Biophysical characterization of LNPs: effects of pka on Encapsulation Efficiency and Cytotoxicity

A dissertation submitted by

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Abstract

Lipid nanoparticles (LNPs) have emerged as highly promising vehicles for efficient drug delivery, exhibiting the potential to significantly enhance therapeutic outcomes while minimizing unwanted side effects. Their versatility has been demonstrated in successfully delivering a wide range of novel therapeutics, including nucleic acids and chemical compounds, opening doors for exciting future applications. However, despite these advancements, further research is necessary to comprehensively investigate the biophysical properties of LNPs, as this will contribute to a deeper understanding of how these properties influence their efficacy and potential cytotoxic effects. By diving into the complex details of LNPs' biophysical characteristics, such as size, and charge we can gain valuable insights that will aid in the development of optimized LNPs for targeted and efficient drug delivery. Moreover, measurements of the pKa values of LNPs hold significant importance in this research endeavor. Determining the pKa values of the lipids used in LNPs provides crucial insights into their ionization behavior and protonation states under physiological conditions. This knowledge enables the design of LNPs that can effectively respond to the acidic environment within endosomes, facilitating successful intracellular drug release and minimizing premature drug leakage during circulation. Exploring the relationships between these properties and the performance of LNPs will lead to enhanced therapeutic strategies. The properties of the tested LNPs have shown promising results. Initial investigations have revealed favorable characteristics in terms of their size, charge, encapsulation and cytotoxicity indicating their potential for efficient drug delivery. These initial findings provide an optimistic outlook on the suitability of these LNPs as carriers for therapeutic agents.

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1. Introduction

1.1 Gene therapy

Gene therapy holds great promise for treating disorders caused by incorrect gene expression since it can give specificity, precision, and personalization of treatment [1-5]. RNA therapeutics and especially small interference RNA (siRNA) have already started showing their potential as a powerful tool for cell treatments and have revolutionized the field by providing a mechanism to selectively silence or modulate the expression of disease-causing genes [6]. The ability of siRNAs to selectively target and silence specific gene mRNA products underscores their therapeutic promise [7-9].

The ability of RNAi to achieve specific and potent gene silencing has opened new avenues for precision medicine, where therapies can be tailored to individual patients based on their genetic profiles [6]. Creating effective and secure delivery systems that can accurately distribute small interfering RNAs (siRNAs) to particular cell types and tissues is essential for effectively applying this vast potential to clinical applications, which will enable personalised therapeutic treatments [9, 10, 11]. Ongoing research and clinical trials are focused on further understanding the safety, efficacy, and long-term effects of gene therapy and RNA-based therapeutics, paving the way for their widespread adoption and integration into mainstream medical practice [4]. RNAi therapeutics have shown promising results in preclinical and clinical studies for a wide range of conditions, including genetic disorders, viral infections, and certain types of cancers, and by now 4 siRNA drugs have already been approved (Patisiran, Givosiran, inclisiran, and lumasiran) [12].

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1.2 siRNA mechanism of action

Once siRNA reaches the cytoplasm it interacts with the RNA-induced silencing complex (RISC) [1]. The siRNA molecule consists of a sense and antisense strand, with the latter playing a key role in gene silencing [4]. In the cytoplasm, the argonaut 2 protein acts as a catalyst, unwinding the siRNA molecule and eliminating the sense strand. The antisense strand, harboring the desired targeting sequence, is then seamlessly loaded into the RISC complex allowing it to recognize and bind to complementary mRNA sequences [13, 14]. The guide strand directs RISC to the target mRNA with complementary sequences, ultimately leading to the cleavage and cellular nuclease degradation [5]. This sophisticated mechanism allows the effective silencing of genes and suppress the production of proteins encoded by targeted mRNA [15].

1.3 Challenges and Limitations of Naked siRNA

The utilization of naked and unmodified nucleic acid encounters significant challenges that profoundly impact its stability, delivery, and therapeutic efficacy in vivo. In particular, siRNA encounters multiple obstacles originating from its unique characteristics [16]. The high molecular weight and size of siRNAs, typically ranging from 19 to 25 base pairs, hampers their cellular uptake and diffusion across biological barriers [17]. Moreover, the size and hydrophilicity of the siRNA make transportation across the cell membrane incredibly challenging, blocking efficient cellular uptake [14,17]. The polyanionic properties of siRNAs, arising from negatively charged phosphate groups along their backbone, further lowers cell interaction and cell uptake due to electrostatic repulsion with negatively charged cell surfaces [18]. Additionally, naked siRNA has a half-life of less than 10 minutes, as it is susceptible to degradation by serum endonucleases and subsequent

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elimination by the kidneys [19]. Moreover, proteins wrap bare siRNA, making it a target for phagocytosis by reticuloendothelial system macrophages and the presence of foreign RNA molecules, such as siRNAs, can trigger an immune response in the body [5]. Off-target effects have been observed during such treatments that could lead to inhibiting the expression of genes other than those targeted, which is potentially dangerous in the treatment of disease [19].

1.4 LNP-Based Drug Delivery for RNA Therapeutics

The field of drug delivery encompasses a wide range of strategies and technologies aimed at efficiently transporting therapeutic agents to their intended sites of action in the body. These methods include various nanoparticle-based systems, liposomes, polymeric nanoparticles, viral vectors, and other specialized carriers [20, 21]. In recent years, considerable progress has been made in optimizing the delivery systems for RNA therapeutics, including the use of lipid nanoparticles (LNPs) to enhance stability, cellular uptake, and targeted delivery of siRNA molecules [8]. The combination of gene therapy and RNA therapeutics holds immense potential to revolutionize the treatment landscape, offering hope for patients with previously untreatable or poorly managed conditions [16]. LNPs have emerged as a promising carrier system due to their ability to protect its cargo from degradation, facilitate efficient cellular uptake, and enable controlled release of the therapeutic agent [14]. LNP systems are at the forefront of non-viral methods for delivering genetic drugs to patients. These systems have the potential to treat diseases by silencing pathological genes, expressing therapeutic proteins, or through gene-editing applications [19, 22]. The major ingredients of LNPs are ionizable lipid or lipid-like materials, cholesterol which improves LNP stability and encourages membrane fusion, and polyethylene

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glycol (PEG)-lipid which lessens LNP aggregation and "shields" the LNP from immune cells' nonspecific endocytosis [23, 24].

