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Research Paper

Development of a Novel Method for Formulating Stable siRNA-Loaded Lipid Particles for *In vivo* Use

Sherry Y. Wu,[1,2] Lisa N. Putral,[1] Mingtao Liang,[2] Hsin-I. Chang,[2] Nigel M. Davies,[2] and Nigel A. J. McMillan[1,3]

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Purpose. A simple yet novel method was developed to prepare stable PEGylated siRNA-loaded lipid particles which are suitable for *in vivo* use.
Methods. PEGylated siRNA-loaded lipid particles were formulated by hydration of a freeze-dried matrix. The effect of various formulation parameters on the size and homogeneity of resulting particles was studied. Particles prepared using this method were compared to those prepared using an established post-insertion procedure for the entrapment efficiency, stability, *in vitro* biological activity as well as *in vivo* biodistribution.
Results. Using this hydration method, a particle size of less than 200 nm can be obtained with high siRNA entrapment efficiency (>90%) and high gene-silencing efficiency. Following intravenous administration into mice, these particles achieved a similar degree of accumulation in subcutaneous tumours but displayed less liver uptake compared to the post-insertion formulations. Importantly, in contrast to post-insertion preparations, particles made by hydration method retained 100% of their gene-silencing efficiency after storage at room temperature for 1 month.
Conclusions. This paper describes a simple method of formulating PEGylated siRNA-loaded lipid particles. Given the ease of preparation, long term stability and favourable characteristics for *in vivo* delivery, our work represents an advance in lipid formulation of siRNA for *in vivo* use.

KEY WORDS: cancer; liposomes; PEGylation; siRNA; systemic gene delivery.

INTRODUCTION

The discovery of RNA interference (RNAi) has opened up an entirely new field of biology and medicine (1). The ability of RNAi to specifically silence target genes has yielded not only a new tool for basic research but also raised the concept of developing medicines based on RNAi. RNAi works through the targeting of mRNA via sequence-specific matches and results in degradation of target mRNA or its translational inhibition, leading to the loss of protein expression. This is pharmacologically achieved via the introduction of small 19–21 bp dsRNA molecules called small interfering RNA (siRNA). Since its discovery, siRNA has been widely investigated *in vitro* for its utility in treating various diseases, such as cancer (2), neurodegenerative (3) and infectious diseases (4).

A major barrier to its further development has been the inability to effectively deliver siRNA *in vivo* due to the size, charge, and the generally instability of these molecules. Indeed, various delivery systems have been developed to enhance the uptake of siRNA into the target tissues after systemic administration (5–7). These include the use of polymers (8), lipids (2) or nanoparticles (9). Most of these vectors are cationic to ensure efficient interaction of particles with negatively-charged siRNA nucleotides and to facilitate their cell entry. However, the ability of these cationic particles to deliver siRNA systemically is often poor due to rapid uptake by reticuloendothelial (RES) organs (10–12), thereby hindering the delivery of these particles to the site of interest. To overcome this problem, polyethylene glycol (PEG) has been used extensively in the formulations as it decreases RES uptake of these particles (reviewed in (13)). This PEGylation also permits the accumulation of the particles in sites where defective

[1] Diamantina Institute for Cancer, Immunology and Metabolic Medicine, University of Queensland, Princess Alexandra Hospital, Ipswich Rd, Buranda, QLD 4102, Australia.
[2] School of Pharmacy, University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia.
[3] To whom correspondence should be addressed. (e-mail: n.mcmillan@uq.edu.au)

