Proof of concept studies for siRNA delivery by non-ionic surfactant vesicles: *in vitro* and *in vivo* evaluation of protein knockdown

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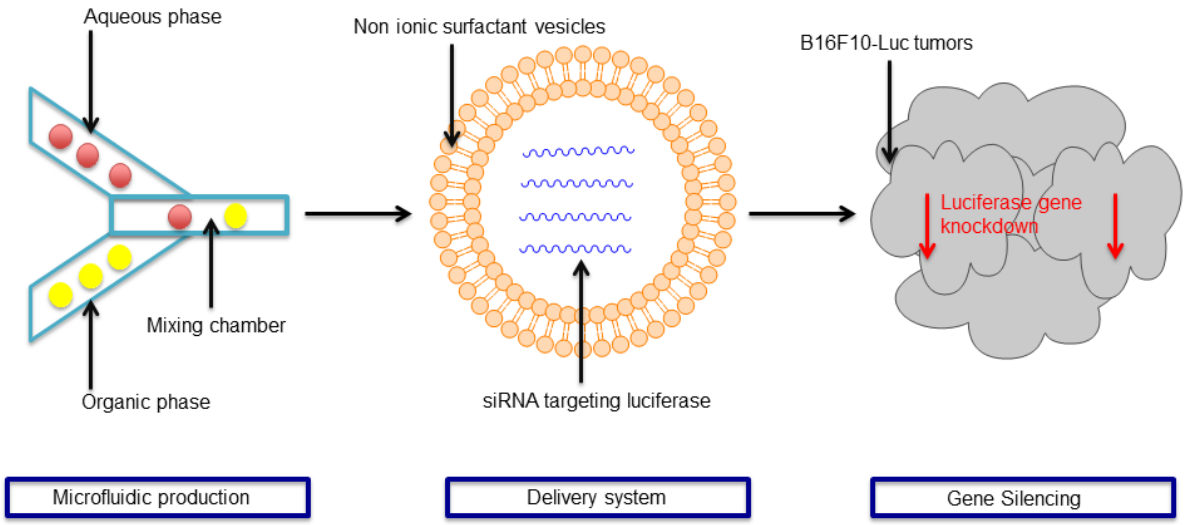
Abbreviations

NISV: Non-ionic surfactant vesicles; CN: cationic niosomes; PBS: Phosphate buffered saline; Chol: Cholesterol; T85: Tween 85; PDI: Polydispersity index; ZP: Zeta potential; siRNA: Small interfering RNA; RNAi: RNA interference; FACS: fluorescence-activated cell sorter.

Graphical abstract



Abstract

RNA interference (RNAi) is an effective and naturally occurring post-transcriptional gene regulatory mechanism. This mechanism involves the degradation of a target messenger RNA (mRNA) through the introduction of short interfering RNA (siRNA) that is complementary to the target mRNA. The application of siRNA-based therapeutics is limited by the development of an effective delivery system, as naked siRNA is unstable and cannot penetrate the cell membrane. In this study, we investigated the use of cationic niosomes (CN) prepared by microfluidic mixing for siRNA delivery. In an *in vitro* model, these vesicles were able to deliver anti-luciferase siRNA and effectively suppress luciferase expression in B16-F10 mouse melanoma cells. More importantly, in an *in vivo* mouse model, intratumoral administration of CN-carrying anti-luciferase siRNA led to significant suppression of luciferase expression

compared with naked siRNA. Thus, we have established a novel and effective system for the delivery of siRNA both *in vitro* and *in vivo,* which shows high potential for future application of gene therapeutics.

Key Words

Niosomes, Nanoparticles, Microfluidics, RNA interference, Drug delivery

1. Introduction

Short interfering RNA (siRNA) has gained substantial interest as a promising therapeutic agent as it has the ability to silence specific upregulated genes through a RNA inhibitory (RNAi) mechanism and therefore hindering corresponding protein expression. However, the efficacy of siRNA therapy is significantly hampered by poor cellular membrane penetration, rapid degradation by RNase enzymes, non-specific tissue distribution, and short circulating time [1, 2]. Therefore, therapeutic application of siRNA requires the use of an efficient delivery vehicle that can carry, protect, and efficiently deliver siRNA into target cells [3]. In this regard, lipid-based nanoparticles, including liposomes, have been widely investigated as possible siRNA carriers because of their advantages such as high loading efficiency and biocompatibility [4, 5]. Through their membrane bilayer structure, lipid nanoparticles can protect siRNA that is embedded in the aqueous core or adsorbed on the surface of the nanoparticles [6]. Although liposomes have been demonstrated to be successful in siRNA delivery, they have limitations for widespread use such as stability and high production costs. Non-ionic surfactant vesicles (NISV, niosomes) are one type of lipid-based nanoparticle that have a membrane bilayer structure similar to liposomes [7, 8]. NISV consist of non-ionic surfactants, in addition to cholesterol and charging species in place of the phospholipids used in liposomes [9]. These non-ionic surfactants are composed of a hydrophilic head and a hydrophobic tail that will spontaneously arrange in a bilayer structure upon hydration [10]. The use of non-ionic surfactants in NISV improves the stability of these particles and decreases the production cost compared to liposomes. Cholesterol is another component in the NISV structure that modulates the mechanical strength and water permeability of the bilayer structure [11]. Other additives in NISV include charged molecules which enhance the stability of the formulated vesicles by inducing electrostatic repulsion between individual particles [9]. NISV have gained

considerable interest as a drug delivery system and there are many successful reports for their application in cancer [12, 13], diabetes [14, 15], and transdermal drug delivery [16, 17]. Nevertheless, there are limited reports about the use of NISV as a delivery vehicle for siRNA [18], which has significant potential and needs to be investigated and developed in order to mediate efficacious gene silencing for therapeutic applications.

