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Co-delivery of siRNA and etoposide to cancer cells using an MDEA esterquat based drug delivery system.

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Abstract
Cancer has become the leading cause of death in many countries. Chemotherapy is a key component in the treatment of most cancers but has limited efficacy if the cancer develops resistance to the treatment over time and recur. RNA interference may be used to reduce the production of the proteins responsible for chemotherapeutic resistance. Small interfering RNAs (siRNA) may be used to induce RNA interference but the application of these to cancer cells is hampered by poor serum stability and delivery to their cytoplasmic site of activity.

This work introduces a novel nanoparticle delivery system for siRNA and hydrophobic anticancer drugs. The system is based on a cationic MDEA esterquat, which is widely and safely used in personal care products but has never been assessed for drug delivery applications. We show that MDEA forms spherical compact nanoparticles when combined with siRNA that delivers the siRNA to cancer cells where it induces gene silencing. By combining DOPE and MDEA in ratios of 2:1 and 3:1, even higher gene silencing levels (>90%) may be achieved. The system is capable of combinational therapy by co-delivering siRNA and the chemotherapeutic drug etoposide to cancer cells and these particles both induce gene silencing and chemotherapy induced cell death. We believe the present system may be used for intra-tumoral injection of chemotherapy in solid chemotherapy resistant tumors and for systemic delivery with further development.

Key words: MDEA, DOPE, siRNA, etoposide, cancer, RNA interference, nanoparticles

1. Introduction
Even though there is a remarkable progress in cancer prevention, detection and treatment, the disease continues to be among the leading causes of death worldwide. Around 90% of cancer-related deaths are due to metastases that happen if cancers are not treated early [1]. Lung cancer is the deadliest cancer form because of its high incidence and low survival rate. Around 87% of lung cancers are non-small cell lung carcinomas (NSCLC) and the 5 years survival rate for this cancer group is still below 20%, due to lack of effective treatments [2]. One of the main cancer treatments is chemotherapy where drugs, commonly small hydrophobic molecules, are used to kill cancer cells. Hydrophobic drug delivery is hindered by low solubility, bioavailability, and absorption in the body. Even so, chemotherapy is usually effective at fast shrinking tumors and in some cases curing the patient. In many cases, unfortunately, the cancer cells develop resistance to the chemotherapy drugs which causes the cancers to recur in an untreatable form.

There are different chemotherapy resistance mechanisms but very often resistance develops when the cancer cells start to over express genes that disable or exclude the chemotherapy drugs. A common method is for cancer cells to over express efflux proteins like the multidrug resistance (MDR) proteins that pump these chemotherapy drugs from the cells. RNA interference (RNAi) provides a method for reducing the
expression of such proteins. RNAi may be effected by small interfering RNAs (siRNAs). When the synthetic double stranded siRNA enters the cells, it binds the RNA induced silencing complex (RISC). The guide antisense strand stays in the complex, while the passenger strand leaves. The antisense strand then guides the RISC complex by base pairing to complementary mRNA which the RISC complex then cleaves [3] [4]. After transfection, gene silencing (knockdown) of mRNA due to RNA interference commonly lasts from 3 to 7 days in dividing cells, and 3-4 weeks in non-dividing cells [5]. The stability of RNAi is affected by the RNA phosphodiester’s structure which is more prone to nuclease degradation than that of DNA. Cancer cells have previously been re-sensitized to chemotherapeutic treatment by silencing MDR1 with siRNAs [6] [7]. The expression of proteins from other cancer related genes like oncogenes and migration genes EGFR [8], BCR/ABL1 [9], K-ras [10], EphA2 [11], TMEM98 [12] could also be silenced in a similar fashion using siRNAs. Combinational therapy with gene therapy and anticancer drugs has also been proven effective against cancer cells [13].

The biggest impediment to siRNA based treatment is serum stability and cellular internalization of siRNA to the cytoplasm – as it is easily degraded, negatively charged and large. siRNA is usually comprised of about 40 nucleotides, weighs 12-13 kDa, and is about 10 nm long. To deliver siRNA into the cytoplasm and protect it from nucleases the development of new carriers is required. Nanoparticles may be used as such siRNA carriers as well as carriers of hydrophobic drugs to improve their pharmacokinetic properties [14]. Many nano-particulate systems have been developed for siRNA delivery including liposomes, cationic lipid and polymer complexes and solid polymer particles, these systems have different advantages and limitations. Cationic lipid complexes are one of the most common non-viral carriers of both DNA and RNA into the cells and their use is attractive as lipid/siRNA complexes are simple and fast to produce and typically yield high transfection rates. They can be applied for co-delivery of anticancer drugs as well [15]. Quaternary amine lipids like DOTAP and lipofectamine use a clathrin dependent pathway for cellular entry [16]. This is possible if the diameter of the particles is below 500 nm [17].

This study aims at improving combinational therapy with siRNA and anticancer chemotherapy drugs. The anticancer drug used in this study is etoposide which is representative of many other hydrophobic anticancer drugs with poor bioavailability, low solubility and high resistance [18] [19]. Etoposide is a topoisomerase inhibitor that finds application in the treatment of cancers like refractory testicular tumors, small cell lung carcinoma and glioblastoma. Etoposide and siRNA is delivered to lung and oral cancer cells using the cationic quaternary amine lipid MDEA. MDEA is a biodegradable and inexpensive lipid currently used in a wide variety of personal care products such as hair conditioners with apparent safety in external applications [20] [21]. But MDEA has not, to our knowledge, been applied to drug delivery studies before nor has its cytotoxicity profile been established. We demonstrate that it forms nanoparticles with siRNA and that these can deliver functional siRNA and etoposide to cancer cells in the presence of serum.