ABBREVIATIONS: bp, Base pair; CMV, Cytomegalovirus; DEPC, Diethylpyrocarbonate; dH_2O, Distilled water; DiR, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide; DMEM, Dulbecco's modified Eagle medium; DNA, Deoxyribonucleic acid; DODAP, 1,2-Dioleoyloxy-3-(dimethylamino)propane; DOTAP, Dioleoyl trimethylammonium propane; dsDNA, Double-stranded DNA; dsRNA, Double-stranded RNA; EDTA, Ethylenediaminetetraacetic acid; FACS, Fluorescence activated cell sorting; FBS, Fetal bovine serum; GFP, Green fluorescence protein; HFDM, Hydration of freeze-dried matrix; i.v., Intravenous; LP, Lipid particle; mRNA, Messenger RNA; N/P, Nitrogen/phosphate; ODN, Oligodexoynucleotides; PBS, Phosphate buffered saline; PEG, Polyethylene glycol; PI, Post-insertion; RES, Reticuloendothelial system; RNA, Ribonucleic acid; RNAi, RNA interference; RT, Room temperature; SD, Standard deviation; siRNA, Small interfering RNA; UV, Ultraviolet.

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vasculature is present, such as tumours, owing to the "Enhanced Permeability and Retention" phenomenon (14,15).

For lipid-based delivery vectors, various methods for formulating polynucleotide-loaded PEGylated particles have been reported to date, including post-insertion (16), reverse-phase evaporation (17), detergent dialysis (18) and ethanol dialysis (19–21). However, most of these methods, though effective, require relatively complicated and lengthy formulation procedures with the resulting particles suspended in an aqueous state. This has lead to long term storage issues including aggregation and/or fusion of the particles (22,23), hydrolysis of the lipids (22), and instability of siRNA nucleotides in an aqueous environment. Moreover, these formulations are also prone to be affected by stresses occurred during transport, such as agitation or temperature fluctuation (23,24). These problems, along with the significantly increased effort required for large-scale production of these particles using the existing formulation procedures will limit the widespread adoption of siRNA-containing lipid-based products in the clinics. Clearly, there is a need to develop a simple and effective method to formulate these PEGylated siRNA-loaded lipid particles where the final product is also suitable for long term storage.

In 2004, Li and Deng reported on a method for the preparation of liposomes involving hydration of a freeze-dried matrix of lipid and sugar (22). This preparation method not only has the advantage of ease of preparation but the end freeze-dried product is also ideal for long term storage. This method has so far been modified to entrap various bioactives in liposomes, such as ketoprofen (a small hydrophobic drug; MW 254) (22) and lipopeptides (small amphiphilic molecules; MW≈1,000) (25) and, in both instances, neutral lipids were used in the formulations. Here, we investigated the feasibility of adapting this technique to prepare siRNA-loaded (large anionic molecules; MW≈13,000) cationic lipid particles. We determined the effect of various formulation parameters on the size and homogeneity of resulting particles and importantly, compared these particles to those prepared using an established post-insertion procedure (16) for the entrapment efficiency, stability, *in vitro* biological activity as well as *in vivo* biodistribution. Overall, our results suggested that the hydration of freeze-dried matrix (HFDM) method is a simple yet efficient method of formulating stable siRNA-loaded lipid particles for *in vivo* use.

MATERIALS AND METHODS

Materials

Dioleoyl trimethylammonium propane (DOTAP) and cholesterol were purchased from Sigma (St Louis, MO). Polyethylene Glycol $(PEG)_{2000}$-C16Ceramide conjugate was from Avanti Polar Lipids (Alabaster, AL). HeLa and TC1 cells were obtained from the American Type Culture Collection (Manassas, VA). Green Fluorescence Protein (GFP)-expressing HeLa cells were prepared according to protocols described in Gu *et al.* (26) and TC-1 luciferase^{+ve} cells were prepared by transfecting TC-1 cells with lentivirus expressing luciferase and GFP in cytomegalovirus (CMV) promoter. All cell lines were maintained in 0.2% primocin (Invivogen, San Diego, CA)-containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bovogen, Keilor East, Australia) and 2mM L-glutamine (Invitrogen).

