Chapter 7

Determination of Optimum Ratio of Cationic Polymers and Small Interfering RNA with Agarose Gel Retardation Assay

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Abstract

Nanomaterials have aroused attention in the recent years for their high potential for gene delivery applications. Most of the nanoformulations used in gene delivery are positively charged to carry negatively charged oligonucleotides. However, excessive positively charged carriers are cytotoxic. Therefore, the complexed oligonucleotide/nanoparticles should be well-examined before the application. In that manner, agarose gel electrophoresis, which is a basic method utilized for separation, identification, and purification of nucleic acid molecules because of its poriferous nature, is one of the strategies to determine the most efficient complexation rate. When the electric field is applied, RNA fragments can migrate through anode due to the negatively charged phosphate backbone. Because RNA has a uniform mass/charge ratio, RNA molecules run in agarose gel proportional according to their size and molecular weight. In this chapter, the determination of complexation efficiency between cationic polymer carriers and small interfering RNA (siRNA) cargos by using agarose gel electrophoresis is described. siRNA/cationic polymer carrier complexes are placed in an electric field and the charged molecules move through the counter-charged electrodes due to the phenomenon of electrostatic attraction. Nucleic acid cargos are loaded to cationic carriers via the electrostatic interaction between positively charged amine groups (N) of the carrier and negatively charged phosphate groups (P) of RNA. The N/P ratio determines the loading efficiency of the cationic polymer carrier. In here, the determination of N/P ratio, where the most efficient complexation occurs, by exposure to the electric field with a gel retardation assay is explained.

Key words Small interfering ribonucleic acid (siRNA), Agarose gel retardation assay, siRNA/cationic polymer carrier complex, Nanoparticles, N/P ratio, Gene delivery

1 Introduction

Regulation of a specific gene has been used for the treatment of a wide range of diseases such as cardiovascular diseases [1], neurodegenerative diseases [2], and cancer [3]. RNA Interference (RNAi) has become a powerful tool for gene silencing studies due to its advantageous properties such as high specificity, effectiveness, a minimum amount of side effect, and easy preparation [4]. RNAi mechanism was first determined by Andrew Z. Fire, Craig

C. Mello, and their colleagues [5]. As a result of their studies, they received the Nobel Prize in Physiology or Medicine in 2006. Interfering RNAs have the ability to silence target genes in cells [6]. At this silencing process, 18–31 nucleotides length small RNA molecules are introduced into cells and induce a sequence-specific gene silencing at the post-transcriptional level by blocking mRNAs containing a matched sequence.

siRNA is the most commonly used interfering RNA and has shown high potential as a therapeutic RNA for gene-based treatments [7]. It regulates the expression of various genes by binding to mRNAs in the cell cytoplasm and causing degradation of their mRNA target. The siRNA is double stranded in nature and is about 22 nucleotides in length. Its precursor is initially recognized by Dicer RNase and then incorporated with the RNA-induced silencing complex (RISC). The siRNA-RISC complex can bind the targeting region of the mRNA and lead to a sequence-specific cleavage with endonuclease Argonaute-2 (AGO2), thereby reducing the expression of the targeted protein [8].

Although siRNA has had particular interest in research, there are some limitations. The major limitations of siRNAs-based therapeutics are their rapid degradation by serum nucleases, poor cellular uptake due to the negatively charged backbone, rapid renal clearance following systemic delivery, off-target effects, and induction of immune responses [9]. In addition, even after siRNA is released from the endosome without being exposed to the lysosome and released into the cell cytoplasm, gene silencing of the siRNA may not be immediately observed [10]. Thereby, before the silencing therapeutic effect of siRNA begins, there is always an induction period due to the intracellular half-life of the target protein. The silencing effect of the given siRNA decreases over time owing to the natural degradation of the siRNA molecules in the cell. Moreover, the therapeutic effect persists for a limited time in rapidly dividing cells such as cancer cells, due to the continuous dilution of siRNA in the replication.

Bare siRNA molecules have poor cellular internalization and they need a carrier to enter cells, where their mechanism of action occurs. The major challenge in the delivery of nucleic acids is the availability of a suitable carrier for transferring siRNA to target cells. To do that, there are two main approaches such as viral and non-viral vectors [11]. The suitable vectors should provide a high degree of transfection for a long period without causing systemic toxicity and immunogenicity [12]. Despite the high transfection activity of viral vectors, possible damage to host genes, immune system stimulation, and infection potential limit its application for gene therapy [13].