2. Materials and methods

2.1 Materials

MDEA esterquat (commercially known as Varisoft® EQ 100 and Quaternium 98) which is chemically Bis-(Isostearoyl/Oleoyl Isopropyl) Dimonium Methosulfate) with CAS number: 1474044-71-7 was a gift from Evonik (DE). DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) solution in dichloromethane is purchased from Avanti Polar lipids (AL, USA). HEPES, Etoposide, Cholesterol, Nuclease free water, DAPI, Resazurin, PBS, 37% formaldehyde, DiI, Nile red and FBS (fetal bovine serum) were purchased from Sigma Aldrich. Penicillin-Streptomycin (10,000 U/mL), DMEM and RPMI 1600, were purchased from Thermofisher Gibco – BLR (UK). Cal27 and H1299 cells were a gift from Professor Jørgen Kjems at Aarhus University, Denmark. siRNA targeting GFP protein match and mismatch are purchased from Biosearch Technologies (DK):

siGFP sense RNA 5′GUUCACCUCUGAUGCCGUUCdTdT3′
siGFP-RNA (antisense) 5′GAACGGCAUCAAGGUGAAACdTdT3′
siMM-RNA (sense) 5′CCGUCUUAUUUGCGGUCCUdTdT3′
siMM (antisense) 5′AGGACCGCAAUAAGACGGdTdT3′.

2.2 Particle preparation
The particles were prepared with two different methods. The particular particle preparation step is indicated in the results. Firstly, particles were formed using a stirring method. A 1 mg/ml solution of MDEA esterquat was prepared in nuclease free water with 20 mM HEPES (pH 7.4). Particles were then prepared by combining this solution with 20 μM siRNA under vigorous stirring, at room temperature, to form particles at different amine to phosphate (N:P) ratios that roughly equate charge ratios at physiological pH. Secondly, particles were prepared by thin film hydration. Here MDEA was first dissolved in ethanol to 0.83 mg/ml, this solution was then dried under nitrogen before being hydrated and vortexed to form particles of 5 mg/ml and 2.5 mg/ml concentrations. Hydration was done with nuclease free water unless otherwise stated. A helper lipid DOPE was dissolved in chloroform to 25 mg/mL, this solution was then added to the MDEA solution prior to dehydation in required DOPE to MDEA molar ratio (D:M) by vortexing and the combined solution was then dried and rehydrated. Nile red and etoposide was added to the particles like DOPE but were dissolved in ethanol at 0.1 mg/ml and 2.5 mg/ml, respectively. Some samples, indicated in the text, were hydrated with 20 mM HEPES (pH 7.4). The D:M ratio is controlled in all samples and indicated as 3:1 (3), 2:1 (2), 1:1 (1) and 0:1 (0) for pure MDEA. Similar to many protocols for commercial siRNA transfection reagents that are based on cationic lipids (e.g. TransIT-TKO and lipofectamine), the siRNA is added to the pre-formed particles by vortexing. This is done 30 minutes before transfection at room temperature, and the particles are stored at room temperature until transfection. N:P ratio of 6 is used for all experiments, unless specifically marked, and the final siRNA concentration in wells is always 50 nM. In all experiments MDEA/DOPE particle formulations formed aggregates and did not get completely dissolved after hydration, the supernatant was used for transfections. Ethanol addition increased solubility of the samples.

1.3 Cell culture
The study used H1299 cells which are human non-small cell lung carcinoma (NSCLC) cells from a lymph node metastasis stably expressing eGFP protein and Cal27 cells which are from a human epithelial squamous cell carcinoma from tongue. The H1299 and Cal27 cells were maintained in RPMI and DMEM medium, respectively. All media were supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were incubated at 37 °C and in 5% CO₂ and split 2-3 times each week.

2.4 Transfection
Transfections were performed in 6, 24 and 96 well plates wherein cells were seeded at a cell density of 15000 - 25000 cells/cm² as indicated below. Cells were transfected 24 hours after seeding at a siRNA concentration of 50nM. The transfections were done in normal medium in the presence of serum. The cell medium was replaced 24 hours after transfection. At 24 or 48 hours (as indicated in the text) after transfection, cells were fixed for flow cytometry and microscopy or incubated with resazurin for evaluation of cell viability.

2.5 Flow cytometry
Transfections were performed in 24 well plates seeded with 50000 cells. Flow cytometry experiments were performed at Odense University Hospital on a BD™ LSR II Flow Cytometer. Before analysis, transfected cells were first washed with PBS, trypsinized, transferred to centrifugation tubes with media containing
serum and centrifuged at 1200 RPM for 5 minutes. The cell pellet was then dissolved in PBS, centrifuged and then fixed with 1% formaldehyde in PBS and stored at 4°C, wrapped in aluminium foil. On the flow cytometer, the cell population was selected with forward and side scatter. The geometric mean fluorescence was assessed in a histogram of the green fluorescence (FITC) channel to determine the degree of GFP fluorescence in the H1299 cells. For determining Nile red uptake into cells, the geometric mean fluorescence of the red fluorescence (Pe-Cy5.5) channel was used. At least 3 replicates and 2000 cell events per replicate were used to obtain the mean numbers for each group. Graphs display the average geometric mean values of fluorescence intensity with standard deviation on the error bars. Knockdown efficiency of GFP protein in H1299 cells is expressed as a percentage, normalized to the control samples or mismatch siRNA formulation which are set to 100%.