Encapsulation of the nucleic acid within LNPs offers several advantages in terms of delivery [25]. Firstly, it provides protection from degradation, ensuring the integrity and stability of the nucleic acid cargo throughout the delivery process. Additionally, it facilitates passive targeting, allowing for the accumulation of LNPs in target tissues through the bloodstream. Moreover, LNPs offer the potential for active targeting by attaching ligands or antibodies to the nanoparticle surface. This active targeting approach enables specific recognition and binding to target cells or tissues, further enhancing the delivery precision and efficacy of the nucleic acid payload [26]. Also, LNPs offer the advantage of evading endosomal Toll-like receptors (TLRs) [25]. These receptors, known as pattern-recognition receptors (PRRs), constitute a vital class of molecules involved in innate immunity. They play a crucial role in recognizing specific molecular patterns associated with pathogens (PAMPs) and initiating immune responses against them. Activation of PRRs triggers a cascade of events leading to the production and release of pro-inflammatory cytokines, such as interleukins and interferons, as well as the recruitment of immune cells to the site of infection or inflammation. [27].

Some of the most important challenges for successful delivery are the dissociation of nanoparticles via serum proteins, the ability of cellular uptake, and the intracellular disassembly of the cargo [25]. In vivo, LNP-siRNA systems that incorporate ionizable cationic lipids exhibit a notable accumulation within target hepatocyte cells through the process of endocytosis. The presence of ionizable lipids is crucial in LNP formulations as it plays a pivotal role in enhancing their efficacy [28]. The absence of a significant positive charge in the particles contributes to

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improved pharmacokinetics, primarily by slowing down their clearance by the reticuloendothelial system (RES) at physiological pH [26]. This phenomenon allows for an extended circulation time in the body, enhancing the potential for targeted delivery and therapeutic efficacy. The reduced interaction with the RES, which is known to recognize and eliminate positively charged particles more rapidly, promotes the accumulation of the particles at the target site [5]. Overall, the absence of a substantial positive charge in the particles provides a favorable pharmacokinetic profile, facilitating their effective utilization in various biomedical applications. [26].

The optimization of ionizable cationic lipids in LNP siRNA systems is driven by the rationale that the dissociation constant (pKa) which is used to describe the strength of an acid of these lipids should be carefully considered [28]. It is crucial for the pKa to be sufficiently high to ensure a significant proportion of the lipids becomes protonated at endosomal pH values. This protonation enables their interaction with endogenous anionic lipids, facilitating the formation of non-bilayer structures that disrupt the endosomal membrane [22]. By achieving this, the ionizable cationic lipids enhance the escape of siRNA from the endosomes into the cytoplasm, where it can effectively engage with the RNA-induced silencing complex (RISC) and initiate gene silencing as described before [15]. An effective delivery system should have the ability to detect slight changes in pH in its surroundings, such as the difference between the neutral pH of the blood and the acidic pH of endosomes within cells [25]. It should also be able to alter its charge to a cationic state within the endosome to facilitate its escape [26]. At the same time, the pKa must also be sufficiently low so the LNP surface charge does not result in clearance by the immune system before accumulation by target cells [22].

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1.5 Aims

The investigation of these ionizable cationic lipid pKa values and their impact on the performance of LNPs in siRNA delivery represents a novel and unexplored area of research. While previous studies have explored the impact of lipid pKa values on the performance of lipid nanoparticles (LNPs) in gene delivery, a significant gap remains in our understanding of the specific effects within the context of particular lipids. To fill the gap in research the objective of this project is to investigate the influence of lipid pKa values on the performance of LNPs in siRNA delivery. The study involves characterizing the lipids used in LNP formulations and determining their pKa values through experimental and theoretical methods. Additionally, the biophysical properties of LNPs, including size, charge, and polydispersity index (PDI), will be assessed. The impact of ionizable lipids on LNP size will be examined, as smaller particles generally exhibit enhanced internalization compared to larger ones [(29)]. Furthermore, the encapsulation efficiency of DNA within LNPs and the cytotoxicity of LNPs on target cells will be evaluated. By understanding the relationship between pKa values, LNP characteristics, and their performance, this research aims to contribute to the development of optimized LNPs for effective gene delivery.

2.Materials and Methods

2.1 Materials

The TNS (6-p-toluidinyl-2-naphthalenesulfonic acid) solution was obtained from Sigma-Aldrich(T9792-250mg), and the PrestoBlue reagent was obtained from Invitrogen, UK. The HEPES reagent was obtained from Gibco, UK. The lipids 1,2-dioleoyl-snglycero-3-phosphoethanolamine

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(DOPE), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000), were purchased from SIGMA Aldrich (St. Louis, MO). The Quant-it PicoGreen Assay kits were purchased from Thermo Fisher Scientific (Loughborough, UK). The plasmid was obtained from Addgene (Watertown, MA)

2.2 Nanoparticle Formulation

The lipids including Ionizable cationic lipid, DOPE, and DMG-PEG2000 were prepared in 90% t-BuOH at a final volume of 400 μL and a molar ratio of 50:50:1. 100 μL of DNA (0.4 mg mL^{-1}) were mixed by rapid mixing to obtain the final nanoparticles with the lipids to give an N/P ratio of 6.