Control oligodeoxynucleotides (ODN) with sense sequence of 5′-GTCAGAAATAGAAACTGGTCATC-3′ and antisense sequence of 5′-GATGACCAGTTTCTATTTCTGAC-3′ were obtained from Invitrogen (Carlbad, CA). Anti-GFP siRNA (5′-GCACGACUUCUUCAAGUCCUU-3′; 5′-GGACUUGAAGAAGUCGUGCUU-3′) and control siRNA (5′-GCAACAGUUACUGCGACGUUU-3′; 5′-ACGUCGCAGUAACUGUUGCUU-3′) were purchased from Sigma-Aldrich (St Louis, MO) in annealed form.

All other chemicals and solvents used were of at least analytical grade.

Liposome Formulations

Lipoplexes Prepared by Hydration of Freeze-Dried Matrix (HFDM) Method

A flow diagram summarizing the process for preparing polynucleotide-loaded lipid particles by hydration of freeze-dried matrix (HFDM) method is shown in Fig. 1. Firstly, required amounts of DOTAP, cholesterol and PEG_{2000}-C16Ceramide were dissolved in 1 mL of *tert*-butanol. Forty micrograms of ODN or siRNA was added to 1 mL of filtered sucrose solution before mixing with the lipid solution. The resultant formulation was then snap-frozen and freeze-dried overnight (ALPHA 1–2 LDplus, Martin Christ, Germany) at a condensing temperature of −80°C and pressure of less than 0.1 mbar. dH_2O was then added to the lyophilised product with gentle shaking. The amount of lipids and sucrose used in each experiment as well as the rehydration volumes investigated are summarised in Table I. A Nitrogen/Phosphate (N/P) ratio of 4:1 was used for all formulations and three separate batches were made for each formulation condition. (n=3) When comparing with the post-insertion method of formulating

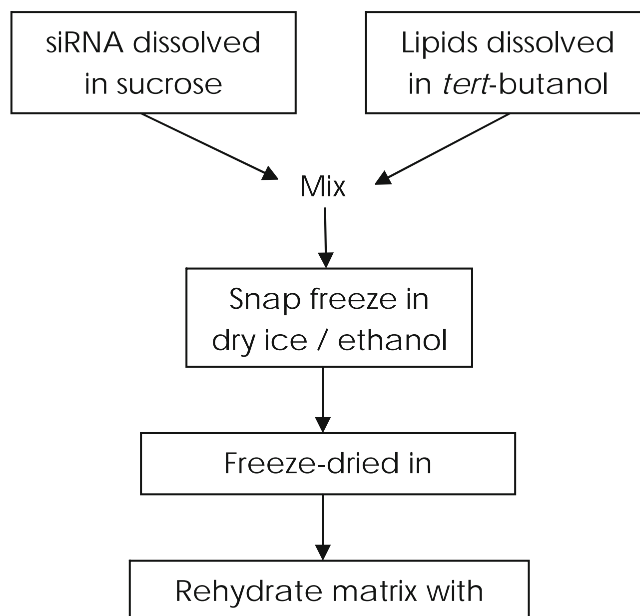

Fig. 1. Flow chart of the hydration of freeze-dried matrix (HFDM) method for preparing siRNA-loaded lipid particles.

Table I. Summary of Experimental Parameters Investigated for the Hydration of Freeze-Dried Matrix (HFDM) Method for Preparing Polynucleotide-Loaded Lipid Particles

	Lipid compositions (molar ratio)						
Exp	DOTAP	Cholesterol	PEG_{2000}-C16Ceramide	ODN (µg)	N/P ratio	Sucrose (mg)	Rehydration volume (µL)
A	50	40	10	40	4:1	38	250 500 1000
B	50	40	10	40	4:1	7.6 38 190	500
C/D	50	50 45 40	0 5 10	40	4:1	27.75	300

Results are presented in Fig. 2

PEGylated lipoplexes, DOTAP, cholesterol and PEG_{2000}-C16Ceramide with a molar ratio of 45:45:10 was used. For those formulations, the final product contained 40 µg siRNA in 300 µL isotonic sucrose solution.