To overcome these limitations different types of delivery systems have been designed such as lipid [14, 15], polymer [16], peptide [17, 18], dendrimer [19–21], and micelle [22] based

vehicles. An ideal siRNA delivery system should be non-toxic, safe, and effective. Thus, many studies have focused on the development of non-viral vectors with minimal toxicity. Furthermore, the carrier systems should assure entrance of siRNA cargos to the cell cytoplasm without being interrupted by biological barriers such as serum, cell membrane, and endosome/lysosome [23]. In detail, the cell entry of siRNA/cationic polymer carriers is mostly facilitated by the mechanism known as “endocytosis” [10]. In particular, siRNA/cationic polymer carriers should be able to have endosomal escape. Otherwise, siRNA could be degraded in the acidic and enzymatic milieu of endosome/lysosome [24].

Cationic polymers and lipids have frequently been employed in research due to their advantages in gene delivery such as biodegradability, low cytotoxicity, structure variety, and easy scale-up production [25]. Therapeutic nucleic acid cargoes are loaded into the carrier systems mostly through the positive–negative charge interactions between positive charges of carrier and negative charges of phosphate groups in RNA. However, cationic polymer carriers with excess of positive charge may cause toxicity. Cationic carriers cause considerable disruption of cellular membrane integrity because of the negatively charged constitution of cell membrane [26]. Moreover, cationic nanocarriers induce cell necrosis due to the positive charge [27]. They also cause mitochondrial and lysosomal damage and formation of a high number of autophagosomes [28]. In order to overcome this problem, smart carriers have been developed not to cause cell membrane hydrolysis and necrosis so that they can deliver the therapeutic agents to the target site [29]. Although the benefits of nanocarriers in drug delivery have attracted much attention and great efforts have been made to investigate better cationic carriers, toxicity has always been the main problem of cationic carrier applications [30]. Because of this toxicity issue, the number of positive charges of polymer carriers should be kept low. In that case, the required therapeutic concentration of siRNA could not be achieved. Thus, N/P ratio has a great importance for gene delivery. Consequently, it is essential to keep the N/P ratio low, which indicates the complexation efficiency of the anionic therapeutic agents and the cationic carriers, in order to prevent cytotoxicity from excess amount of cationic polymer carriers. As a result, the main aim for optimum N/P ratio is to carry the most efficient number of siRNA with minimum number of cationic polymer carriers.

To identify the optimum ratio of N and P, the gel retardation assay commonly used for nucleic acid separation could be chosen for determination of N/P ratio [31]. This technique is frequently utilized for the determination of DNA or RNA fragments based on their molecular weight [32]. The phosphate backbone of the DNA or RNA is negatively charged, and therefore RNA fragments

migrate to the positively charged anode when placed in the electric field for separation, identification, and purification [33].

Agarose which, is a pure linear polymer obtained from seaweed, is frequently used for gel electrophoresis [34]. The polymer is boiled to dissolve in a buffer solution and polymerized in gel form by hydrogen bonding when left to cool at room temperature. There is no other component such as catalysts required than agarose. Therefore, preparing agarose gel is simple and fast. The advantages of agarose gel electrophoresis are being non-toxic gel medium, rapid, and easy to cast of gels and providing well separation of high molecular weight nucleic acids [35]. In addition, the samples can be recovered from the gel by melting or digesting the gel with any agarose enzyme or by treating a chaotropic salt [36].

The movement of molecules in an agarose gel depends on their size, charge, the type of electrophoresis buffer, and the pore size of the gel. In this method, siRNA is forced to migrate through an extremely cross-linked agarose base in response to an electric field. In the solution, the phosphate groups of the siRNA are negatively charged so the siRNA molecule migrates to the positive pole (Fig. 1).

This technique is also being used for determination of the complexation efficiency of siRNA/cationic polymer carriers. By adding a positively charged polymer to the siRNA, the overall charge of the complex is neutralized. Because of decreasing the negative charge density of siRNA complex, the movement of siRNA/cationic polymer carriers in the gel is getting difficult. If the complexation does not happen completely, for example, there are free forms of siRNA with siRNA/cationic polymer carriers, the free siRNAs travel far away than the complexation forms [37].

