & Khan 2003). Since c-Myc has a significant role in the regular cellular processes and tumorigenesis, developing c-Myc as a novel therapeutic requires more comprehensive research to avoid adverse events due to its inhibition (Lin et al. 2010). However, the inhibition of c-Myc due to its ubiquitous nature has been under

investigation due to its attractive therapeutic aspect for various malignant diseases, which currently have only a few treatment alternatives and a poor prognosis (Madden et al. 2021). The mechanisms of c-Myc and how its dysregulation is implicated in inflammation remain poorly understood.

Although much can be studied in *in vitro* experiments, the off-target effects remain a limitation since *in vivo* experiments are required to achieve more reliable results and knowledge (Yang et al. 2021). The immunogenicity toxicity studies require *in vivo* studies, but the *ex vivo* studies may possess more accurate and reliable results of the toxicity and off-target effects. Ex *vivo* studies regarding immunogenicity may be a greater option to reduce the risks and avoid causing unnecessary risks. In conclusion, c-Myc has become an attractive novel therapeutic target due to its versatile functions inside the cells and tissues. After optimizing its downregulation, it could be utilized in the different cancers and their novel therapy alternatives.

6 CONCLUSION

Hepatocellular carcinomas have a poor prognosis without efficient therapy for the more advanced stages of HCC. The current therapeutics cause resistance and severe side effects for most patients with long-term drug use. The inefficient treatment for HCC has raised interest among researchers since HCC will become more common worldwide yearly. C-Myc is responsible for regulating up to 20 % of all human genes. It also plays a key role in versatile cancers by leading to malignancy in up to 70 % of all liver cancers due to its overexpression. The cell cycle, protein synthesis, apoptosis, and cell proliferation are highly regulated by c-Myc, which indicates the importance of c-Myc as the novel therapeutic target.

Zif-8s have been widely studied with remarkable results. Zif-8 provides stable and pH-mediated delivery of its cargo into the target cell. Zif-8 can be easily used as a nanocarrier in a cancerous environment due to its controllable pH-mediated release, great protection, and ability to target the movement of its target. The nanoparticle-mediated delivery of CRISPR/Cas9 edited plasmids provides potential in future cancer therapies with the unique characteristics of nanoparticles, such as controllable sizes, easy modification of the surface, and low immunogenicity. CRISPR/Cas9 enables simple and specific human gene editing, which can be utilized in enhancing personalized medicine by providing more efficient and safer therapeutics for cancer patients with poor prognoses. CRISPR/Cas9 gene editing enables easy editing of the wanted DNA sequences to enhance the validation of the targets and the identification of the genetic disease mechanisms.

In this thesis, the delivery of the Double Nickase Plasmid into HCC was successful with Zif-8 nanoparticles. The Zif-8-mediated transfection was almost as efficient as the positive control transfection with Lipofectamine ™ 3000 transfection into HepG2 and Huh-7 cells. The cellular uptake of Zif-8 is successful and efficient in HepG2 and Huh-7 cells. High ratio of the endosomal escape occurred after 4 hours of transfection. Nuclei targeting was after 6 hours of the transfection.

In HepG2 cells, Zif-8 provided almost as remarkable results of downregulating c-Myc expression compared to the Lipofectamine-mediated transfection. Since the amount of the loaded plasmid is related to the c-Myc expression level, the higher the amount of transfected Zif-8 is, the lower the c-Myc expression. Downregulation of c-Myc expression throughout gene editing provided adequate results in this thesis, which may lead to the potential future utilization as the therapeutic target for HCC therapy with further studies and optimization. Furthermore, the c-Myc downregulation can be utilized in different cancers with the optimization regarding the target cell, tissue delivery, and editing method. The results represented in this thesis indicate the future potential of intracellular c-Myc knockout CRISPR/Cas9 plasmid delivery ZIF-mediated in liver cancer cells.

Future novel therapeutics must overcome the issues regarding the acidic environment, moisture, oxygen levels, and high temperature to achieve as efficient therapy alternative as possible without causing oxidation, degradation, or crystallization. Most of the issues may be overcome by utilizing nanoparticle-mediated therapeutic protection, although it requires much more optimization and further experiments to achieve the ideal safety level. This study was conducted using *in vitro* experiments, which may not produce results as accurate and reliable as in an *in vivo* environment. Therefore, future experiments should have more information from the *in vivo* studies since *in vitro* experiments do not provide accurate knowledge of the possible off-target effects, toxicity, or mutations, although it is necessary to optimize the discussed issues previously before proceeding into *in vivo* experiments to avoid the unnecessary risks and harms.

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