Post-insertion Method of Formulating PEGylated Lipoplexes

A dried lipid thin film consisting of DOTAP and cholesterol (molar ratio 1:1) was prepared as previously described (27). The lipid film was hydrated with sterile isotonic sucrose solution to give a final concentration of 5×10^{-6} mol DOTAP per millilitre. After stabilizing at room temperature for 2 h, liposomes were then extruded through 0.4 µm pore size polycarbonate membranes, followed by 0.2 µm pore size membranes using Lipex extruder (Northern lipids, Vancouver, Canada). The resultant small unilamellar liposomes were then complexed with 40 µg siRNA in 275 µL sterile isotonic sucrose solution at N/P ratio of 4:1. PEG coating of lipoplexes was performed according to the protocol described in Li *et al.* (16). Briefly, 295 µg of PEG_{2000}-C16Ceramide was added to the preformed lipoplexes and the resultant formulation was incubated at 60°C for ten minutes before allowed to cool to room temperature. The final product had a lipid compositions of DOTAP/Chol/PEG of 45:45:10 (molar ratio) and siRNA concentration of 40 µg per 300 µL. Three separate batches were made for each study. (n=3).

Particle Characterisation

Size, polydispersity and zeta potential of the resultant lipoplexes were measured using a Zetasizer Nano ZS™ (Malvern Instruments, Malvern, UK) following appropriate dilution in distilled water. Measurements were carried out at room temperature.

siRNA Entrapment Efficiency

siRNA entrapment efficiencies were determined using the Quant-iT™ PicoGreen® reagent (Invitrogen). Briefly, samples were diluted in 1× Tris–HCl–EDTA buffer (pH 7.5) to the concentration that falls within the linear range of the standard curve (1 ng/mL–0.75 µg/mL). One hundred µL of diluted PicoGreen reagent (1:200 dilution) was then added to 100 µL samples in a 96-well plate and was incubated in the dark for 2–5 min. Fluorescence intensity was subsequently measured using a Fluostar® plate reader at 485 nm excitation and 520 nm emission wavelengths. A standard curve was generated using 1 ng/mL to 0.75 µg/mL siRNA and sample concentrations were then calculated accordingly. Fluorescence intensities were also detected after treating samples with 0.5% Triton-X 100 (Sigma), which destabilised lipid particles and allowed the release of entrapped siRNA. siRNA entrapment efficiencies were then calculated using the formulae: ([siRNA] with Triton-X-100−[siRNA] without Triton-X-100)/[siRNA] with Triton-X-100.

Protection of siRNA by Lipid Particles in Fetal Bovine Serum (FBS)

siRNA (2.5 µg), which were present in 18.75 µL aqueous solution, were incubated with 50 µL non-heat inactivated FBS at 37°C for various times (<10, 30, 60, 90 and 120 min). The siRNA were either naked or associated with PEGylated lipid particles. Diethylpyrocarbonate (DEPC; Sigma)-treated dH_2O was then added to give a final volume of 200 µL. The samples were then vortexed with 200 µL phenol/chloroform (1:1 v/v) and were subsequently spun at 14,000 rpm at 4°C for 10 min. Twenty-five microlitres from the aqueous layer was then mixed with 5 µL loading buffer and was loaded onto 12% non-denaturing polyacrylamide gel. Following electrophoresis, the gel was stained with 1 µg/mL ethidium bromide (Sigma) for 2–5 min before viewing using Bio-Rad® UV transilluminator.