All in all, the movement of a siRNA/cationic polymer carrier complexation through a gel depends on (a) size of the complexation structure, (b) agarose concentration, (c) type of agarose, (d) applied voltage, (e) presence of staining dye, and (f) electrophoresis buffer type [38]. After running the samples in a suitable dye-containing gel, the complexed and free siRNA can be visualized under UV light.

2 Materials

1. siRNA.
2. Agarose.
3. Tris-HCl.
4. Acetic acid or boric acid.
5. EDTA.
6. DNA Ladder.

Fig. 1 Agarose gel retardation assay to evaluate siRNA binding efficiency of 15 kDa cationic polymer complexation with siRNA at different N/P (amine/phosphate) ratios. The gel retardation assay is set as the number of carriers is increasing, siRNA is kept constant. The orange-colored rectangle marking in the figure shows the optimal N/P ratio (2/1) which is siRNA completely complexed with the carriers

7. Loading Dye.
8. RedSafe nucleic acid staining solution.
9. Ethidium bromide.
10. dH₂O.
11. Microwave oven.
12. Gel casting device.
13. Glass beaker.
14. Graduated cylinder.

Prepare solutions with distilled water and nuclease-free water. Store all reagents (except siRNA and loading dye) at room temperature.

2.1 Agarose Gel

1. Preparation of tris acetate-ethylenediaminetetraacetic (TAE) buffer reagents: Prepare 50× TAE buffer (pH 8.0) with 40 mM ethylenediaminetetraacetic (EDTA) disodium salt (M_W : 336.21 g/mol), 2 mM tris base (M_W : 121.14 g/mol), 1 M Acetic Acid (M_W : 60.05 g/mol) (*see Note 1*).
2. Preparation of stock solution 5.0 l 50× TAE buffer (pH 8.0): Weight 1.21 g of Tris base, 67.24 g of EDTA, and draw 285.95 mL of acetic acid and dissolve all of them in 5.0 L distilled water, carefully. Perform this step on the magnetic stirrer and adjust pH 8.0 with hydrochloric acid (HCl) (*see Note 2*). Store the solution at room temperature.

3. Preparation of $1\times$ TAE buffer: Draw 20.0 mL of $50\times$ TAE buffer solution and complete it to final 1000.0 mL in a glass beaker (*see Note 3*).
4. Preparation of 1% agarose gel: Weight 1.0 g agarose powder, add 100.0 mL of electrophoresis buffer. Agarose powder is dissolved in the electrophoresis buffer to the desired concentration (*see Note 4*).

2.2 Polymer and siRNA

1. Preparation of polymer solution: Add 1.0 mg of polymer in 1.0 mL of nuclease-free water. Store at 4°C .
2. Stock siRNA solution: Dissolve siRNA in nuclease-free water at a concentration of $50\text{ }\mu\text{mol}$. Afterwards, allocate 800.0 μL of stock siRNA solution into microcentrifuge tubes (*see Note 5*) and store at -20°C .

3 Methods

3.1 Calculation of Nitrogen to Phosphate (N/P) Ratio for Complexation for siRNA/Cationic Polymer Carriers

Following the steps below, the number of siRNA, cationic polymers, and N/P ratios will be calculated. It is good to create a table containing the amounts of siRNA, polymer, loading dye, and distilled water, which will be helpful for setting the experiment (*see Table 1*). After the calculation, the complexation siRNA/cationic polymer carriers at the different N/P ratios are loaded into gel wells, run in the electrophoresis, and visualized under UV light.

To sum up, the procedure can be simply separated into four steps: (1) preparation of materials to be loaded into wells and carried out of complexation, (2) preparation of agarose gel and loading of samples into wells, (3) running the samples at appropriate voltage and time, and (4) obtained data and identification of the optimum N/P ratio (Fig. 2).

3.1.1 Calculation of the Amine Groups of Polymer

1. Calculate the molecular weight and the number of amine groups of the cationic polymer (or look at the datasheet of your polymer if you purchase from a vendor) (*see Note 6*), i.e.;
 - (a) The molecular weight of a cationic polymer: 180,000.0 g/mol.
 - (b) Amine groups per the cationic polymer: 235 amines.
2. Determine the initial dose of the cationic polymer to be used and calculate the mole of the cationic polymer, i.e.;
 - (a) The cationic polymer dose: 1.0 mg.
 - (b) The number of the cationic polymer moles: 5.5×10^{-9} mol.