Previously, we have reported the *in vitro* efficacy of cationic niosomes (CN) formulation in suppressing green fluorescent protein (GFP) expression in copGFP-A549 cells by anti-GFP siRNA [19]. In the present study, to confirm the gene silencing results observed on copGFP-A549 cells and to further explore the *in vivo* efficacy, the biological activity of the CN formulation was tested on a different cell model using another protein reporter. B16-F10 mouse melanoma cells stably expressing luciferase enzyme were used. The CN formulation were loaded with either AllStars Alexa Fluor® 488 (AF488)-labelled Negative Control siRNA to confirm cellular uptake of siRNA or with anti-luciferase siRNA (siLUC) to confirm the effectiveness of these formulations in delivering siRNA and suppressing luciferase enzyme expression. An *in vivo* experiment was then carried out to assess the luciferase suppression in an animal model.

2. Materials and methods

2.1. Materials

Polyoxyethylenesorbitan trioleate (Tween 85), cholesterol (Chol), dimethyldioctadecylammonium bromide (DDAB), resazurin powder, serum-free and antibiotic-free Roswell Park Memorial Institute medium (RPMI 1640), L-glutamine, penicillin–streptomycin were purchased from Sigma–Aldrich (Irvine, UK) (all at cell culture grade). Foetal bovine serum (FBS) was purchased from Biosera (East Sussex, UK). Sodium pyruvate (100mM) and minimum essential medium non-essential amino acids (MEM NEAA)

were purchased from Life Technologies (Loughborough, UK). Mouse melanoma B16-F10-luc-G5 luciferase expressing cells and D-luciferin were obtained from Caliper life Science (Hopkinton, USA). Sterile, RNase-free phosphate buffered saline 1M and sterile, RNase-free water were purchased from LONZA (Slough, UK). AllStars AF488-labelled Negative Control siRNA and HiPerFect transfecting reagent were purchased from Qiagen (Manchester, UK). The anti-luciferase siRNA (siLUC) duplex sequence (*Sense*: rGrArGrGrCrUrArArGrGrUrGrGrUrGrGrArCrUrUrGrGrACA, *Antisense*: rUrGrUrCrCrArArGrUrCrCrArCrCrArCrCrUrUrArGrCrCrUrCrGrA) were synthesised by Integrated DNA Technologies (Leuven, Belgium). ONE-Glo™ luciferase assay system was purchased from Promega Corporation (Southampton, UK).

2.2. Formulation of the cationic niosomes (CN)

CN composed of T85:Chol:DDAB (at a molar ratio of 40:40:20) were prepared by microfluidic mixing as described previously [20] using sterile RNase-free 5 % (w/v) glucose as an aqueous medium. Briefly, a specific volume of the aqueous medium was mixed with the lipid phase in ethanol at a volumetric flow rate of 3:1 (aqueous: lipid) in the microfluidic micromixer at a total flow rate of 12 mL/minute (9 mL/minute for the aqueous phase and 3 mL/minute for the lipid phase) at 50ºC. The mixing process was carried out using a NanoAssemblr^{TM} (Benchtop, Precision NanoSystems Inc., Vancouver, Canada).

2.3 Characterisation of the CN

Particle size, polydispersity index (PDI) and zeta potential (ZP) were measured with a Zetasizer Nano-ZS (Malvern Instruments, UK). These measurements were carried out at 25°C at a 1/20 dilution in the same medium used for the particle preparation. All samples were prepared in triplicate and the $Z_{Average}$, PDI, and ZP reported.

2.4. Cell viability assay

To evaluate the cytotoxicity of the CN, B16-F10 cells were seeded into a 96-well plate at a density of $1x10^4$ per well in 100µl RPMI 1640 medium supplemented with 10% (v/v) FBS, 1 % (v/v) L-glutamine and 1 % (v/v) penicillin-streptomycin and incubated at 37 °C, 5 % CO_2 and 100 % humidity. Twenty-four hours later, cells were treated with CN at concentrations from 9.77-1250 µg/ml. Ten-percent dimethyl sulphoxide (DMSO) was used as positive control and untreated cells as negative control. The treated cells were incubated for a further 24 h and then 20 µl of resazurin (0.1 mg/ml) was added to each well and incubated for a further 24 h. The quantity of resorufin produced resulting from metabolism of resazurin by viable cells, was measured on a SpectraMax M5 plate reader (Molecular Devices, USA) at 560 nm - 590 nm. Cell viability was expressed as a percentage of the untreated control cells. The results were expressed as a mean and standard deviation obtained from three experiments.

2.5. *In vitro* cellular uptake

Cellular uptake of CN by B16-F10 cells was quantified by fluorescence-activated cell sorter (FACS). B16-F10 cells were seeded in 12-well plates at $1x10^5$ cells/well in 1100 µL of RPMI 1640 culture media, supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) MEM NEAA (without antibiotics), 24 h before experiments at 37ºC, 5% CO_2 and 100% humidity. CN/siRNA complexes (termed nioplexes) were prepared as follows: an appropriate volume of siRNA (from 10 µM stock) was mixed with the CN formulation (from a 625 µg/ml stock) with pipetting up and down to ensure optimal mixing. The nioplex samples were incubated at room temperature for 30 min to allow the formation of transfection complexes.

Cells were then treated with CN encapsulating AF488-labelled Negative Control siRNA at a final concentration of 20 nM/well. The results were compared to those obtained with the

positive control HiPerFect transfecting reagent and the experiments were done with the use of siRNA alone, CN alone and untreated cells as controls. Cells were incubated for 72 h and then the media was removed, cells were trypsinised and diluted with PBS to 1 ml. The cell suspension was then centrifuged at 1200 rpm for 5 min and then the pellet re-suspended in 1 ml of FACS buffer (10%, v/v, FBS in PBS). Quantitative cellular uptake was measured using a FACSCanto flow cytometer, BD Biosciences (UK) using FACS Diva software. Upon acquisition, the cells were gated using forward scatter versus side scatter (FCS vs SSC) to eliminate dead cells and debris. Cells (10,000) were collected for each sample and the data were analysed with FACS Diva software. The results are presented as a mean and standard deviation obtained from three samples.