2.3 TNS assay

The protocol for measuring the pKa of lipids and nanoparticles was developed based on Journal of Controlled Release by Heyes, J. in 2005 [30].20mM citric acid/NaOH buffer (with 150mM NaCl, pH 3.0, 3.5, 4.0, 4.5, 5.0, 5,5), 20mM sodium dihydrogen phosphate/NaOH buffer (with 150mM NaCl, pH 6.0, 6.5, 7.0, 7.5, 8.0) and 20mM Tris/HCL buffer (with 150mM NaCl pH 8.5, 9.0, 9.5, 10) were prepared. TNS (6-p-toluidinyl-2-naphthalenesulfonic acid) dissolved at 0.6 mM in water as a stock solution. In a 96-well black plate, 2μL of the TNS solution, 12 μL of the LNP solution (0.5mM total lipid), and 186μL of each buffer above mixed in triplicates. After shaking the incubation mixture (300rpm, 10min), the fluorescence of the TNS (Ex:321/447) will be measured on the FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK). The apparent pKa of the surface will be calculated as the pH at which the LNP showed 50% of the maximum fluorescence (IC50) [30]. Blank samples were included in the experimental setup as a control.

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2.4 Nanoparticle Size and Zeta Potential

The protocol for measuring nanoparticle size, charge and polydispersity index was performed as described on international journal of nanomedicine by Tagalakis, A. D. in 2015 [31]. Nano ZS Zetasizer (Malvern Instruments, Malvern, UK) was used to measure the size and zeta potential of the nanoparticles by dynamic light scattering (DLS) and laser Doppler anemometry, respectively. Measurements were performed in triplicate for each sample and the results were analyzed using the built-in software (DTS version 7.12).

2.5 DNA Encapsulation

The DNA encapsulation efficiency was determined using the Quant-it PicoGreen assay. 100 μL of nanoparticles or DNA solution (for standard curve) were diluted in 100 μL of 10 mM HEPES buffer (pH 7.4) containing PicoGreen, in the presence or absence of 20 μg mL^{-1} dextran sulfate and 0.1% w/v Triton X-100 and were then added to a 96-well plate (Falcon, Fisher Scientific UK). Fluorescence was measured using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK) [32]. Measurements were performed in triplicate for each sample and the DNA concentrations were determined in reference to a DNA standard curve. The DNA encapsulation efficiency was then calculated by the following formulas:

$$DNA\ encapsulation\ \% = \frac{Encapsulated\ DNA - Unencapsulated\ DNA}{Total\ DNA\ concentration} \times 100 \quad (1)$$

$$DNA\ encapsulation\ \% = 1 - \frac{Unencapsulated\ DNA}{Encapsulated\ DNA} \times 100 \quad (2)$$

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2.6 Cell viability assay

The protocol for evaluating cell viability was performed as described on international Toxicology in vitro by Luzak, B. in 2022 [33]. HEK-293 Cells were washed with 1X Phosphate-buffered saline (PBS) 24 h following transfection with LNPs containing DNA and 100 μL of fresh media were added in each well, as well as 10 μL of a 10X PrestoBlue solution. The cells were then incubated at 37 °C for 10 minutes. Following this, fluorescence readings were recorded using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK) set at 550 nm excitation and 590 nm emission. Specifically, the assay relies on the reduction of resazurin, a non-fluorescent blue dye, to resorufin, a red fluorescent dye, by metabolically active cells. The resulting fluorescence intensity is directly proportional to the number of viable cells. The cell viability was calculated as a percentage of the viability of the control untreated cells. Triplicate wells were used and measured for each condition to ensure a better statistical outcome.

2.7 Statistical Analysis

All data were subjected to one-way ANOVAs with multiple comparisons followed by Bonferroni's posthoc correction except TNS, which was subjected to NEM p value style. Values that were statistically significant were expressed as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All graphs used in the paper display the mean and standard error of the mean (SEM). Graph-Pad Prism 8.4.2. was used for all the analyses and the generation of the graphs.

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3. Results

In this study, the influence of key factors on the performance of lipid nanoparticles (LNPs) as siRNA delivery systems were investigated. The focus is on evaluating the pKa values of lipids and their impact on LNP functionality, optimizing the biophysical properties of LNPs, assessing DNA encapsulation efficiency, and evaluating cytotoxicity. To determine the pKa values, a TNS assay was used for the experimental estimation and computational software for the theoretical estimation. The LNP size, charge, and polydispersity index (PDI) were characterized using dynamic light scattering (DLS) and zeta potential measurements. Additionally, fluorescence-based assays to assess DNA encapsulation efficiency within LNPs were employed. To evaluate the safety and biocompatibility of LNPs, cytotoxicity assays using cell viability measurements were conducted to assessed any potential adverse effects. By elucidating the relationships between pKa values, LNP properties, DNA encapsulation, and cytotoxicity, these findings have the potential to enhance the design and development of safe and effective siRNA delivery systems for gene therapy applications.

3.1 Theoretical estimation of pKa of lipids.

The pKa values of the lipids used in the LNP formulations were initially estimated theoretically. This estimation was performed using computer models and predictive algorithms for the physicochemical properties of compounds, utilizing software ChemDraw and chemicalize.

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Lipid name	Structure	Theoretical pKa (ChemDraw)	Theoretical pKa (Chemicalize)
J1		10.2	9.8
J2		11.1	9.8
J3		10.1	9.8
J4		9.2	8.3
J5		10.2	10.8
KCAT1		9.1	10