2.6 Toxicity assay
Resazurin based toxicity assays were performed on cells seeded in 96 well plates at 5000 cells per well together with a reference sample that had no cells. At the end of the culture and transfection period, the medium was replaced with 100 µl of media with 10% resazurin. The cells were incubated for 2-4 hours at 37 °C and 5% CO2 after which the absorbance was measured at 600 and 690 nm on an Epoch plate reader. Scattering absorbance at 690 nm is subtracted from absorbance at 600 nm to acquire the actual absorbance. Viable cells reduce the color and recorded lower absorbance values. Viability values were therefore found by subtracting the absorbance values of the wells with non-treated cells from that of the “no cells” reference sample. The viability values of samples were then expressed as a percentage of this reduction in absorbance, normalized to the non-treated control sample which is set to 100%. Graphs display average viability values with standard deviation on the error bars.

2.7 Confocal Microscopy
Confocal microscopy was performed with a Zeiss LSM510 Meta Confocal Microscope in the Danish Molecular Biomedical Imaging Center (DaMBIC), at University of Southern Denmark. Cells (250000) were seeded on 25 mm round coverslips # 1,5 in 6 well plates. Transfections were performed 24 hours after cell seeding and the cells were fixed 48 hours after transfection. Cells were fixed with paraformaldehyde (4% in PBS). DAPI staining was performed by permeabilizing the cells with 1% triton-100 for 30 minutes, then washing with PBS. DAPI stock solution of 0.3 µM is used for the cell nuclei staining and cells are incubated for 5 minutes before washing again with PBS. For imaging DAPI the two photon laser tuned to 760 nm was used for excitation.

2.8 Dynamic light scattering
Dynamic light scattering to determine the hydrodynamic diameter of the particles was performed at the Center for Biomembrane Physics (MEMPHYS) at University of Southern Denmark, on a DelsaMax Core and DelsaMax Pro, Beckman Coulter (USA).

2.9 Transmission Electron Microscopy (TEM)
Particles with D:M ratio of 1, 2 and 3 were prepared and 100 µL of the particle suspensions was then diluted with 900 µl HEPES. 50 µl of this solution was added 50 µl of a 2% osmium tetroxide solution and the mixture was left to react for 10 min. A drop of the particle-osmium tetroxide suspension was loaded on to a copper grid coated with a polyvinyl film, whereupon it was left to air-dry at room temperature. TEM images were acquired by use of JEM - 1400 Plus electron microscope, equipped with Quemesa TEM CCD camera.

2.10 Nile Red Release Study
The release profiles of the Nile red loaded particles formed by the thin film method were evaluated in vitro using the conventional dialysis bag method [22]. 0.5mL of Particle containing Nile red with D:M ratios 1, 2 and 3, were placed in a dialysis bag (cellulose membrane, molecular weight cut-off 14 kDa) containing 2.5 ml 2 mM HEPES buffer. The dialysis bag was placed in a beaker containing 25 mL of 2 mM HEPES buffer (release buffer). The beaker was stirred constantly at room temperature and protected from light for 7 days. Samples were taken at time 0, 2, 4, 8 and 24, 48, 86 and 124 hours. At these intervals, 400 µl of the release medium was removed and replaced with fresh HEPES buffer. The 400 µl samples were evaporated and resuspended in a mixture of 1:1 ethanol:HEPES solution and the Nile red concentration was determined by means of fluorescence on a Varioskan LUX Multimode Microplate Reader (Thermo Fischer). All experiments were performed in triplicate.

2.11 Nile Red Incorporation
Nile red incorporation was tested by filtration on different columns. Particles with D:M ratio of 1 were prepared with the thin film method, after rehydration the particle suspension was either passed through a Minisart® 0.2 µm filter (Sartorius), a PD10 desalting column (Sephadex G-25, GE healthcare) or an Amicon Ultra Centrifugal Filter (3 KDa MWC). The filtrate was hereafter evaporated and redissolved in 1:1 EtOH:HEPES. Samples of free Nile red in 1:1 EtOH:HEPES solutions with the same concentrations as in the particles were used as controls. The Nile red concentration before and after filtration was determined by means of fluorescence on a Varioskan LUX Multimode Microplate Reader (Thermo Fischer).

2.12 Statistical analysis
Analysis of the data was performed with Microsoft Excel (Version 2016) (WA, USA). All graphs show means and standard deviation, calculated in Excel. Difference between the means of two or more groups was tested using Excel’s Students T-Test and ANOVA with Tukey HSD Test performed with R (Version 3.4.0), respectively. Groups with p-value < 0.05 are considered significantly different. We have chosen not to remove any outliers in the datasets, this gives a high standard deviation for some of the samples.