2.6. *In vitro* luciferase gene silencing assay

B16-F10 cells were plated in a 96-well plate (7500 cells/well) in 75 µl RPMI medium containing 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) MEM NEAA (without antibiotics) at 37ºC, 5% CO_2, 100% humidity for 24 h prior to transfection. Cells were then treated with CN or the positive control HiPerFect, containing siLUC at final concentrations of 0, 10, 25, 50, 100, and 200 nM per well. siLUC alone (naked siLUC) and particles alone (mock transfection) were used as controls. The expressed luciferase level in the cells was measured after 24, 48, and 72 h of transfection using a ONE-Glo™ luciferase assay system following the manufacturer's protocol. Briefly, at each time point, 100 µl of ONE-Glo reagent from the assay kit was added to the cells in each well and incubated at 25 °C for 3 min. The bioluminescence expressed in Relative Luminescence Unit (RLU) was measured using an *in vivo* imaging system (IVIS) (IVIS Spectrum®, PerkinElmer, UK). Luciferase activity of a sample was expressed as the percentage of luminescence intensity compared to untreated cells. The percentage of luciferase expression was calculated by the equation:

$$\% \text{ expression} = (RLUluc/RLUctl)*100$$

Where RLUluc is the mean of RLU for luc in treated cells and RLUctl is the mean of RLU for untreated cells. The results were reported as the mean and standard deviation of four different experiments.

2.7. *In vivo* silencing study

2.7.1. Animals

Female BALB/c nude mice, 42-49 days old (average weight of 20 g), were purchased from Charles River Laboratories (UK). Mice were housed in groups of three at 19°C to 23°C with a 12-h light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, UK), with mains water provided *ad libitum*. The *in vivo* experiments described below were performed in accordance with the UK Home Office regulations.

2.7.2. Determination of the most tolerable dose of CN/siLUC

Two female BALB/c nude mice were injected intraperitoneally (i.p.) every day respectively with increased concentrations of empty nanoparticles (0.2 ml, starting from 39 µg/ml nanoparticles) and siLUC nanoparticles (0.2 ml, from 625 nM siLUC) in order to determine the maximum tolerable dose of formulations. The mice were monitored daily for any changes in body weight as a surrogate marker of toxicity.

2.7.3. *In vivo* luciferase gene silencing study

BALB/c female nude mice were injected subcutaneously with $1x10^6$ B16-F10-Luc cells. Seven days later, when the tumours became vascularised and palpable, a single dose of siLUC (625 nM) loaded in CN (0.2 ml, at 39 µg/ml) was injected intratumorally. Naked siLUC, particles alone, and untreated mice were used as controls. Three mice were used in each group. The light emitted as a result of luciferase gene expression was visualised in mice using quantitative whole-body imaging [21]. To this end, mice received an i.p. injection of the luciferase substrate

D-luciferin (150 mg/kg body weight) after 4, 12, 24, and 48 h of treatment and were anesthetised by isoflurane inhalation. Ten min post-injection, bioluminescence was measured for 2 min using the IVIS Spectrum®. Data were analysed using Living Image® software (PerkinElmer, UK). The resulting pseudo-colour images represent the luciferase expression within the animal. Identical illumination settings were used for acquiring all images. Luciferase expression in the treated mice was expressed as a percentage of luminescence intensity compared to the untreated mice.

2.8. Statistical analysis

Results were expressed as means ± standard deviation of three readings. Statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey multiple comparison test and t-test was performed for paired comparisons using Minitab® software, State College, PE. Differences were considered statistically significant for p values < 0.05.

3. Results

3.1 CN characterisation

CN prepared by microfluidic mixing, using sterile RNase-free 5 % (w/v) glucose for particles preparation, were characterised by DLS. The average particles size was 61.37 ± 0.16 nm with a PDI value of 0.18 ± 0.01 indicating homogeneous distribution. Moreover, the average ZP was 55.80 ± 6.55 mV. Further physicochemical properties were reported previously [19].

3.2. Cell viability assay

Toxicity of the CN was assessed on B16-F10 cells using various concentrations of CN (9.77-1250 µg/ml) to quantify cell viability and determine the concentration that caused 50% cell death (EC_{50}). Figure 1 shows the dose-response curves for the cells treated with the CN and the calculated EC_{50}. The cell viability decreased significantly by increasing the CN concentration especially at concentrations of 312.5 µg/ml and above where the cells showed

minimal viability. The EC_{50} value was 84.03 ± 7.39 µg/ml. However, the CN formulation was not toxic at or below 40 µg/ml in which the cells were 100% viable. Therefore, all subsequent experiments that included siRNA transfection were carried out so the final NISV concentration was below 40 µg/ml.

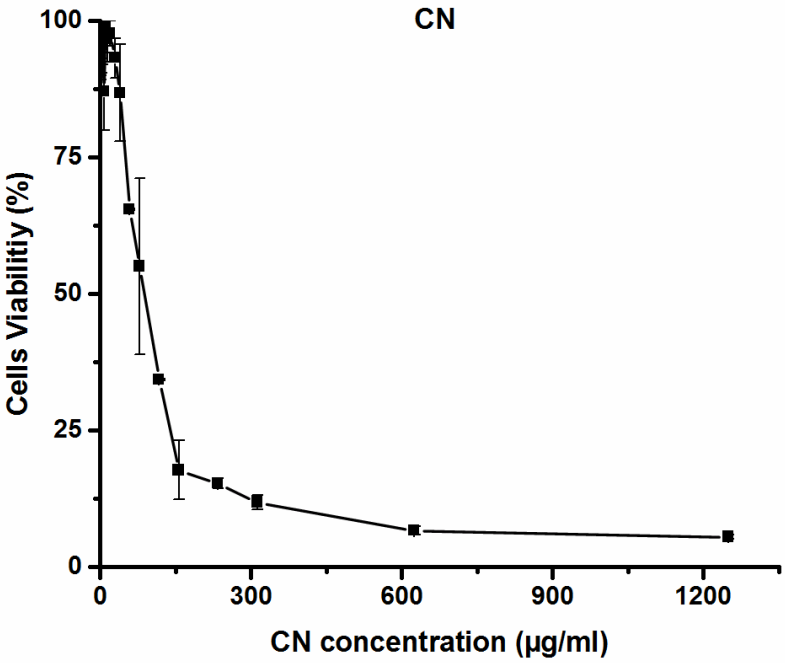


Figure 1 Cytotoxicity of the CN Formulation on B16-F10 cells. The data represents the mean ± SD (n=3).