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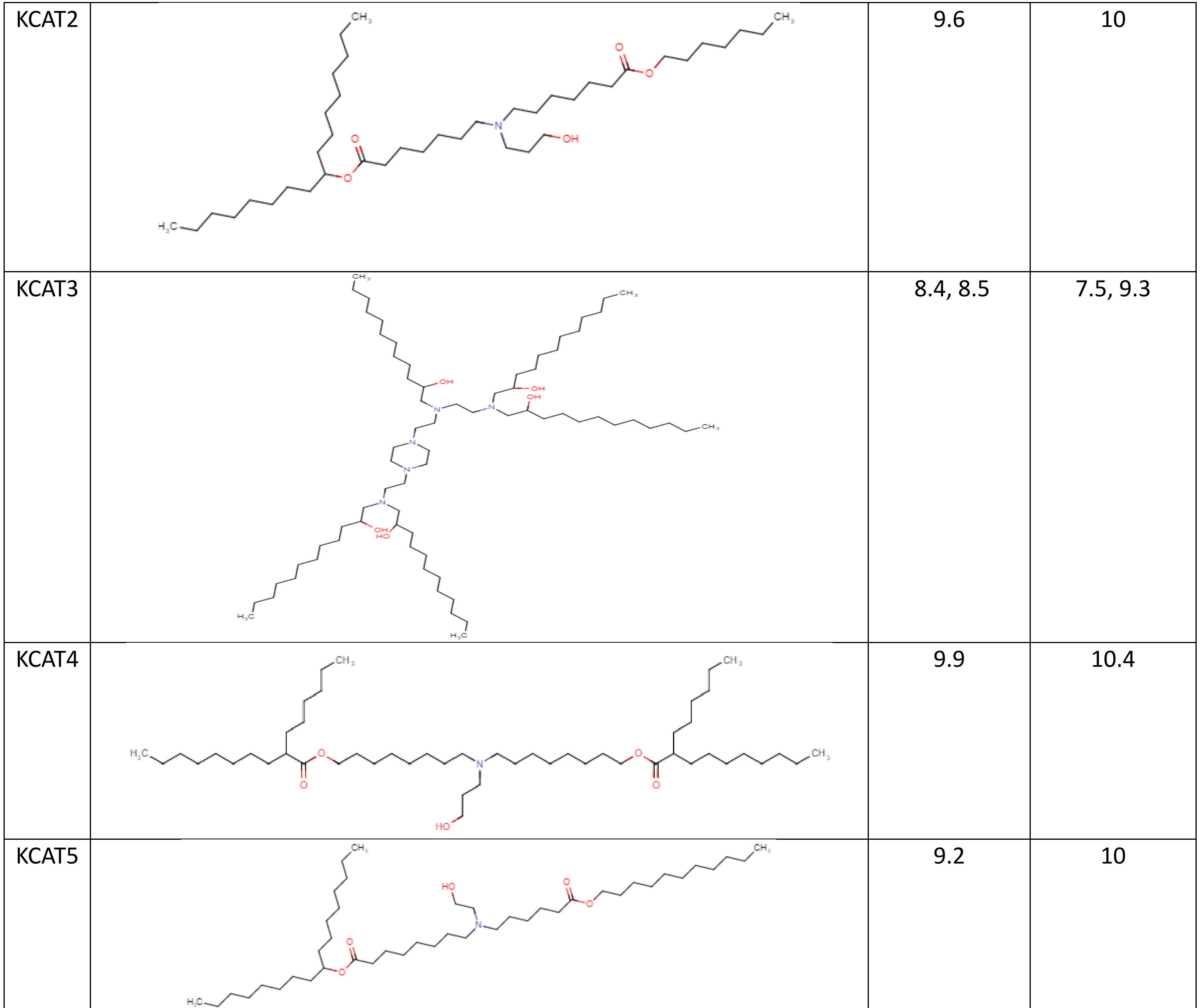

KCAT2		9.6	10
KCAT3		8.4, 8.5	7.5, 9.3
KCAT4		9.9	10.4
KCAT5		9.2	10

Table 1: Chemical structures of the ionizable cationic lipids and their theoretical pka as estimated by ChemDraw and Chemicalize.

The structural information and the estimated pKa values of the lipids investigated in this study are presented in Table 1 By taking into consideration the molecular structure and chemical composition of the lipids, approximate values of their pKa were obtained. These estimated pKa

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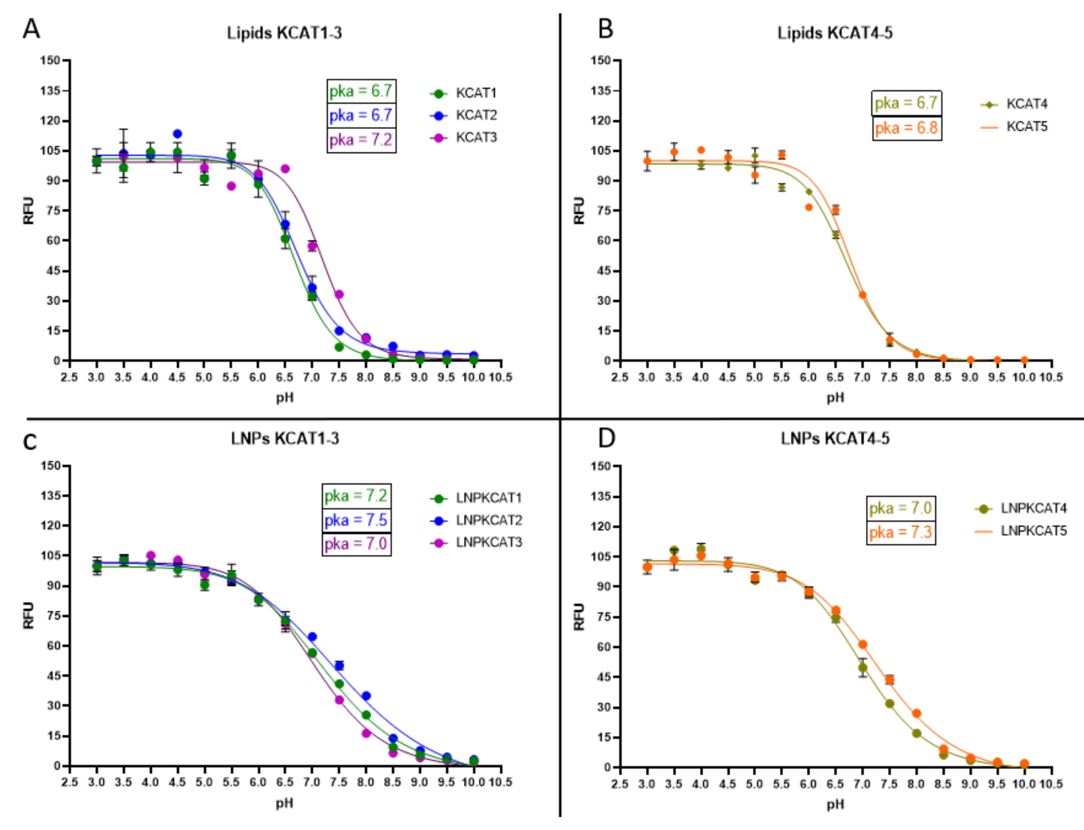
values served as a starting point for further analysis and investigation using experimental techniques.