siRNA Transfection

HeLa PLL3.7 GFP^{+ve} cells were seeded in six-well plates the day before the experiment at the density of 75,000 cells/well. Four microlitres of PEGylated lipoplexes (40 µg siRNA/300 µL) was used for each millilitre of Dulbecco's Modified Eagle Media (DMEM), which was supplemented with 10% FBS and 0.2% primocin, resulting in 40 nM final siRNA concentration. Three millilitres of siRNA-containing media was then added to each well and was incubated at 37°C for 3 days. For both formulation methods, siGFP, control siRNA, as well as empty liposomes were examined for GFP-silencing efficiency. Oligofectamine (Invitrogen) was used according to

the manufacture's instruction as a positive control. One millilitre 40 nM Oligofectamine–siRNA complex dispersed in Opti-Mem I reduced-serum media (Invitrogen) was applied to each well and was incubated for 8 h at 37°C. After incubation, Oligofectamine–siRNA complex was removed and was replaced with fresh primocin-containing DMEM media. All formulations were tested in triplicates.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells treated with siRNA or empty liposomes were trypsinized, collected in 15 mL tubes and washed with phosphate buffered saline (PBS) before resuspending in 2% paraformaldehyde (Sigma-Aldrich) in PBS. Cells were kept at 4°C in the dark before the fluorescence intensities of cells being quantified by BD-FACSCanto® flow cytometry. Results were analysed using the CellQuest® software.

Biodistribution of siRNA-containing PEGylated Lipoplexes

Five-month-old female C57BL/6 mice (ARC, Perth) were inoculated subcutaneously at the right abdominal side with 1×10^6 TC-1 luciferase^{+ve} cells suspended in 100 μL PBS. On day 16 post inoculation, 300 μL of DiR-labelled PEGylated lipoplexes which contained 40 μg of siRNA was injected intravenously (i.v.) into each mouse via tail vein. Formulations made by either HFDM or post-insertion method were both tested and three mice were used per treatment group. DiR (155 ng; Invitrogen) which was dissolved in 1 μL of 10% ethanol solution was added to each formulation. At 24 h post i.v. administration, each mouse received intraperitoneal injection of 180 μL D-luciferin firefly (10 mg/mL; Caliper Life Sciences, Hopkinton, MA) before being anaesthetised using isofurane (Abbott, Kurnell, Australia). Luciferase activities were examined using Kodak® In Vivo Imager to locate the tumours and the biodistribution of DiR-labelled PEGylated lipoplexes were investigated using excitation and emission wavelengths of 720 and 790 nm, respectively. Results were analysed using Kodak® molecular imaging software. All experiments were approved by the University of Queensland Animal Ethics Committee.

Stability Studies

Stability of siRNA-containing PEGylated lipid particles formulated using either post-insertion or HFDM method was assessed. HFDM preparations were stored as lyophilised powders and were rehydrated prior to analysis. Particle size, zeta potential and siRNA entrapment efficiencies of the formulations were examined after samples being stored under normal atmospheric condition at either 4°C or room temperature. Tests were performed at 2 and 4 weeks post manufacture and three separate batches were prepared for each storage condition. (n=3) Gene-silencing efficiencies for all formulations were also assessed at the end of 4 weeks using GFP-expressing HeLa cells.

Statistical Analysis

Data are presented as mean±SD. Unpaired t-test (two-tailed, $p<0.05$) was used to analyse statistical significance.

RESULTS

Characterisation of Lipoplexes Prepared by HFDM Method

Various formulation parameters were investigated for their impact on the size of the polynucleotide-loaded particles which were formed upon hydration of the freeze-dried matrix (HFDM) (Table I). For these initial experiments, a 23-bp dsDNA oligonucleotide was used instead of siRNA. All formulations contained 40 μg of polynucleotides and an N/P ratio of 4 was used. The effect of different rehydration volumes on particle size was firstly studied when 10% (molar ratio) PEG-C16Ceramide and 38 mg of sucrose were included in the formulation. (Exp A, Table I) As shown in Fig. 2A, no difference in particle size was observed between different rehydration volumes, ranging from 250 to 1,000 μL. We next investigated changes in the sucrose level on final particle size. Various sucrose levels (7.6, 38 or 190 mg per 40 μg of polynucleotides) were tested, which corresponded to 10, 50 and 250 times total lipid weight, respectively. (Exp B, Table I) All of these formulations were rehydrated with 500 μL of dH_2O. The results showed a statistically significant difference between particles formulated using each dose of sucrose with particle size being inversely proportional to the amount of sucrose used (Fig. 2B).