3.3. B16-F10 cellular uptake

To evaluate the cellular uptake, B16-F10 mouse melanoma cells were treated with CN formulation loaded with AF488-labelled negative control siRNA. The treated cells were analysed by FACS for quantitative cellular uptake (Figure 2). As can be seen in Figure 2A, B16-F10 cells did not show any siRNA uptake after being treated with siRNA alone, which can be confirmed by the very low MFI (Figure 2B) and the histogram curve (Figure 2C) compared to untreated cells. The cellular uptake for cells treated with AF488-labelled negative

control siRNA encapsulated in the CN formulation was 88.28 ± 2.29% (Figure 2A). This cellular uptake was significantly ($p < 0.05$) higher than the cellular uptake achieved by HiPerFect (70.77 ± 4.35%) and the MFI was significantly higher for cells treated with AF488-labelled negative control siRNA encapsulated by CN or HiPerFect compared to naked siRNA (Figure 2B). When the CN formulation alone without AF488-labelled negative control was used, the MFI values were low, indicating no auto-fluorescence (Figure 2B) and the histogram curves had slightly shifted compared to untreated cells (Figure 2C).

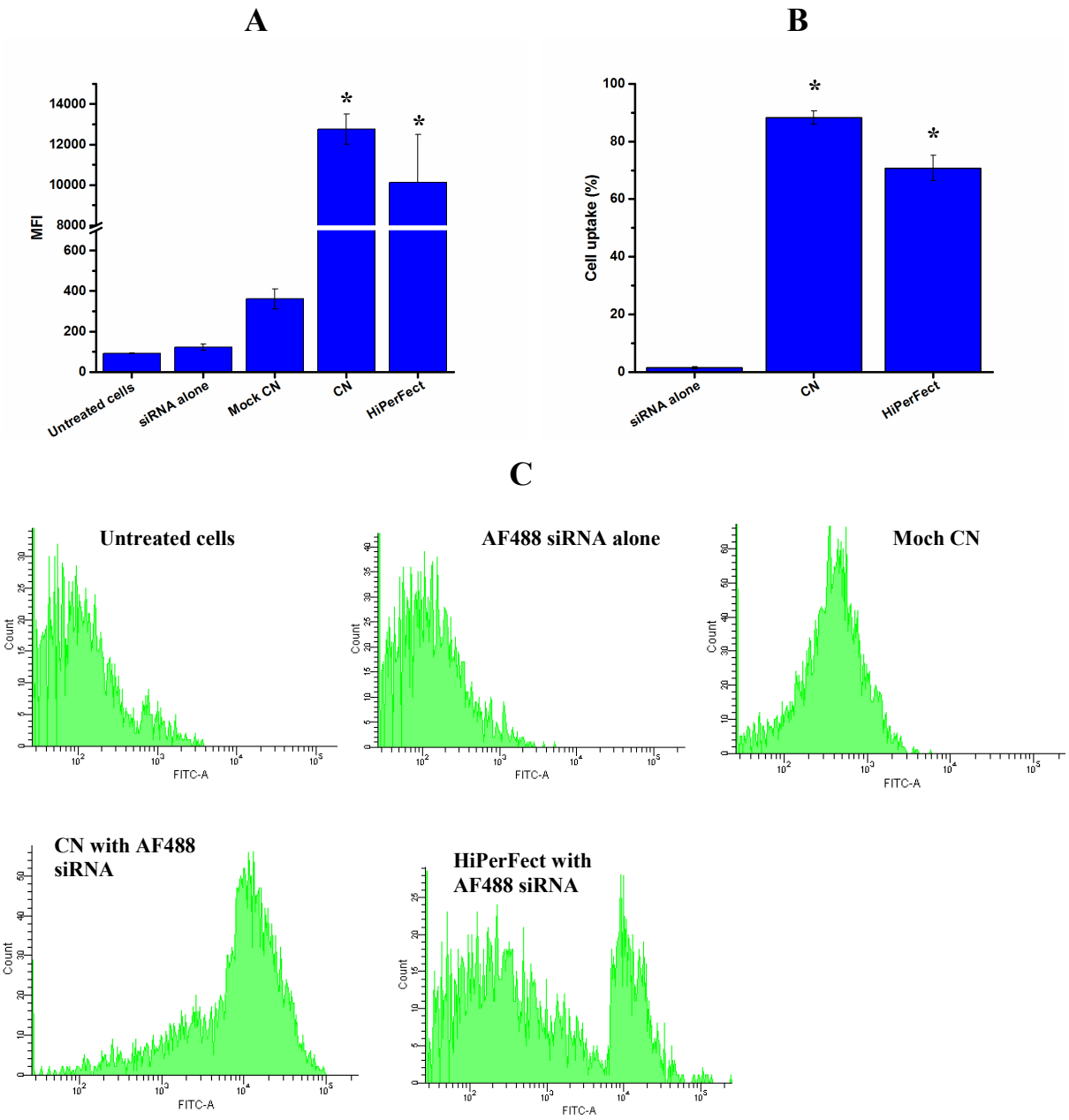


Figure 2 FACS results for (A) MFI, (B) percentages of cellular uptake, and (C) flow cytometry histograms of B16-F10 cellular uptake when treated with CN or HiPerFect loaded with AF488-labelled negative control siRNA. Images are representative of three independent images from each sample. The data represents means ± standard deviation (n = 3). *p <0.05 significant difference from cells treated with naked siRNA. CN: cationic niosomes, Moch CN: empty cationic niosomes.