Table 1
The siRNA/cationic polymer carrier complexation for different N/P ratios

Well number	Treatment (N/P)	Stock siRNA solution (μL)	Stock polymer solution (μL)	Loading dye (μL)	Nuclease free H ₂ O (μL)
1	DNA ladder	–	–	–	–
2	Free siRNA	1.0	–	4.0	15.0
3	1.0/1.0	1.0	1.6	4.0	13.4
4	1.5/1.0	1.0	2.4	4.0	12.6
5	2.0/1.0	1.0	3.2	4.0	11.8
6	2.5/1.0	1.0	4.0	4.0	11.0
7	4.0/1.0	1.0	6.4	4.0	8.6
8	6.0/1.0	1.0	9.6	4.0	5.4
9	Free polymer	–	10.6	4.0	5.4

Fig. 2 Brief summary of the creation stages of gel retardation assay

3. Multiply the number of amine groups in the cationic polymer by the number of cationic polymer moles. This number will be the number of total amine moles.

(a) The mole of amine groups: 1.3×10^{-6} mol.

3.1.2 Calculation of Phosphate Groups of siRNAs

1. Calculate the average molecular weight of siRNAs (*see Note 7*), i.e.;

(a) The average molecular weight of a siRNA molecule: 340.0 g/mol.

(b) siRNA consists of double stranded 21 bp so it has a total of 42 bp unit/siRNA molecule.

(c) The molecular weight of 21 bp siRNA (siRNA molecule * number of bp * 2) 14,280.0 g/mol ($340.0 * 21 * 2$). This is the value used in the subsequent calculations.

2. Determine the initial dose of the siRNA to be used and calculate the mole of the siRNA.

(a) The siRNA dose: 0.7 µg.

(b) The number of siRNA moles: 4.9×10^{-11} mol.

3. Multiply the number of Phosphate (PO_4^{3-}) groups in the siRNA to get the total number of phosphate groups.

(a) The mole of PO_4^{3-} groups (siRNA moles * nr PO_4^{3-} groups): 2.06×10^{-9} mol (4.9×10^{-11} mol * 42).

3.1.3 Calculation of N/P Ratios

One milligram of cationic polymer (1.3×10^{-6} mol obtained from Subheadings 3.1.1–3.1.3) dissolved in 1.0 mL of distilled water as a stock solution. For the calculation of 1:1 N/P ratio, calculate the amount of amine groups in the polymer which equals to the same mole of PO_4^{3-} groups (2.1×10^{-9} mol obtained from the Subheadings 3.1.2 and 3.1.3). Based on this calculation, 1.60 µL of the polymer solution is taken. For 1.5:1, 2:1, 2.5:1, 4:1, 6:1 N/P ratios, this volume is multiplied with 1.5, 2.0, 2.5, 4.0, and 6.0, respectively (*see Table 1*).

3.2 Preparation of 1% Agarose Gel Electrophoresis

1. To prepare $1\times$ TAE buffer, get 50.0 mL of $50\times$ TAE buffer stock solution and complete to 1000.0 mL volume with distilled water in a beaker.
2. Add 1.0 g of agarose powder into 100.0 mL of $1\times$ TAE buffer and mix it to make 1% agarose gel. After that, boil the agarose mixture in a microwave oven to melt it (*see Note 8*).
3. Cool the solution to approximately 50–60 °C.
4. Add the RedSafe nucleic acid staining solution (5.0 µL) (*see Note 9*).

5. Poured into a casting tray containing a sample comb and allowed to solidify at room temperature (*see* **Note 10**).
6. Remove the sample comb from the cooling casting tray (*see* **Note 11**).

3.3 Loading siRNA/ Cationic Polymer Carrier Complex into 1% Agarose Gel

1. Prepare mixture of nuclease-free water, polymer, and siRNA in separated microcentrifuge tubes as given in Table 1.
2. Allow 30 min for the complexation of siRNA/cationic polymer carrier.
3. Add 4.0 μL of DNA gel loading dye to each centrifuge tube. The loading dye ($6\times$) is added to the gel for the visualization of siRNA during sample loading and running.
4. Load a total 20.0 μL volume from each sample into agarose gel wells.
5. Set power supply at 60 V for 60 min (*see* **Note 12**).
6. The electrophoretic mobility of the siRNAs of each sample is visualized under UV light (*see* Fig. 1).