3. Results
MDEA/siRNA particles were prepared using the stirring method at different N:P ratios with mismatched (siMM) or GFP targeted (siGFP) siRNA. The particles and unformulated siRNA were then used to transfect GFP expressing H1299 cells (Fig. 1). The GFP knockdown was evaluated using flow cytometry. At N:P = 5, median GFP fluorescence was significantly reduced compared to the control group (45%, p = 1.7 x 10⁻⁷). Silencing at N:P = 10 was similar to that of N:P = 5. Lower NP ratios and the controls made with pure siGFP (no MDEA, N:P = 0) did not result in any knockdown.
Figure 1. GFP silencing induced by MDEA/siRNA particles made using the stirring method. The figure shows the mean normalized GFP fluorescence intensity of H1299 cells 48 hours after transfection with MDEA/siRNA containing 50nM siRNA at different NP ratios. For siRNA, G is siGFP and M is siMM. N:P = 0 corresponds to unformulated siRNA.

To test if MDEA particles mediate toxicity we performed a resazurin assay on transfected H1299 cells (Fig. 2). The cells were added MDEA alone or MDEA/siRNA particles formed using the stirring method at different N:P ratios in a 96 well plate. The cell viability was reduced by up to 40% by MDEA with and without siRNA.
Figure 2. Cytotoxicity of unformulated MDEA and MDEA/siRNA particles made using the stirring method. The graph shows the average cell viability normalized to the non-treated control samples in H1299 cells 48 hours after treatment with 1 mg/ml unformulated MDEA or MDEA/siRNA particles at different N:P ratios. For MDEA alone the N:P ratios refer to the same amount of MDEA as if added siRNA. For siRNA, G is siGFP and M is siMM.

The diameter of MDEA/siRNA particles formed using the stirring method at different N:P ratios was measured using dynamic light scatter (Table 1). Particles form at N:P = 1 and particle size appears to be similar at all tested N:P ratios with diameters ranging from 119.1 nm to 161.1 nm. Additionally, the polydispersity index was maintained with an average of 0.238 for NP 1-10 for both siMM and siGFP indicating monodispersed samples. The particle size of below 170 nm is within the range previously reported for MDEA based uni-lamellar vesicles [20] [21].

Table 1. Dynamic light scattering, DelsaMax Core, 40 acquisitions per sample - Z average cumulative analysis, intensity distribution of 1mg/ml MDEA particles in nuclease free water with siRNA. NP is N:P ratios.

<table>
<thead>
<tr>
<th>MDEA/siRNA particles</th>
<th>Diameter (nm)</th>
<th>PD Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 0, siMM</td>
<td>378.8</td>
<td>0.571</td>
</tr>
<tr>
<td>NP 1, siMM</td>
<td>161.1</td>
<td>0.238</td>
</tr>
<tr>
<td>NP 2, siMM</td>
<td>119.1</td>
<td>0.225</td>
</tr>
<tr>
<td>NP 5, siMM</td>
<td>122.2</td>
<td>0.238</td>
</tr>
<tr>
<td>NP 10, siMM</td>
<td>144.8</td>
<td>0.238</td>
</tr>
<tr>
<td>NP 0, siGFP</td>
<td>2247</td>
<td>0.571</td>
</tr>
<tr>
<td>NP 1, siGFP</td>
<td>127.9</td>
<td>0.57</td>
</tr>
<tr>
<td>NP 2, siGFP</td>
<td>144.1</td>
<td>0.194</td>
</tr>
<tr>
<td>NP 5, siGFP</td>
<td>135.3</td>
<td>0.207</td>
</tr>
<tr>
<td>NP 10, siGFP</td>
<td>142.6</td>
<td>0.316</td>
</tr>
</tbody>
</table>

The achieved degree of silencing of 45% is low compared to what we have previously achieved in this cell line using commercial as well as experimental transfection reagents that regularly achieve degrees of silencing of 80% at 50nM siRNA [23]. We hypothesized that this was caused by cationic MDEA binding the anionic siRNA too strongly inhibiting its release to RISC or alternatively, by poor membrane penetration. To optimize transfection, neutral helper lipids were therefore added to the particles. We further hypothesized that a thin film hydration method might produce better particles. Cholesterol addition to MDEA was tried but did not increase knockdown efficiency (data not shown). The addition of DOPE and using a thin film hydration method for particle formation, however, led to improved knockdown efficiency. With the same method we tested the effect of including small molecules such as the fluorophore Nile red and the chemotherapeutic etoposide in the particles; these were added to the DOPE/MDEA mix before dehydration. DOPE was added at different molar ratios to MDEA and particles were formed by rehydrating the thin film to 5 mg/ml and 2.5 mg/ml with water or HEPES. After particle formation, siRNA was added to
give N:P = 3, 6 or 9 particles and these were used to transfect H1299 cells, the data was collected in 3 separate successive experiments (Fig. 3a-c). All reported degrees of silencing with siGFP are compared to the siMM transfected control cells for the particular sample as Nile red and etoposide inclusion increased the fluorescence of the cells in the GFP channel. Concentrations of Nile red and etoposide refer to final well concentrations.