3.4. *In vitro* luciferase gene silencing study

We examined the extent of gene silencing by siLUC loaded in the CN formulation. To evaluate the gene knockdown efficiencies, B16-F10 cells stably expressing luciferase were incubated with CN formulation, loaded with various concentrations of luc siRNA, for 24, 48, and 72 h. The specificity of the siLUC was confirmed using scrambled negative control siRNA. Luciferase expression was measured by quantifying the luciferase luminescence intensity at each time point in the cells treated with CN loaded with various concentrations of siLUC (Figure 3). The luciferase enzyme knockdown was compared to untreated cells. CN demonstrated significant ($p< 0.05$) transfection efficiency, which was concentration- and time-dependent. After 24 h incubation, the percentage of luciferase expression (compared to untreated cells) decreased significantly ($p< 0.05$) from 77.01 ± 2.22% to 54.42 ± 2.06% by increasing the siLUC concentration from 10 – 200 nM. After 48 h incubation, all the siLUC concentrations induced the same level of luciferase expression inhibition resulting in a luciferase expression of around 30% (Figure 3). Longer incubation times with CN formulation did not result in higher luciferase inhibition. This significant luciferase inhibition, achieved by the CN formulation, was much higher than the inhibition demonstrated by HiPerFect. With HiPerFect, the luciferase inhibition was dose-dependent at all time points with the highest knockdown achieved after 48 and 72 h with no significant difference between both time points. After 24 h incubation, the highest luciferase inhibition achieved when the cells were transfected with 200 nM siLUC using HiPerFect was 75.54 ± 0.56% luciferase expression. This was significantly ($p <0.05$) higher than the luciferase expression when the cells were treated with the CN formulation using the same siLUC concentration (luciferase expression 54.42 ± 2.06%). Longer incubation time for cells transfected with HiPerFect resulted in higher luciferase inhibition, where the maximum effect seen after 48 h using 200 nM of siLUC resulted in luciferase expression of 48.35 ± 4.48% with no further effect at longer incubation times (Figure

3). No effect was noticed for CN formulation at 0 nM siLUC (mock transfection) at all time points. Similarly, no effect was seen when using siLUC alone at all time points (data not shown). This indicates that the decrease in luciferase expression using siLUC was indeed caused by the sequence specific gene silencing of siLUC.

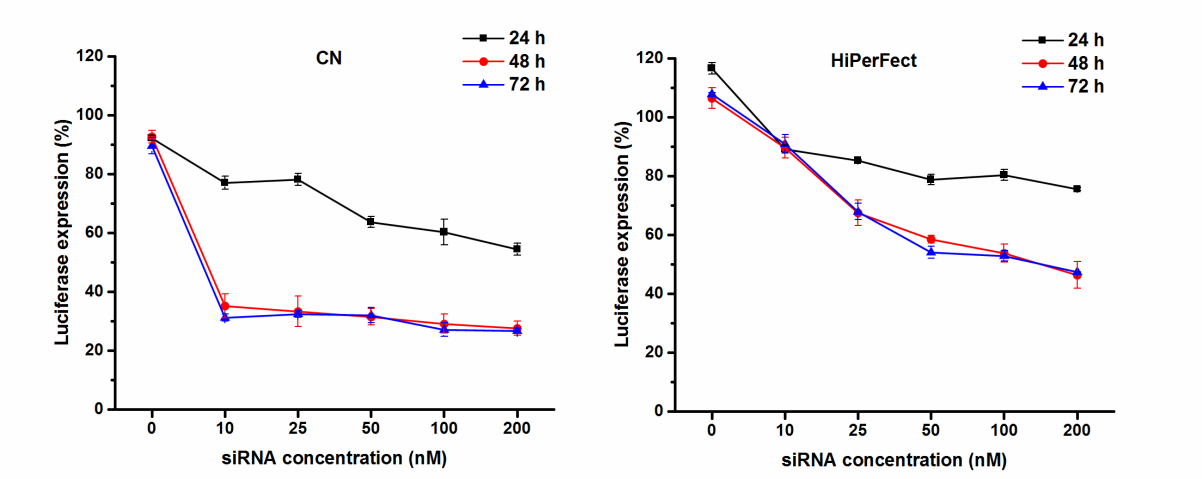


Figure 3 Percentage of luciferase expression in B16-F10 cells after being transfected by various siLUC concentrations using CN formulation and HiPerFect. Luminescence was measured after 24, 48, and 72 h of incubation. Data represents mean ± SD (n=3).

3.5. *In vivo* silencing study

3.5.1. Determination of the most tolerable dose of CN/siLUC

In a pilot study, empty particles at a concentration of 39 µg/mL was found to have no effect on the weight of the animal, whereas at concentrations higher than 156 µg/mL, there was a decrease in weight (Table 1). This concentration of empty particles was therefore selected and used to encapsulate various concentrations of siLUC. A siLUC concentration at 625 nM was found to be well tolerated by the mouse while concentrations higher than 1250 nM caused

weight loss (Table 2). Therefore, in the following *in vivo* experiments, CN/siLUC nioplexes were prepared using 39 µg/ml of CN encapsulating 625 nM of siLUC.

Table 1 Change of the mouse weight when given increasing doses of empty particles of CN.

Empty particles					
Days	1	2	3	4	5
Particle concentration (µg/ml)	39	156	315.5	-	-
siRNA concentration (nM)	0	0	0	-	-
Animal weight (g)	19.2	19.3	18.7	18.6	18.4

Table 2. Change of the mouse weight when given increasing doses of siLUC encapsulated in CN at concentration of 39 µg/ml.