3.2 TNS assay to measure the pKa of lipids and LNPs.

The pKa values of the lipids incorporated into LNPs were experimentally determined using the TNS assay, which is a well-established method for measuring the pKa value of lipids and LNPs. In this assay, the pKa values were precisely quantified by employing the fluorescent probe TNS, which exhibits pH-dependent fluorescence properties. The interaction between anionic TNS and positively charged ionizable lipids renders TNS lipophilic. As the pH approaches the pKa of each LNP, TNS becomes less lipophilic, and the presence of water molecules leads to the quenching of TNS fluorescence [23]. The pKa values of the lipids and LNPs were determined by monitoring the fluorescence intensity of TNS at different pH levels.



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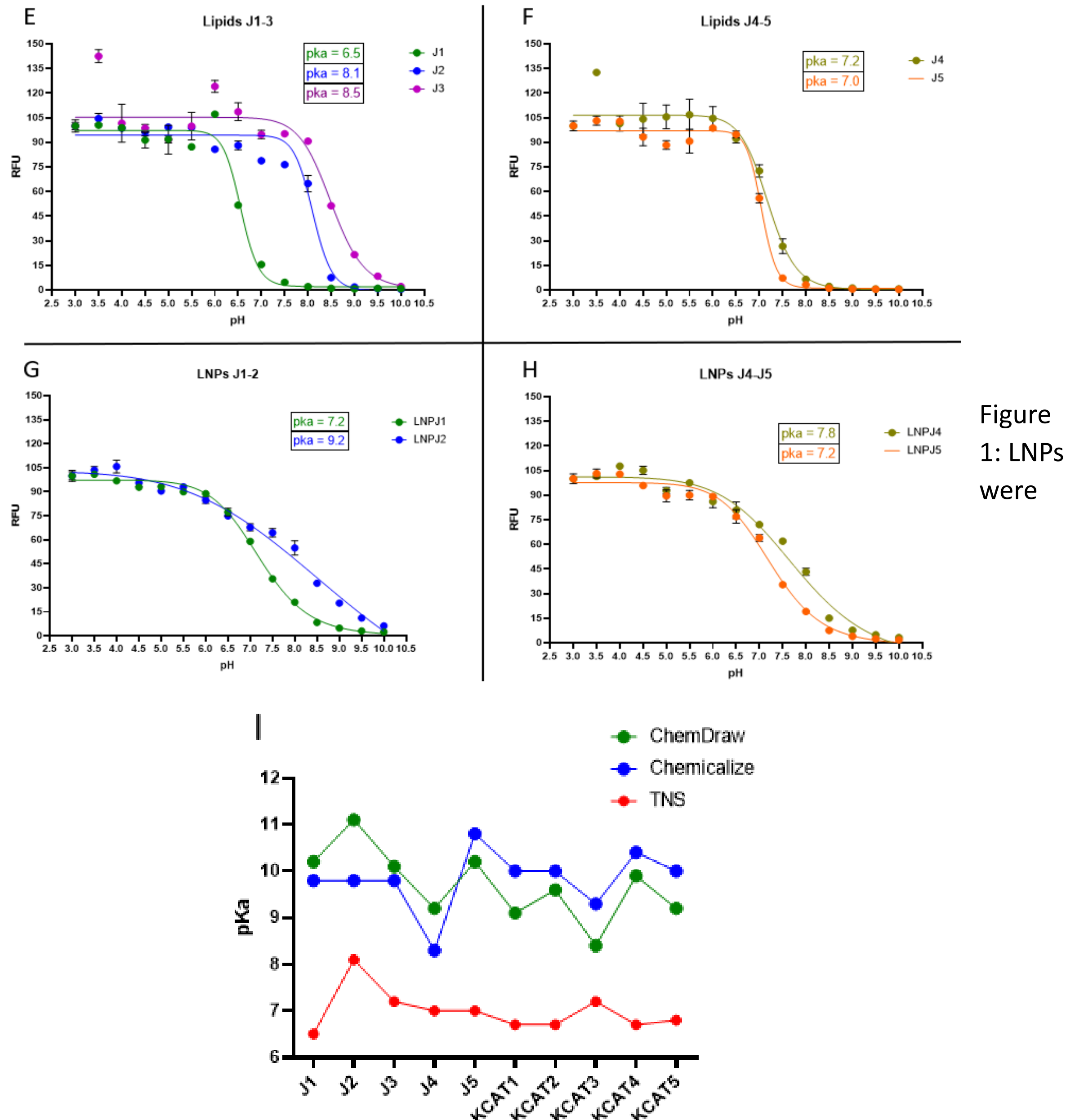

Figure 1: LNPs were assembled with the mole ratios 50:50:1.0 (ionizable lipid: cholesterol: PEG-DMG2000). **A)** TNS assay measurements of p*K*a of ionizable lipids KCAT1-3. **B)** TNS assay measurements of p*K*a of ionizable lipids KCAT4-5. **C)** TNS assay measurements of p*K*a of LNPs containing ionizable lipids KCAT1-3. **D)** TNS assay measurements of p*K*a of LNPs containing ionizable lipids KCAT4-5. **E)** TNS assay measurements of p*K*a of ionizable lipids J1-3. **F)** TNS assay measurements of p*K*a of ionizable lipids J5-5 **G)** TNS assay measurements of p*K*a of LNPs containing ionizable lipids J1-2. **H)** TNS assay measurements of p*K*a of LNPs containing ionizable lipids J4-5. **I)** Graph representing the theoretical and experimental pka values. Measurements were performed in

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triplicates for every condition (N = 3). All observed TNS graphs display a p-value smaller than 0.0001 except Lipids KCAT1 and KCAT2(0.003, 0.009) respectively.