The DOPE to MDEA ratios (D:M) were tested for their degree of silencing in the absence and presence of Nile red. When rehydrating to 5 mg/mL and using a D:M ratio of 1 and a N:P ratio of 6, a reduction in GFP fluorescence of 85% ($p = 5 \times 10^{-7}$) was obtained (Fig. 3a). Despite successful silencing, the method yielded aggregates upon rehydration and therefore the rehydration concentration was reduced to 2.5 mg/ml and tested with HEPES and water, reducing the rehydration concentration reduced aggregation, using HEPES made no difference. Rehydrating in HEPES at a D:M ratio of 3 and a N:P ratio of 6 resulted in 93% silencing (Fig. 3b). Rehydrating in water or adding 3.26 μM Nile red did not lower the transfection efficiency significantly and resulted in degrees of silencing of 86% and 83%, respectively.

Next, the Nile red concentration was fixed to the lipid content while the D:M and N:P ratios were varied (Fig. 3c). At a D:M ratio of 2 with 9.5 μM Nile red, 91% silencing was obtained ($p < 1 \times 10^{-8}$). Lower D:M ratios, however, resulted in less silencing. Particles formed with D:M ratios of 3 and N:P = 6 and 12.7 μM Nile red resulted in 83% gene silencing ($p = 2 \times 10^{-4}$). A lower D:M ratio of 1 at N:P = 3 and N:P = 9 was also tried. At N:P = 9, 91% gene silencing was achieved ($p = 4 \times 10^{-4}$) whereas at N:P = 3, no silencing was observed. A sample with N:P = 3 was tested at an D:M ratio of 3, this resulted in 73% silencing ($p = 2 \times 10^{-4}$). Most importantly, 77% silencing ($p = 1 \times 10^{-5}$) was achieved with a sample made at an D:M = 3, an N:P = 6 and containing 13.72 μM etoposide.

Combined, this indicates that optimal parameters for gene silencing is rehydrating to 2.5 mg/ml, using a D:M ratio of 2 or 3, and a N:P ratio of 6 or more. Possibly, the use of a higher N:P ratio may allow a lower D:M ratio. Finally, we found that the inclusion of small molecules like Nile red and etoposide in the particles only yields a small drop in gene silencing.
Figure 3. Mean GFP fluorescence intensity of H1299 cells normalized to non-treated controls at 48h after transfection with MDEA:DOPE particles prepared by thin film hydration to 5mg/ml and 2.5 mg/ml. In (a) and (b), the N:P ratio is 6, whereas the D:M ratio is 1 in (a) and 3 in (b).

We included Nile red as a hydrophobic small molecule which could represent chemotherapeutic drugs as it has similar logP. Additionally, Nile red can act as a diagnostic tool that enables fluorescent tracking of cellular uptake of such small molecules. Cellular Nile red fluorescence induced in H1299 cells after 48 hours of transfection with different MDEA/DOPE particles, rehydrated to 2.5 µg/µl, was measured using flow cytometry (Fig. 4). These experiments were run in parallel to those presented previously (Fig. 3c). Samples containing Nile red induced a cellular fluorescence that was between 23 and 443 times higher than non-transfected control cells. Particles not containing Nile red also increased fluorescence but much less so (between 1.9 and 8.3 times more fluorescence). Interestingly, the particles that yielded the highest gene silencing (those made with N:P = 6 & D:M = 2 and N:P = 6 & D:M = 3) seemed to yield less Nile red fluorescence despite being added more Nile red than those made at lower D:M ratios. It should be noted, however, that cellular Nile red fluorescence is only indicative of delivery as the fluorescence of Nile red depends on its molecular surroundings [24]. More stable particles that encapsulate Nile red in less
fluorescent surroundings may, for example, yield less fluorescence even if they deliver more Nile red to a cell compared to a less stable particle. We do not believe that GFP contributes to measured Nile red fluorescence as the sample with N:P = 9 and D:M = 1 displays 91% GFP silencing when made with siGFP compared with siMM whereas siGFP and siMM particles display similar Nile red fluorescence.

Figure 4. Mean Nile red fluorescence intensity of H1299 cells 48 hours after transfection with MDEA/DOPE particles containing siRNA and Nile red that were prepared using the thin film method. Samples were normalized to the non-treated control. Note the logarithmic scale.

To further explore Nile red delivery, transfected H1299 cells were observed using a confocal fluorescence microscope 48h after transfection (Fig. 5). Nile red, colored red, was observed to accumulate in the cytoplasm of the cells confirming its intracellular delivery. GFP fluorescence of H1299 cells, colored green, was almost non-detectable for siGFP particle formulations, which confirms the flow cytometry observation of GFP silencing (Fig. 3). Hence co-delivery of Nile red and GFP targeted siRNA has taken place.
Figure 5. Fluorescence microscopy images of H1299 cells 48h after transfection with MDEA/DOPE particles containing siMM or siGFP siRNA and 3.26µM Nile red. The particles were made using the thin film method and at N:P and D:M ratios of 6 and 3, respectively.

The uptake of Nile red delivered by MDEA/DOPE particles was further studied using a Cal27 cell line. Particles made with D:M ratios of 1 and 2 were transfected into the cells. Flow cytometry and confocal fluorescence microscopy was used to evaluate Nile red uptake into cells after 48 hours (Fig. 6). All samples increased Cal27 Nile red fluorescence compared to the control (p-values < 0.5), the increase was highest for the free Nile red controls that increased fluorescence 10 times, the particles increased the fluorescence 5.4 and 4.3 times for D:M ratios of 1 and 2, respectively. Uptake of free Nile red and particles with D:M ratios of 1 in Cal27 cells was further visualized using confocal microscopy. The images again suggest the delivery of Nile red to the cytoplasm.
Figure 6. Nile red fluorescence intensity of a Cal27 cell line measured 48 hours after transfection with pure Nile red or MDEA/DOPE particles made with siRNA and Nile red at two different D:M ratios. The particles were made using the thin film method and at a N:P ratio of 6. Shown are average fluorescence intensity normalized to the non-treated control as measured by flow cytometry (left) and fluorescence microscopy images of red Nile red fluorescence for pure Nile red (top right) and particles with Nile red (lower right). Note the logarithmic scale.