Particles + siLUC					
Days	1	2	3	4	5
Particle concentration (µg/ml)	39	39	39	-	-
siRNA concentration (nM)	625	1250	2500	-	-
Animal weight (g)	19	19.3	18.8	18.6	18.8

3.5.2. *In vivo* luciferase gene silencing study

To investigate whether CN could release its encapsulated siRNA and inhibit gene expression in tumours, CN loaded with siLUC (39 µg/ml of CN encapsulating 625 nM of siLUC) were injected intratumorally in nude mice bearing subcutaneous B16-F10-Luc melanoma. Luciferase expression in the tumour was evaluated qualitatively and quantitatively in

anaesthetised animals. Mice injected with siLUC alone, CN alone, or left untreated were used as controls. Figure 4 shows the average bioluminescence measured for each group. A representative mouse whose emitted light was closest to the average for that group (3 mice per group) is shown (Figure 5).

Luciferase expression in mice injected with siLUC encapsulated in CN was significantly ($p < 0.05$) decreased, 4h after injection, by about 50% (Figure 4). The maximum luciferase expression knockdown (by 70%) was obtained 12 h after injection. This inhibition was reversible and the luciferase expression returned to normal after 24 h. In contrast, the bioluminescence signals in the mice treated with naked siLUC increased over the same time period, suggesting no inhibition of luciferase expression at any time point. Luciferase expression did not appear to be affected by the CN formulation, as the bioluminescence signal increased over time following treatment with empty CN. These results suggest that the inhibition of luciferase expression resulted from siLUC delivery by the CN into the cytoplasm of the cells and the subsequent RNAi mechanism. This demonstrates that siRNA can be released from the CN to the cancer cells and can inhibit target gene expression *in vivo*.

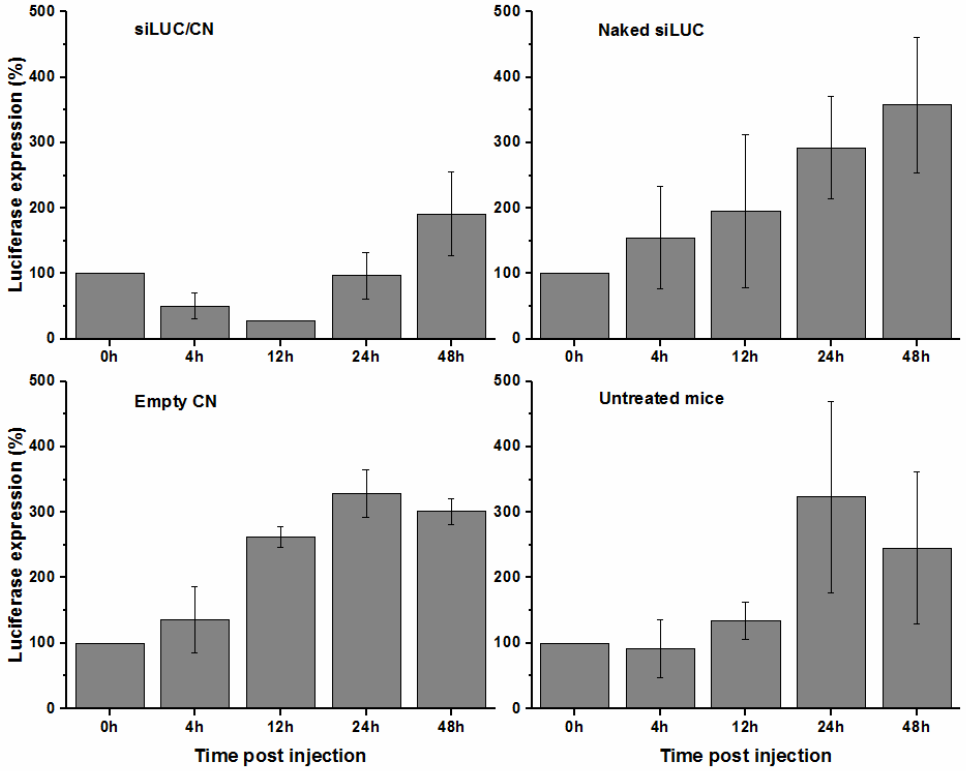


Figure 4 Bioluminescence of mice injected with siLUC/CN nioplexes, naked siLUC, empty CN, or left untreated. Readings were taken at t = 0h, 4h, 12h, 24h, and 48h post intra-tumour injection. Results represent the average of readings taken from three mice in each group ± SD.