The experimental pKa values of the investigated lipids in this study provide valuable insights into the ionization behavior and protonation states of these lipids under physiological conditions. By determining the pKa values, we can validate and refine the estimations, enhancing our understanding of the relationship between lipid structure, pKa, and the physicochemical properties of the LNPs. As shown in Figure 1. a notable pattern emerged from the comparison between the pKa values of the individual lipids and the corresponding LNPs. It was observed that the pKa values of the LNPs generally tended to be higher than those of the individual lipids. While most of the determined pKa values for the LNPs in this study fell within the range of 7-7.5. Also, shown in Figure 1, I. the difference between the experimental pKa values and the theoretical values. It was found that the experimental pKa values of the lipids generally exhibited a difference of 2-4 pka values compared to the theoretical values.

3.3 Size charge and PDI of LNPs

The physicochemical properties of the LNPs were comprehensively evaluated by employing dynamic light scattering (DLS) and zeta potential measurements. These analyses allowed the thorough characterization of the size, charge, and polydispersity index (PDI) of the LNPs, providing valuable insights into their biophysical properties. DLS measurements provided information on the particle size distribution, while zeta potential measurements offered insights into the surface

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charge of the LNPs. By thoroughly investigating these biophysical properties, we gained a deeper understanding of the physical characteristics of the LNPs.

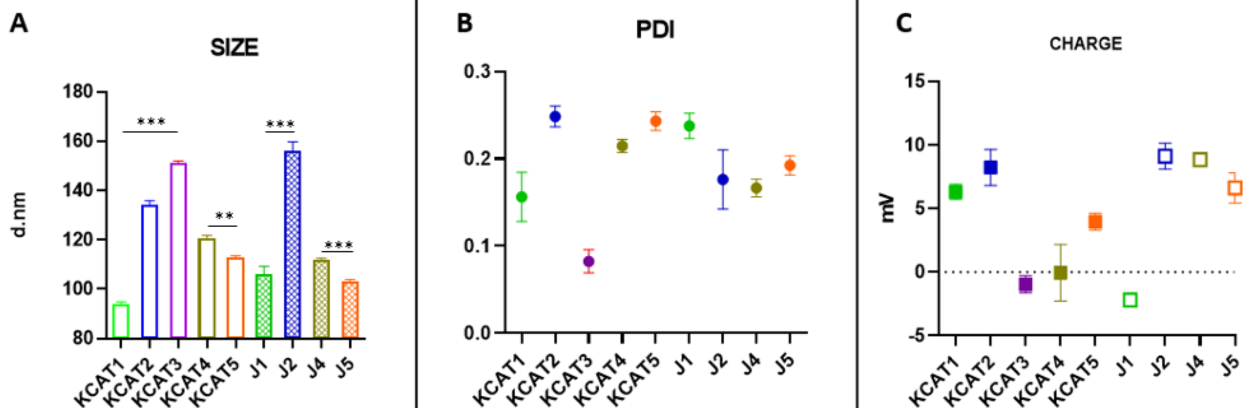

Figure 2: Physicochemical properties (size, charge, PDI) of freshly prepared LNPs+DNA Measurements were performed in triplicates for every condition (N = 3). **A**) Graph represents the size. **B**) Graph represents the charge and **C**) Graph represents the PDI. Measurements were performed in triplicates for every condition (N = 3). Results represent mean ± SEM. Statistical significance is displayed only for size. $^{**}p < 0.01$, $^{***}p < 0.001$.

The physicochemical properties of the freshly prepared nanoparticles were evaluated to gain insights into their characteristics. Figure 2. illustrates the results obtained from this analysis, focusing on key parameters such as size, charge, and polydispersity index (PDI). Size measurements of the LNPs revealed a consistent and controlled particle size distribution, ranging from 90 to 160 nm. This indicates that the LNPs exhibit a uniform size, which is important for their stability and efficient drug delivery. The charge characterization of the LNPs demonstrated low surface charges, with values ranging from -3 to +10 mV. These findings suggest that the LNPs possess a relatively neutral or slightly positive charge, which is desirable for enhancing their stability and reducing potential cytotoxic effects. The polydispersity index (PDI) values obtained

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for all LNPs were below 0.3, indicating a narrow size distribution reflecting the homogeneity and uniformity among the particles.

3.4 Encapsulation of DNA

After the preparation of the LNP formulations and characterization of their physicochemical properties, the DNA encapsulation efficiency of the LNPs was evaluated, instead of siRNA due to its high cost, to assess their potential as carriers (Figure 3). Two different formulas were employed in this study to determine the DNA encapsulation efficiency.

The Quant-it PicoGreen assay was utilized as a reliable method to quantify the amount of DNA present in the LNPs. By employing these formulas and assay, we gain valuable insights into the efficiency of the LNP formulations in encapsulating DNA, which is crucial for the successful delivery of therapeutic agents.

In the first formula (Formula 1), the amount of DNA successfully incorporated into the LNPs was quantified by comparing it to the total amount of DNA used during formulation. This comparison provides a measure of the extent to which DNA has been effectively incorporated into the LNPs.

In the second formula (Formula 2), the percentage of DNA successfully encapsulated within a particular system was determined by comparing the amount of unencapsulated DNA to the total amount of encapsulated DNA. This calculation allows for a precise assessment of the proportion of DNA that has been encapsulated within the LNPs.