We next investigated the influence of the level of polyethylene glycol (PEG) on particle size distribution (Exp C, Table I). PEG_{2000}-C16Ceramide was added into each formulation at a molar ratio of 0%, 5% and 10%. Sufficient sucrose was added to allow end products that were isotonic upon hydration of the freeze-dried matrix with 300 μL of dH_2O. Both particle size (Z_{ave}) and polydispersity were examined. It was found that in the absence of PEG-lipid conjugates, large (~400 nm), polydispersed lipid particles were formed upon hydration (Fig. 2C). However, the addition of either 5% or 10% PEG resulted in a smaller particle size of 200–280 nm. Furthermore, a significant decrease in polydispersity was observed upon PEG-lipid addition (Fig. 2D).

Comparison of PEGylated siRNA-Loaded Lipoplexes Prepared by HFDM and Post-insertion Methods

Particle Characterisation

Based on the results above, we used the HFDM method to prepare PEGylated siRNA-containing lipid particles. DOTAP, cholesterol and PEG_{2000}-C16Ceramide were used at a molar ratio of 45:45:10. For comparison we compared our particles with those prepared used an established post-insertion method (16). It should be noted that for these experiments all formulations used the same lipid composition, lipid/siRNA ratio, and siRNA concentration, irrespective of the preparation method. The size, polydispersity index and zeta potential of the resulting lipid particles, as well as the entrapment efficiency of siRNA, are summarized in Table II. The results showed that both methods resulted in particles of similar size, zeta potential, and siRNA entrapment efficiencies (~90%). Due to the absence of the extrusion process in the HFDM method, the polydispersity index of these particles was around 0.3, which was slightly higher than particles made by the post-insertion method.