To further analyze the encapsulation of Nile red in the particles, particles with Nile red (thin film method, D:M ratio = 1, N:P ratio = 6) and free Nile red at the same concentration was passed through various filters and fluorescence was measured before and after (Table 2). Free Nile red was unable to pass through a SEC column but passed freely through both a 200nm filtration column and an ultrafiltration column (cutoff: 3kDa) with no loss of fluorescence. When particles were formulated with Nile red, their fluorescence before filtration was lower than for free Nile red but fluorescence was still easily detectable, the lower level is likely to be because Nile red exhibits different levels of fluorescence when placed in different environments [24]. When particles were passed through any of the filters, the filtrate did not contain Nile red fluorescence above the level of detection. This concentration level was determined by a standard curve for Nile red to be a detection limit of 100ng/mL Nile red (supplementary figure 1). With the ultrafiltration columns, we were able to resuspend the retentate after filtration, and this retentate showed fluorescence. These experiments strongly indicate that all Nile red is incorporated into the particles and that these samples contain no free Nile red. That free Nile red is more fluorescent than formulated Nile red may explain the observation in figure 6 that free Nile red induces greater cellular fluorescence than formulated Nile red.

Table 2. Filtration of free Nile red and particles incorporating Nile red (D:M ratio 1, N:P 6), fluorescence was used to quantify Nile red before and after filtration. LOD is level of detection (~0.1 µg/ml).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>Before [µg/ml]</th>
<th>Standard Deviation</th>
<th>After [µg/ml]</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Nile red</td>
<td>Desalting column (SEC)</td>
<td>31.21</td>
<td>9.14</td>
<td>Below LOD</td>
<td>0.09</td>
</tr>
<tr>
<td>Particles</td>
<td>Desalting column (SEC)</td>
<td>0.67</td>
<td>0.01</td>
<td>Below LOD</td>
<td>0.36</td>
</tr>
<tr>
<td>Free Nile red</td>
<td>Nanofiltration (&lt; 200nm)</td>
<td>31.21</td>
<td>9.13</td>
<td>31.08</td>
<td>5.35</td>
</tr>
<tr>
<td>Particles</td>
<td>Nanofiltration (&lt; 200nm)</td>
<td>0.71</td>
<td>0.03</td>
<td>Below LOD</td>
<td>0.15</td>
</tr>
</tbody>
</table>
To investigate the degree to which the Nile red was released from the particles in aqueous environments we performed a dialysis release assay over 7 days. No Nile red fluorescence was observed in the dialysis medium (detection limit of 100 ng/mL, supplementary figure 1) at any time point despite the membranes molecular weight cut-off of 14 kDa (the molecular weight of Nile red is 318 Da). This indicates that the Nile red is strongly bound to the particles.

To investigate whether the MDEA/DOPE particles with siRNA and with or without etoposide are toxic to the cells, resazurin based viability assays were performed on H1299 and Cal27 cells (Fig. 7) treated with these particles. The viability of H1299 cells 24h after transfection with MDEA/Dope siRNA particles without etoposide at D:M ratios of 1 and 2 was reduced by 28% and 19%, respectively, when compared to the control (p = 2 x 10^{-5} and p = 9 x 10^{-3}, respectively). Particles with etoposide also reduced viability. Particles formed at D:M ratios of 1 with 10.3 µM and 15.5 µM etoposide led to a 20% and 47% reduction in viability, respectively (p = 3 x 10^{-3} and p < 1 x 10^{-8}, respectively). Particles formed at a D:M ratio of 2 with 10.3 µM and 15.5 µM etoposide reduced viability by 34% and 56%, respectively (p = 9 x 10^{-7} and p < 1 x 10^{-8}, respectively). In Cal27 cells, MDEA/DOPE siRNA particles formed at D:M ratios of 1 and 2 were not toxic at 24h when compared to the non-treated control group (p = 0.54 and p = 0.07 respectively). Cell viability decreases when etoposide was introduced into the particles. Particles formed at a D:M ratio of 1 and with 10.3 µM or 15.5 µM etoposide reduced viability by 43% and 63 %, respectively. Particles formed with D:M ratios of 2 and with 10.3 µM or 15.5 µM etoposide reduced viability by 68% and 72%, respectively. Overall, of the tested conditions D:M ratios of 2 and 15.5 µM etoposide seemed to yield the highest degree of toxicity.

A repeat of the experiment in Cal27 cells with siRNA particles made at D:M ratios of 1, 2 and 3 and different levels of etoposide yielded a similar result (supplementary figure 2), in this experiment, none of the particle samples without etoposide were toxic compared to the control samples, but all particles with etoposide showed statistically significant toxicity except for those made with a D:M ratio of 1 and a 3.4 µM and 6.8 µM etoposide (the lowest etoposide levels tested).
Figure 7. Normalized viability [%] of H1299 and Cal27 cells 24h after treatment with MDEA/DOPE siRNA particles at different D:M ratios and with or without etoposide at different concentrations. The particles were made using the thin film method and at a N:P ratio of 6.