4 Notes

1. Agarose gel electrophoresis of siRNA is performed using either TAE buffer or tris-borate-EDTA (TBE) buffer [39].
2. Wear a mask and gloves when preparing buffer agents. TAE buffer solution can be stored at room temperature for a month. In our laboratory, we prepare fresh solution every month.
3. The most reliable strategy for TAE usage, prepare a fresh $1\times$ TAE buffer for each time just before the experiment.
4. The percentage of agarose gels used is generally in the range of 0.2–3%. This percentage could be changed based on the size of siRNA and total molecular weight of the carrier [40].
5. Allocate all the samples (i.e. siRNA) to avoid damaging the main source.
6. Calculation/determination of the molecular weight, chemical formulation, surface charge, and size of the polymer are carried out according to ^1H -NMR, zeta potential, and size analysis.
7. The average molecular weight of siRNA base is 340 g/mol. Average molecular weight of a double-stranded DNA (dsDNA) base pair and a single-stranded DNA (ssDNA) base pair are 600 and 330 g/mol, respectively [41].
8. Initially, the agarose solution is boiled for 45–60 s in the microwave at 180 $^{\circ}\text{C}$. If the agarose powder does not dissolve well, repeat the step for another 20–25 s until it totally dissolves.

9. Instead of using RedSafe nucleic acid staining dye, ethidium bromide, bromophenol blue or xylene cyanole could be preferred [35].
10. The casting tray containing sample comb must be on a flat surface. Otherwise, the thickness of the gel may not be uniform. While pouring the solution, one should be careful about the formation of bubbles, which may affect the migration efficiency.
11. To solidify the gel, wait 1–1.5 h before removing the comb from the tray to ensure that the loading wells are intact and well-opened.
12. The optimum running time is 45–90 min to identify the N/P ratio of siRNA/cationic polymer carrier complexation. In the case of gel electrophoresis is performed at a higher voltage, the running time is decreased. However, if the running time is increased that leads to heating up which leading to siRNA banding artifacts [39].

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References

1. Zhang X, Li DY, Reilly MP (2019) Long intergenic noncoding RNAs in cardiovascular diseases: challenges and strategies for physiological studies and translation. *Atherosclerosis* 281:180–188. <https://doi.org/10.1016/j.atherosclerosis.2018.09.040>
2. Conejos-Sanchez I, Gallon E, Nino-Pariente A, Smith JA, De la Fuente AG, Di Canio L, Pluchino S, Franklin RJM, Vicent MJ (2019) Polyornithine-based polyplexes to boost effective gene silencing in CNS disorders. *Nanoscale* 12(11):6285–6299. <https://doi.org/10.1039/c9nr06187h>
3. Liu T, Wang L, Xin H, Jin L, Zhang D (2020) Delivery systems for RNA interference-based therapy and their applications against cancer. *Sci Adv Mater* 12(1):75–86. <https://doi.org/10.1166/sam.2020.3694>
4. Subhan MA, Torchilin VP (2019) Efficient nanocarriers of siRNA therapeutics for cancer treatment. *Transl Res* 214:62–91. <https://doi.org/10.1016/j.trsl.2019.07.006>
5. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391(6669):806–811. <https://doi.org/10.1038/35888>
6. Dong Y, Siegwart DJ, Anderson DG (2019) Strategies, design, and chemistry in siRNA delivery systems. *Adv Drug Deliv Rev* 144: 133–147. <https://doi.org/10.1016/j.addr.2019.05.004>
7. Kapadia CH, Luo B, Dang MN, Irvin-Choy ND, Valcourt DM, Day ES (2020) Polymer nanocarriers for MicroRNA delivery. *J Appl Polym Sci* 137(25):48651. <https://doi.org/10.1002/app.48651>
8. Lin Y-X, Wang Y, Blake S, Yu M, Mei L, Wang H, Shi J (2020) RNA nanotechnology-mediated cancer immunotherapy. *Theranostics* 10(1):281–299. <https://doi.org/10.7150/thno.35568>