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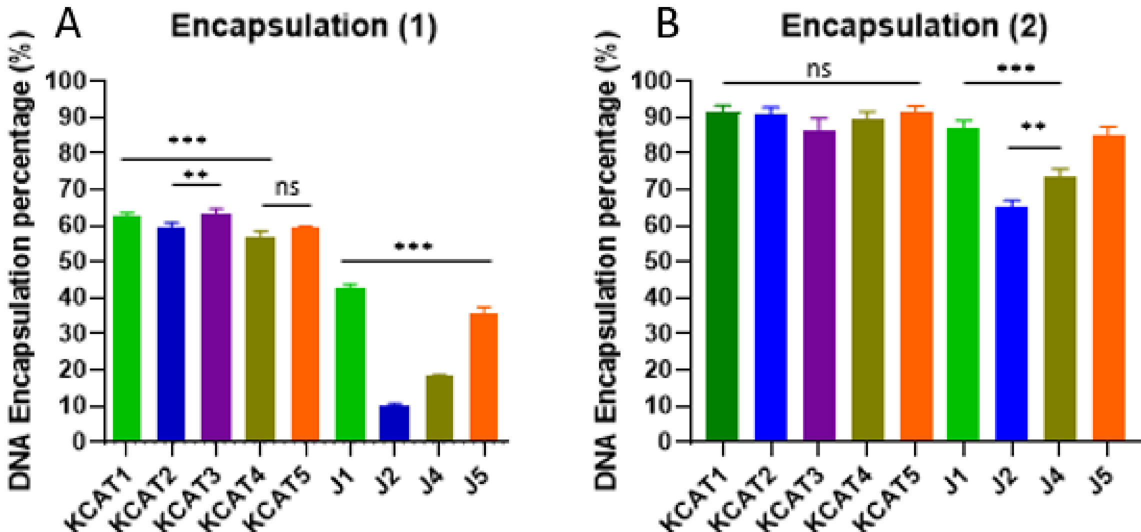

Figure 3: The encapsulation efficiency of the LNPs was assessed to determine the effectiveness of the formulation in encapsulating DNA. The measurements were performed in triplicates for each condition (N = 3). The bar graphs represent the percentage of encapsulation achieved by the LNPs. A) Represents encapsulation efficiency using formula 1. B) Represents encapsulation efficiency using formula 2. The results are presented as mean ± SEM. ns (not significant), **p < 0.01, and ***p < 0.001.

The encapsulation results demonstrated major difference among the different formulas employed in the study with formula (2) providing significantly higher results. LNPs incorporating KCAT lipids demonstrated notably higher encapsulation efficiencies, reaching 63.7% using formula (1) and 91.4% using formula (2). In contrast, certain LNPs incorporating J lipids showed lower average efficiencies, with values as low as 10.4% using the formula (1).

3.5 Cell Viability Assay

After preparing and characterizing the LNP formulations, evaluating their cytotoxicity in vitro became a critical step to ensure their safety for potential therapeutic applications. The cytotoxicity assessment was conducted using a well-established fluorescence-based assay called

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the Presto blue cell viability assay, following a standardized protocol. This assay allows for quantitative measurements of cell viability based on metabolic activity. By analyzing the cytotoxicity of the LNPs through this assay, valuable insights into their impact on cell viability and overall biocompatibility were obtained.

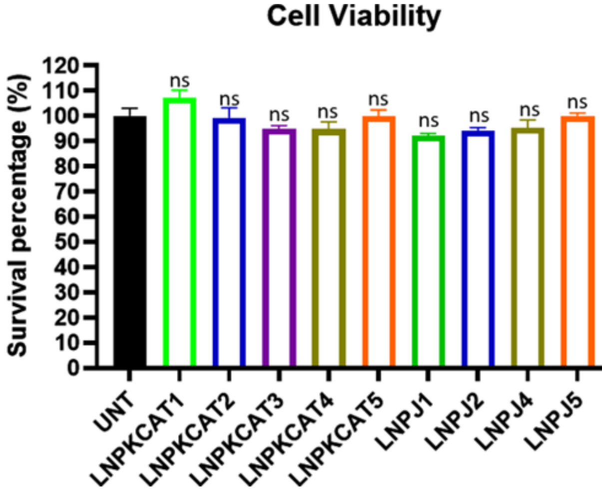

Figure 4. Cell viability of HEK-293 cells, 24 h after transfection with LNPs + DNA. The fluorescent variant of the PrestoBlue cell viability assay was used. Measurements were performed in triplicates for every condition (N = 3). Bar graphs represent cell survivability. Results represent mean ± SEM. UNT represents the untreated cells. ns = not significant (compared to UNT).

The results obtained from the cell viability assay demonstrating the percentage of viable cells after exposure to different LNPs. In summary, as it is shown in figure 4. all formulations tested (LNPs + DNA) proved to be non-cytotoxic in vitro with all LNPs exhibiting cell viability over 92.4%.

4. Discussion

Overall, insights were gained into the size and charge of the different LNPs to assess their suitability for in vivo use. The relationship between pKa values, encapsulation efficiency, cytotoxicity, and the physicochemical properties of LNPs was evaluated. The data obtained from

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the theoretical estimation of pKa, TNS assay, size and charge measurements, encapsulation efficiency evaluation, and cell viability assay provide a comprehensive understanding of the impact of pKa on the behaviour of LNPs.

The optimization of pKa values in the design of LNPs is essential for achieving efficient intracellular delivery and maximizing the therapeutic potential of gene therapy (25, 34, 35). The theoretical and experimental estimations of the comparison of pKa values for the cationic ionizable lipids align closely with the literature, showing a difference of 2-4 points (28). While most of the theoretical and experimental estimations of the pKa values for the cationic ionizable lipids align closely with the expected trend, not all estimations exhibit perfect consistency. However, since the specific names of these compounds have not been provided, a direct and specific comparison in literature of the lipids already tested cannot be made. Nonetheless, the consistency between the theoretical estimations and the experimental values reinforces the reliability and validates the accuracy of these pKa determinations within the expected range. Still, these findings contribute valuable insights into the pKa characteristics of cationic ionizable lipids, underscoring the importance of further investigation and refinement of lipid-based delivery systems for precise and efficient therapeutic applications.