To compare the particles formed using the thin film method to those formed using the stirring method, the intensity averaged diameter of particles made at different D:M ratios with/without Nile red and etoposide was measured using DLS (Table 3). The sizes of most particles were below 500nm but they were larger than those formed using MDEA and the stirring method only.

Table 3. Dynamic light scattering of particles made using the thin film method at different D:M ratios with and without Nile red and etoposide, the N:P ratio is 6. Table shows intensity averaged diameters and standard deviation of three measurements.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Diameter (nm)</th>
<th>Standard Deviation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDEA Only</td>
<td>284</td>
<td>103</td>
</tr>
</tbody>
</table>
The size and shape of particles formed using the thin film methods was also examined using TEM (Figure 8). Particles formed at a D:M ratios of 1 and 2 were round, compact and homogenous in shape and with polydisperse sizes varying between 50nm and 400nm confirming the DLS result. At a D:M ratio of 3, the particles had the same shape but some were larger with sizes above 1000nm, however, the samples still contained smaller particles as well. A control sample containing HEPES only showed no particles.

<table>
<thead>
<tr>
<th>D:M Ratio</th>
<th>SiRNA</th>
<th>Chemotherapeutics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>464</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>265</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>302</td>
<td>81</td>
</tr>
<tr>
<td>1, +Nile Red</td>
<td>435</td>
<td>68</td>
</tr>
<tr>
<td>2, +Nile Red</td>
<td>219</td>
<td>44</td>
</tr>
<tr>
<td>3, +Nile Red</td>
<td>296</td>
<td>88</td>
</tr>
<tr>
<td>1, +Etoposide</td>
<td>516</td>
<td>62</td>
</tr>
<tr>
<td>2, +Etoposide</td>
<td>354</td>
<td>39</td>
</tr>
<tr>
<td>3, +Etoposide</td>
<td>408</td>
<td>163</td>
</tr>
</tbody>
</table>

4. Discussion
Resistance to anticancer drugs is a major clinical problem. Non-small cell lung cancer, for example, is often insensitive to chemotherapeutics and the main treatment is based on surgical resection which is often not possible if the cancer is located near critical nerves or blood vessels [25]. This could potentially be addressed with siRNA targeting cancer resistance genes. We show here that MDEA/DOPE nanoparticles that contain both siRNA and etoposide can induce effective gene silencing and chemotherapy induced cell death in the presence of serum.
To our knowledge, the MDEA esterquat has not been studied for drug delivery applications before. Currently, MDEA esterquat is used in household and personal care applications such as in fabric softener, hair conditioner and shampoo [26]. It has been developed for this purpose to provide a vegetable sourced, safe, biodegradable and environmentally friendly replacement for older cationic quaternary ammonium lipids that were non-biodegradable. Biodegradability was achieved by incorporating hydrolysable ester linkages between the quaternary ammonium and the fatty acid groups, this also makes the molecule more absorbent [26]. An added benefit is that methyl ester ammoniums, like MDEA, reportedly possess antibacterial properties [27]. It was previously reported that liposomal formulations of quaternary ammoniums decrease toxicity, compared to the same concentration of quaternary ammonium [28]. We found that free MDEA resulted in a 30-40% reduction in viability (figure 2) but that several of the MDEA/siRNA particles resulted in no or less toxicity (figure 7 and supplementary figure 2), confirming this finding.

Many quaternary ammonium lipids find application as transfection reagents and drug delivery vectors including DODAB, DOTAP, DOSPA and DOTMA [29], [30]. The thin film hydration technique for DOTAP and DODA [29] results in bilayer vesicles in aqueous solutions. We therefore hypothesized that thin film hydration results in bilayer vesicles for MDEA as well. The DLS and TEM revealed that compact, spherical lipid nanoparticles were formed, likely through ionic interactions between anionic siRNA and cationic MDEA. Some DOPE is likely incorporated into the particles through hydrophobic interaction with MDEA, but adding excess DOPE (at a D:M ratio of 3) seemed to yield a particle fraction with a large size (~1000nm) (figure 8). These could be the MDEA/siRNA particles that have grown bigger or it could be particles made from pure DOPE, we speculate that it is the latter as the sample still contains small particles which we believe are the MDEA/siRNA particles with a finite amount of DOPE. Cationic lipids facilitate drug delivery by binding anionic or hydrophobic cargoes like siRNA and chemotherapy by electrostatic or hydrophobic interactions, respectively. Their cationic and hydrophobic nature also facilitates binding to the anionic phospholipid cell membrane where after they and their cargo can be internalized by endocytosis. Although the particles are polydisperse, the particles are all less than 400nm in diameter at D:M ratios of 1 and 2, and even the sample with a D:M of 3 also contained many particles in this size range. At this size range, the particles are small enough to be endocytosed by a combination of clathrin and caveolae mediated endocytosis [31].