9. Karimi F, Azizi Jalilian F, Hossienkhani H, Ezati R, Amini R (2019) siRNA delivery technology for cancer therapy: promise and challenges. *Acta Med Iran* 57:83–93. <https://doi.org/10.18502/acta.v57i2.1760>
10. Tsouris V, Joo MK, Kim SH, Kwon IC, Won Y-Y (2014) Nano carriers that enable co-delivery of chemotherapy and RNAi agents for treatment of drug-resistant cancers. *Bio-technol Adv* 32(5):1037–1050. <https://doi.org/10.1016/j.biotechadv.2014.05.006>
11. Shirley JL, de Jong YP, Terhorst C, Herzog RW (2020) Immune responses to viral gene therapy vectors. *Mol Ther* 28(3):709–722. <https://doi.org/10.1016/j.ymthe.2020.01.001>
12. Sabouri-Rad S, Oskuee RK, Mahmoodi A, Gholami L, Malaekheh-Nikouei B (2017) The effect of cell penetrating peptides on transfection activity and cytotoxicity of polyallylamine. *Bioimpacts* 7(3):139–145. <https://doi.org/10.15171/bi.2017.17>
13. Rezaee M, Oskuee RK, Nassirli H, Malaekheh-Nikouei B (2016) Progress in the development of lipopolyplexes as efficient non-viral gene delivery systems. *J Control Release* 236:1–14. <https://doi.org/10.1016/j.jconrel.2016.06.023>
14. Parashar D, Rajendran V, Shukla R, Sistla R (2020) Lipid-based nanocarriers for delivery of small interfering RNA for therapeutic use. *Eur J Pharm Sci* 142:105159. <https://doi.org/10.1016/j.ejps.2019.105159>
15. Jorge A, Pais A, Vitorino C (2020) Targeted siRNA delivery using lipid nanoparticles. *Methods Mol Biol* 2059:259–283
16. Liao X (2019) Development of a cationic polymer-based siRNA delivery system for the regeneration of tendon injury. University of East Anglia, Norwich
17. Qiu Y, Tam B, Chung WY, Mason J, Lam JK (2019) Relationship between the secondary structure of the peptide base siRNA carrier and effective gene silencing effect on lung epithelial cells. *J Aerosol Med Pulmonary Drug Delivery* 2:A16–A17
18. Cummings JC, Zhang H, Jakymiw A (2019) Peptide carriers to the rescue: overcoming the barriers to siRNA delivery for cancer treatment. *Transl Res* 214:92–104. <https://doi.org/10.1016/j.trsl.2019.07.010>
19. Pan J, Mendes LP, Yao M, Filipczak N, Garai S, Thakur GA, Sarisozen C, Torchilin VP (2019) Polyamidoamine dendrimers-based nanomedicine for combination therapy with siRNA and chemotherapeutics to overcome multidrug resistance. *Eur J Pharm Biopharm* 136:18–28. <https://doi.org/10.1016/j.ejpb.2019.01.006>
20. Laurini E, Marson D, Aulic S, Fermeleglia M, Priel S (2019) Evolution from covalent to self-assembled PAMAM-based dendrimers as nanovectors for siRNA delivery in cancer by coupled in silico-experimental studies. Part II: self-assembled siRNA Nanocarriers. *Pharmaceutics* 11(7):324. <https://doi.org/10.3390/pharmaceutics11070324>
21. Jain A, Mahira S, Majoral J-P, Bryszewska M, Khan W, Ionov M (2019) Dendrimer mediated targeting of siRNA against polo-like kinase for the treatment of triple negative breast cancer. *J Biomed Mater Res A* 107(9):1933–1944. <https://doi.org/10.1002/jbm.a.36701>
22. Lu Y, Zhong L, Jiang Z, Pan H, Zhang Y, Zhu G, Bai L, Tong R, Shi J, Duan X (2019) Cationic micelle-based siRNA delivery for efficient colon cancer gene therapy. *Nanoscale Res Lett* 14(1):193. <https://doi.org/10.1186/s11671-019-2985-z>
23. Kandil R, Xie Y, Heermann R, Isert L, Jung K, Mehta A, Merkel OM (2019) Coming in and finding out: blending receptor-targeted delivery and efficient endosomal escape in a novel bio-responsive siRNA delivery system for gene knockdown in pulmonary T cells. *Adv Ther* 2(7):1900047. <https://doi.org/10.1002/adtp.201900047>
24. Prantner AM, Scholler N (2014) Biological barriers and current strategies for modifying nanoparticle bioavailability. *J Nanosci Nanotechnol* 14(1):115–125. <https://doi.org/10.1166/jnn.2014.8899>
25. Patil S, Gao Y-G, Lin X, Li Y, Dang K, Tian Y, Zhang W-J, Jiang S-F, Qadir A, Qian A-R (2019) The development of functional non-viral vectors for gene delivery. *Int J Mol Sci* 20(21):5491. <https://doi.org/10.3390/ijms20215491>
26. Chen J, Hessler JA, Putschakayala K, Panama BK, Khan DP, Hong S, Mullen DG, Dimaggio SC, Som A, Tew GN, Lopatin AN, Baker JR, Holl MMB, Orr BG (2009) Cationic nanoparticles induce nanoscale disruption in living cell plasma membranes. *J Phys Chem B* 113(32):11179–11185. <https://doi.org/10.1021/jp9033936>
27. Wei X, Shao B, He Z, Ye T, Luo M, Sang Y, Liang X, Wang W, Luo S, Yang S, Zhang S, Gong C, Gou M, Deng H, Zhao Y, Yang H, Deng S, Zhao C, Yang L, Qian Z, Li J, Sun X, Han J, Jiang C, Wu M, Zhang Z (2015) Cationic nanocarriers induce cell necrosis through impairment of Na⁺/K⁺-ATPase and cause subsequent inflammatory response. *Cell Res*