A noticeable pattern becomes apparent when comparing the pKa values of the individual lipids to those of the related LNPs. The pKa values of the LNPs consistently exhibited a higher range compared to the pKa values of the individual lipids. This observation indicates that the incorporation of different lipids into the LNP formulation significantly affects the protonation behavior and ionization properties of the lipids (28). As a result, the LNPs as a whole exhibit distinct pKa values due to the altered forces and interactions between the molecules.

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In terms of size, the nanoparticles were found to have dimensions ranging from 90 to 160 nm, which fall within the recommended range for effective delivery systems [29]. This size range ensures optimal cellular uptake and distribution, while minimizing the potential for clearance by the immune system. Importantly, the measured sizes were consistent with those reported in previous studies that have demonstrated successful delivery using nanoparticles [31, 36]. These findings provide further evidence of the potential of these nanoparticles as promising carriers for therapeutic applications, as their size characteristics align with established criteria for efficient delivery and are in accordance with existing literature on successful nanoparticle-based delivery systems [37].

The PDI values of all tested nanoparticles consistently remained below 0.3, indicating a narrow size distribution and high uniformity [29, 32, 38]. This narrow range of PDI values reflects the presence of well-controlled manufacturing processes and stable formulations, which are critical for ensuring the consistent and reliable performance of the nanoparticles. The observed uniformity in size distribution suggests that the nanoparticles were successfully prepared using precise techniques, minimizing variations in their physical properties [39]. Regarding charge, the nanoparticles exhibited low to near-neutral charge. These charge properties are desirable for efficient encapsulation and delivery of nucleic acid cargo. The near-neutral charge of LNPs enables their stability and minimizes non-specific interactions [40].

The results obtained from the DNA encapsulation assay reveal promising outcomes, despite the observed variations among the tested formulations. The majority of the formulations exhibited favorable encapsulation efficiency, highlighting the potential of these LNPs as effective carriers for DNA-based therapeutics. Specifically, LNPs containing KCAT lipids and J1 consistently

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demonstrated higher encapsulation efficiencies compared to LNPs containing J lipids. However, it is important to note that the lower average efficiency observed in some cases for J lipid LNPs raises the possibility of issues during the encapsulation assay or formulation process. These variations could be attributed to factors such as lipid composition, formulation parameters, or experimental conditions. Further investigation is necessary to pinpoint the specific reasons for the lower encapsulation efficiency of J lipid LNPs and to optimize their formulation accordingly.

Developing applicable techniques and using consistent language for encapsulation are of great significance in the field of drug delivery. By employing standardized formulas and language, researchers can establish a common framework that facilitates effective communication, duplicability, and comparison of results across different studies ensuring consistency and reliability of the experimental outcomes. This consistency enables accurate comparisons of encapsulation efficiencies and drug release profiles among different formulations, providing valuable insights into the performance and efficacy of drug delivery systems. Moreover, it ensures clarity and comprehension among researchers and readers. It enables accurate interpretation and understanding of the encapsulation process, making it easier to replicate and build upon previous findings. Consistency in techniques and language also aids in the development of robust guidelines and protocols for future research and clinical applications.

Remarkably, the cytotoxicity assessment revealed that the developed LNPs demonstrate low cytotoxicity, underscoring their potential for safe and biocompatible use in gene therapy applications [35, 41, 42]. This finding holds immense significance for the clinical translation of LNPs, as the safety profile of delivery systems plays a pivotal role in their successful application in

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therapeutic interventions. By showcasing low cytotoxicity, these LNPs offer a compelling advantage for their potential application in clinical settings, where ensuring patient safety and well-being is of great importance. This favourable cytotoxicity profile strengthens the overall feasibility and desirability of LNPs as a delivery platform, paving the way for their potential integration in future gene therapy approaches with enhanced safety and efficacy.

The LNPs' biocompatibility, size, and charge characteristics, as well as their potential for targeted delivery, make them a suitable candidate for delivering gene therapies in liver [(24)]. Gilbert Syndrome (GS) is a liver disease characterized by intermittent jaundice and mild unconjugated hyperbilirubinemia [(43, 44)]. In GS, reduced activity of the enzyme uridine diphosphate-glucuronyl transferase (UGT) leads to impaired bilirubin conjugation, resulting in elevated levels of indirect bilirubin. The UGT-1 gene has been identified as the responsible gene for GS, emphasizing its crucial role in regulating bilirubin UGT expression [(44)]. Further research and preclinical studies are required to optimize the formulation of LNPs and establish their safety and efficacy in targeting the UGT-1 gene in liver cells. By harnessing the potential of gene therapy and leveraging the investigated LNPs, there is a promising opportunity to develop innovative treatments that target the underlying cause of GS, providing a potential cure or substantial relief from associated symptoms for affected individuals. In addition, future investigations can explore the correlation between the lipid structure and pKa values in LNPs will provide the necessary knowledge to build better LNP systems. Understanding the relationship between lipid composition, pKa, and LNP performance can pave the way for tailored design strategies and improved control over the encapsulation process.

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4.1 Conclusion

In conclusion, significant contributions have been made to the understanding and optimization of lipid nanoparticles (LNPs) as effective carriers for gene therapy through our research. The investigation of lipid and LNP pKa values, as well as the characterization of size, charge, polydispersity index, encapsulation efficiency, and cytotoxicity, collectively contribute to the advancement of gene therapy. The knowledge gained from this study can be applied to further enhance the design and formulation of LNPs, leading to improved treatment outcomes and the realization of the full potential of gene therapy in treating various genetic disorders. Future research should focus on investigating the underlying mechanisms responsible for the observed changes in nanoparticle size and morphology. Furthermore, the in vitro and in vivo performance of these nanoparticles, including cargo release kinetics, cellular uptake efficiency, and therapeutic efficacy, should be examined to obtain a more comprehensive understanding of their potential applications in the field of biomedicine.

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