Most cationic lipids are coupled with helper lipids for increasing transfection efficiency. Cholesterol and DOPE are, for example, used for increasing stability and safety, as well as siRNA complexation [32]. DOPE that was used in this study is one of the most commonly used helper lipids. DOPE has fusogenic properties [33] [34] and has been found to promote membrane penetration, improve particle stability and lower toxicity [35] [36] [37]. Particles made from DOPE and DOTAP in ratios of 1:1 and 3:1 were found to facilitate endosomal fusion and cargo release [38]. Our study employs the same DOPE to MDEA ratios and we also found that DOPE addition increased transfection efficiency. It has previously been proposed that DOPE pushes the structure of lipid complexes towards a more effective inverted hexagonal phase and this could be responsible for the higher transfection rate. An alternative or complementary explanation may be that the neutral helper lipid DOPE promotes a weaker binding of the siRNA in the particle allowing a faster release of the siRNA to the RISC within the cytoplasm.

The degree of gene silencing in the present study reached up to 93% at a DOPE to MDEA ratio of 3. This confirms a previous study that reported that a DOPE to DOTAP ratio of 3:1 results in high DNA transfection in A549 cells [38]. This study also observed a high transfection efficiency at a DOPE to DOTAP ratios of 1 whereas we observed that a DOPE to MDEA ratio of 1 disrupted silencing efficiency unless the N to P ratio was increased to 9, in which case we also observed knockdown (73%). We found that the particles could be added Nile red and etoposide with a small reduction in gene silencing efficiency.
This confirms previous studies. In one, DOTAP/DOPE/Cholesterol lipoplexes prepared by thin film hydration at a molar ratio of 5:4:1, respectively, demonstrated no loss in DNA transfection efficiency upon drug loading [39]. In another with DOTAP/DOPE particles where etoposide, docetaxel and p53-DNA was co-delivered to H1299 cells, drug inclusion led to lower transfection efficiency [40]. This study suggested that pretreatment with DNA, followed by delivery of anticancer drugs was more effective than co-delivery of anticancer drug and gene therapy in one particle system. Care should be taken to extrapolate too much from these studies, however, as they employed DNA whereas our study focuses on siRNA. Our study (figures 3-6) shows that active siRNA and Nile red can be co-delivered to the same cells that then undergo gene silencing.

Our filtration (table 2) and dialysis release assays demonstrate that Nile red is incorporated into the particles and likely released very slowly in aqueous environments. Further evidence for the incorporation of Nile red into the particles is given by figure 6 where a difference is observed in fluorescence between Nile red with or without particles, if Nile red was not incorporated the fluorescence levels should have been identical. siRNA is likely also incorporated as siRNA alone gave no silencing (figure 1) in contrast to formulated siRNA. That both Nile red and siRNA is incorporated into the particles and delivered to cells with very little free Nile red is important for the final application as any free chemotherapeutic drug would not be able to kill resistant cancer cells but would still be able to damage healthy cells, therefore as much as possible of the drug should be delivery together with siRNA in order for it to be therapeutically active.

It is also impossible to state whether the particles deliver their small molecular weight drug cargo after the particles have been internalized or whether they are directly transferred from external particles to the cell membrane and cytosol or both. Direct transfer of drugs from particles to cells without internalization of the particles has been observed in other studies [41], these studies showed that nanoparticles incorporating Nile red increased Nile red uptake as compared to pure Nile red. In our study, we found that pure Nile red gave higher fluorescence than when incorporated into particles, both when in solution (table 2) and when applied to cells as measured by flow cytometry (figure 6), however, too many things like particle size and composition differ between [41] and our study to make conclusions on why. Further studies are needed to investigate how drugs are incorporated into and released from MDEA based particles. Future studies should also more systematically vary N:P and D:M ratios, rehydration concentrations and other formulation parameters as well as compare MDEA with other cationic lipids like DOTAP to further optimize the delivery system.

The gene silencing and cell death mediating MDEA/DOPE nanoparticles described in this study could potentially be used to treat non-resectable solid tumors, for example, by intra-tumoral injections. The siRNA could be directed towards cancer oncogenes and/or drug resistance genes like MDR1. The penetration of our rather large nanoparticles through a dense solid tumor would need to be studied however.

5. Conclusion
The cationic lipid MDEA forms nanoparticles when combined with siRNA and these induce sequence specific gene silencing in cancer cells. The addition of DOPE increases transfection efficiency to a maximum of 93% which was achieved at a DOPE to MDEA to ratio of 3 and an N:P ratio of 6. Experiments with the model drug Nile red shows that small molecular weight drugs can be stably encapsulated in the particles and be co-delivered along siRNA to the cytoplasm of cells. Sufficient etoposide could also be included in the nanoparticles to give effective well concentrations of over 10 μM while still achieving 83% and 77% gene silencing, respectively. Etoposide inclusion induced cell death in the lung cancer and oral cancer cell lines H1299 and Cal27, respectively. In conclusion, a novel drug delivery system for co-delivery of siRNA and chemotherapeutics was developed; the system may hold value in the treatment of non-resectable chemotherapy resistant tumors.
6. Acknowledgements
The authors would like to thank Jørgen Kjems and Anne Chauchereau who provided the H1299 and Cal27 cells and the technical staff from the University of Southern Denmark. We would also like to thank Aleksandra Rojek for help with transmission electron microscopy, The Danish Molecular Biomedical Imaging Center (DaMBIC) for use of Confocal Microscopy and the Carlsberg foundation for funding (Carlsberg start up grant to EAC).

7. References


Graphical Abstract