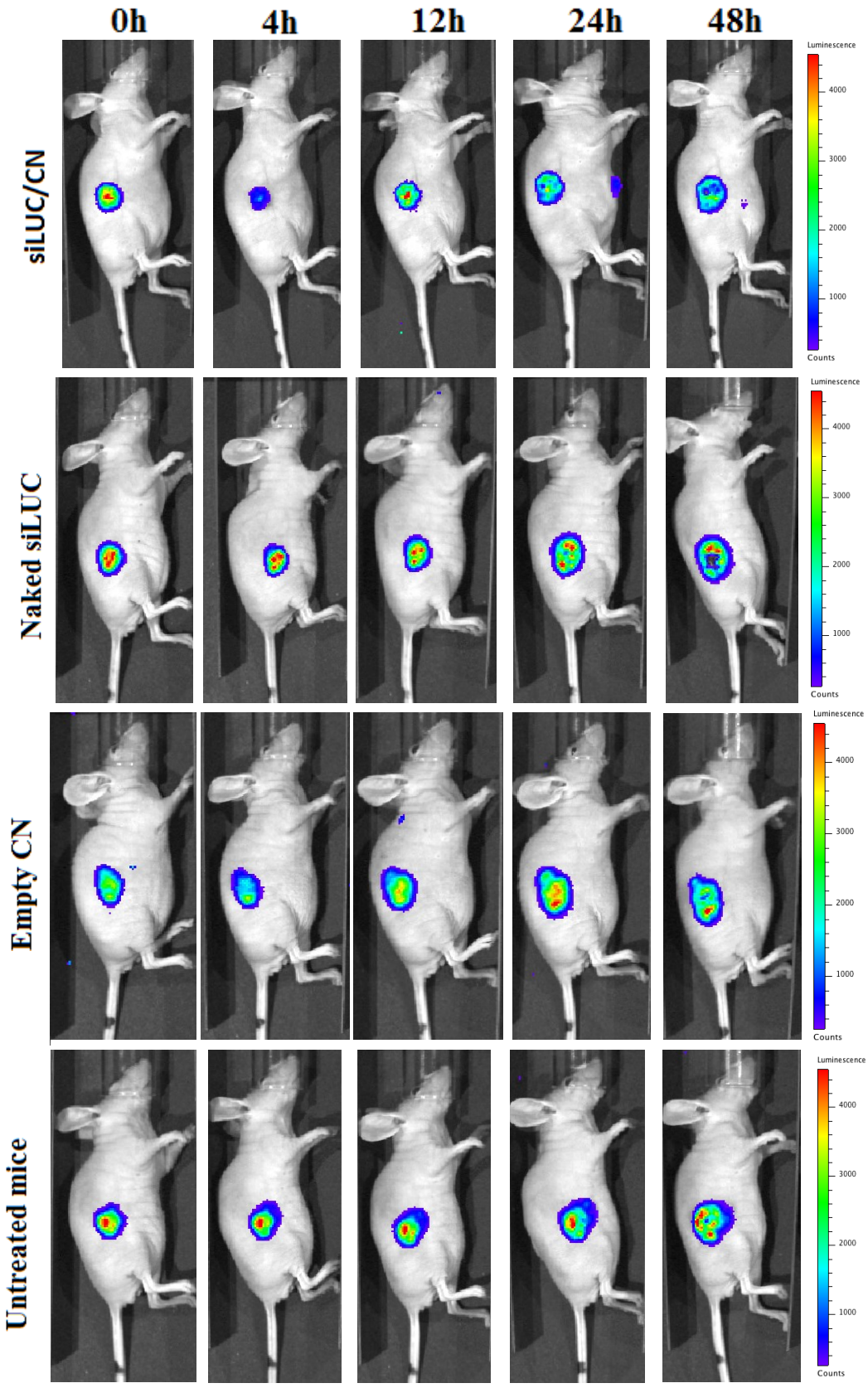


Figure 5 IVIS images of mice receiving siLUC/CN, naked siLUC, empty CN, and untreated mice at zero time and after 4, 12, 24, and 48 hours post intra-tumour injection. A representative mouse whose emitted light was closest to the average for that group (3 mice per group) is shown.

4. Discussion

Numerous barriers have to be overcome for the effective delivery of siRNA into the cytoplasm. The main obstacle to the development of effective therapeutics with siRNA is a suitable delivery system. First, the delivery system has to remain stable over time, to be of minimal toxicity, to be taken up by the cells, and to escape the endosome compartment with the subsequent delivery of the siRNA for its interaction with the RNAi machinery in the cytoplasm [22]. NISV possess attractive properties as a drug delivery system such as biodegradability, biocompatibility, stability, and ease of manufacture. Limited research is available about the use of NISV for siRNA delivery. Previous work for siRNA delivery combined non-ionic surfactant with phospholipids in a formulation called spanosomes [23]. In our previous study, effective various CN formulations were prepared and their efficacy in siRNA transfection and GFP inhibition in copGFP-A549 cells evaluated [19]. In order to confirm the gene silencing observed in copGFP-A549 cells, one formulation was taken forward in this study and the biological activity of the selected CN was tested on a different cell model, the B16-F10 cells, which could then be investigated *in vivo* in a rat model. CN cytotoxicity was evaluated on B16-F10 cells to make sure that any observed luciferase knock-down was a result of the siRNA used rather than as a result of any vesicle-related toxicity. Cytotoxicity results revealed that CN were not toxic at or below 40 μg/ml. Next, the transfection efficiency of the CN was examined by analysing the B16-F10 cellular uptake with FACS after treating these cells with CN encapsulating AllStars AF488-labelled Negative Control siRNA. CN were able to deliver siRNA as confirmed by the high cellular uptake percentages. This high cellular uptake can be attributed to the positive charge on the surface of CN, which enhanced the interaction with the negatively charged cellular membrane. The cellular uptake using CN was compared with HiPerFect transfection reagent. Cells treated with the empty CN or naked siRNA showed no

fluorescence signal, indicating that the above mentioned cellular uptake was a result of the siRNA delivery by the CN formulation.

After evaluating cellular uptake, luciferase gene silencing mediated by siLUC loaded in the CN formulation was evaluated. Luciferase is a bioluminescence producing enzyme widely used for monitoring siRNA delivering efficacy by monitoring bioluminescence changes after anti-luciferase siRNA treatment [24, 25]. After evaluating cellular uptake mediated by the CN formulation, the endosome release after uptake and the subsequent inhibition of the target luciferase enzyme need to be proven. To examine both the extent of gene silencing and the optimal incubation time, B16-F10 cells were transfected by various siLUC concentrations (10-200 nM) using the CN formulation and the changes in bioluminescence intensity was monitored at various time points. Naked siLUC showed negligible gene silencing effects when compared to siLUC with CN. Results indicate that the time of incubation and the siRNA concentrations had effects on the degree of gene knockdown. Increasing the incubation time meant higher exposure of the particles to the cells in order to increase the cellular uptake. Here, the efficacy rose by increasing the incubation time until 48 h, where further incubation did not result in greater knockdown for all siRNA concentrations. This suggests that the observed downregulation effect as a result of siLUC delivery by CN was stable for at least 72 h. Moreover, these experiments were carried out in the presence of serum, which indicates that the CN were able to protect the siLUC from degradation in the presence of serum proteins. Together, these results demonstrate that by using the CN formulation, siLUC is protected against degradation, internalised by the cells, and enabled escape of the endosomes to the cytoplasm where bioactivity was displayed.