- 25(2):237–253. <https://doi.org/10.1038/cr.2015.9>
28. Fröhlich E (2012) The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int J Nanomedicine* 7: 5577–5591. <https://doi.org/10.2147/IJN.S36111>
29. Durmaz YY, Lin Y-L, ElSayed MEH (2013) Development of degradable, pH-sensitive star vectors for enhancing the cytoplasmic delivery of nucleic acids. *Adv Funct Mater* 23(31): 3885–3895. <https://doi.org/10.1002/adfm.201203762>
30. Lv H, Zhang S, Wang B, Cui S, Yan J (2006) Toxicity of cationic lipids and cationic polymers in gene delivery. *J Control Release* 114(1): 100–109. <https://doi.org/10.1016/j.jconrel.2006.04.014>
31. Scott V, Clark AR, Docherty K (1994) The gel retardation assay. In: *Protocols for gene analysis*. Springer, Berlin, pp 339–347
32. Hellman LM, Fried MG (2007) Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions. *Nat Protoc* 2(8):1849
33. Helling RB, Goodman HM, Boyer HW (1974) Analysis of endonuclease R-EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. *J Virol* 14(5):1235–1244
34. Magdeldin S (2012) *Gel electrophoresis: principles and basics*. BoD–Books on Demand, Norderstedt
35. Chawla H (2002) *Introduction to plant biotechnology*. Routledge, Milton Park. <https://doi.org/10.1201/9781315275369>
36. Tietz D (2012) *Nucleic acid electrophoresis*. Springer, Berlin
37. Loening UE (1968) Molecular weights of ribosomal RNA in relation to evolution. *J Mol Biol* 38(3):355–365. [https://doi.org/10.1016/0022-2836\(68\)90391-4](https://doi.org/10.1016/0022-2836(68)90391-4)
38. Rio DC, Ares M Jr, Hannon GJ, Nilsen TW (2010) Nondenaturing agarose gel electrophoresis of RNA. *Cold Spring Harb Protoc* 2010(6):pdb.prot5445. <https://doi.org/10.1101/pdb.prot5445>
39. Sanderson BA, Araki N, Lilley JL, Guerrero G, Lewis LK (2014) Modification of gel architecture and TBE/TAE buffer composition to minimize heating during agarose gel electrophoresis. *Anal Biochem* 454:44–52. <https://doi.org/10.1016/j.ab.2014.03.003>
40. Murphy D, Carter DA, Evans MJ (1993) *Transgenesis techniques: principles and protocols*. Springer, Berlin
41. He F (2011) DNA molecular weight calculation. *Bio-Protocol* 1(6):e46. <https://doi.org/10.21769/BioProtoc.46>

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