CN were able to induce high luciferase suppression by about 68% after 48 h incubation using 10 nM siLUC. CN appeared to be an effective transfection reagent when compared with the commercially available HiPerFect, which exhibited a clear dose-dependent effect on

knockdown efficacy across all the siRNA concentrations. These results for luciferase suppression were consistent with the results reported previously on inhibition of GFP expression in A549 cells by siGFP delivered by various CN formulations [19]. This high luciferase suppression by siLUC delivered by this particular CN can be explained by the CN endosomal escape ability after uptake to the cytoplasm where the RNAi mechanism occurs [26]. CN were able to escape the endosome at a high rate, release the siLUC into the cytoplasm, initiating luciferase RNA interference, and inhibit the luciferase expression. This ability is due in part to the presence of T85 as a non-ionic surfactant in the CN formulation. T85 is believed to have fusogenic properties that enhances endosome escape by promoting instability in the endosome compartment and therefore releasing siRNA into the cytosol [27, 28]. The possibility of the downregulation of gene expression being due to cytotoxic effects of the formulations can be excluded as the non-toxic concentration of CN formulation was used. In addition, no luciferase downregulation was observed when cells were transfected with empty particles of CN alone (0 nM siLUC).

High siRNA concentrations can result in off-target effects which is one of the side effects associated with siRNA therapeutics [29]. Previous reports of siRNA delivery systems targeting luciferase, were able to achieve high luciferase suppression only at high siRNA concentrations, which increases the possibility of siRNA off-target effects. For example, Takemoto *et al.* were able to achieve 80% luciferase silencing with 100 nM siRNA using a siRNA-grafted polymer delivery system [30]. With chitosan nanoparticles, Ragelle *et al.* were able to achieve the maximum of 71% luciferase suppression using 200 nM of anti-luciferase siRNA [31]. Li *et al.* were able to induce around 70% luciferase gene silencing using targeted cationic liposomes using 250 nM anti-luciferase siRNA [32]. In our CN study, comparable high luciferase silencing effects were achieved with a much lower concentration of siRNA at 10 nM,

highlighting the efficiency of this CN formulation. Based on the *in vitro* results, the use of CN for *in vivo* RNA therapeutic applications was then evaluated.

After determining the maximum dose that could be used for the *in vivo* experiments, mice bearing luciferase-expressing tumours were intratumorally injected with siLUC encapsulated in CN. After 4h, the luciferase expression decreased by about 50.77 ± 20.35 %, indicating that the nioplexes were taken up by the cells where the siLUC released into the cytoplasm and incorporated in the RNA induced silencing complex (RISC) followed by luciferase expression knockdown. Twelve hours after the treatment, luciferase expression was significantly decreased by more than 70%. This luciferase suppression was reversible and, 24 h after injection, the luciferase expression was fully recovered. This is in agreement with what has been reported in the literature about the reversibility of the RNAi mechanism [33, 34]. These results provide an insight into the possible required dosing intervals to maintain the target gene suppression by siRNA in a therapeutic application. In mice injected with naked siLUC, there was no luciferase suppression and the luciferase expression increased with time as the tumour size naturally increased, suggesting that the tumour cells did not take up naked siRNA due to their hydrophilic properties [35]. Moreover, mice injected with particles alone showed an increase in the luciferase expression over time, suggesting that the empty particles had no effect on both luciferase expression and tumour growth.

In the work of Minakuchi *et al.*, significant luciferase suppression was achieved with an atelocollagen delivery system using a single injection via the same route of administration and the same tumour type that was used in this study [36]. However, despite the larger doses used by them, based on the tumour size (2.5 μg siRNA/50 μl/50 mm^3 tumor), the luciferase expression was also reversible after 2-3 days [36]. Filleur *et al.* investigated the use of naked siRNA to suppress luciferase expression by intra-tumoral injection and ended up with negative

results, which was similar to the results reported here in which naked siLUC did not induce any gene suppression [37].

These *in vivo* results demonstrate the efficacy of CN in delivering and releasing siRNA into tumour cells. Although further experiments are required such as *i.v.* treatment and biodistribution studies, CN-mediated siRNA delivery possess the potential for *in vivo* delivery of siRNA into tumour tissues. These results are proof of concept of the ability of CN to effectively deliver siRNA into mouse melanoma cells. These CN formulations can be explored with different types of cancer cells in the expectation of similar outcomes. Moreover, these intratumor injection results could be used as a model for localised treatment of siRNA therapeutics delivered by CN and these formulations can also be explored further for topical applications in treatment of different diseases.

5. Conclusions

We successfully formulated CN nanoparticles that could act as an effective siRNA delivery system to deliver siRNA and suppress luciferase expression both *in vitro* and *in vivo*. With these CN formulations, the suppression of over-expressed genes in different cancer types can be investigated through siRNA delivery. We were able to achieve more than 70% of luciferase knockdown through CN both *in vitro* and *in vivo*, which is a promising delivery system in the field of nucleic acids delivery. In conclusion, we have developed CN to efficiently and safely deliver siRNA to tumour cells and demonstrated specific inhibition of luciferase gene expression. To our knowledge, our results present the first evidence that combine *in vitro* and *in vivo* gene silencing data of siRNA delivery by NISV. This suggests that NISV might be used for therapeutic application of siRNA-based therapeutics in cancer treatment.

Acknowledgments

The authors would like to acknowledge the Jordanian Ministry of Higher Education and Scientific Research and Yarmouk University in Jordan for funding this work.

Conflict of Interest

The authors confirm that there is no conflict of interest with this manuscript.

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