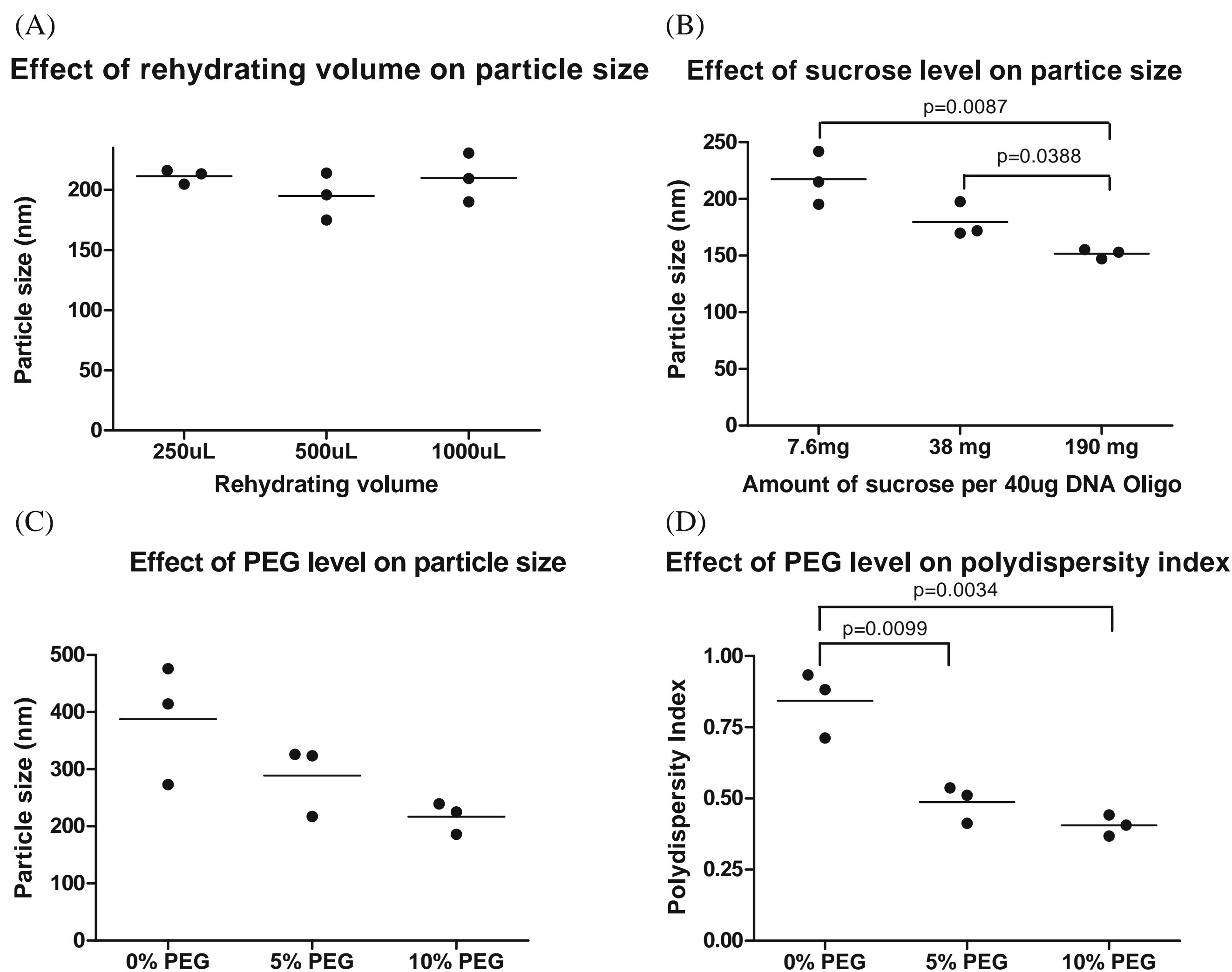

Fig. 2. Hydration of freeze-dried matrix (HFDM) method for formulating polynucleotide-loaded lipid particles. Effects of **A** rehydration volume, **B** sucrose level and **C** presence of PEG on particle size. Figure **D** shows the effect of PEG level on polydispersity index.

Serum Stability and Gene-Silencing In Vitro

An important property of lipid particle designed for *in vivo* use is the ability to protect the siRNA payload from degradation by serum nucleases. Therefore, we determined the ability of our PEGylated lipid particles to protect siRNA by incubating them in fetal bovine serum at 37°C. RNA gel analysis showed that while naked siRNA was broken down in less than 10 min, siRNA molecules entrapped inside the PEGylated lipid particles remained stable for at least 2 h, irrespective of the method of preparation (Fig. 3). We next wished to determine if siRNAs entrapped by each method exhibited any biological activity *in vitro*. Using siRNA directed against the green fluorescent protein (GFP), GFP positive cells were treated with 40 nM siRNA for 3 days before being fixed and the level of GFP was determined using a fluorescent cell sorter (FACS). We observed that GFP-siRNA prepared by both methods were able to knockdown GFP levels by 50% (Fig. 4). The silencing was specific as particles loaded with a control siRNA or empty particles had no effect on GFP levels. As expected, oligofectamine, a commercial *in vitro* transfection reagent that lacks PEG, showed better gene silencing than PEGylated particles made by both methods.

Table II. Summary of Size, Polydispersity Index, Zeta Potential and siRNA Entrapment Efficiency for siRNA-Loaded PEGylated Lipid Particles Formulated Using Either HFDM or Post-insertion Method

	HFDM method	Post-insertion method
Size (nm)[a]	193.2±10.7	178.9±11.8
Polydispersity index	0.32±0.03	0.12±0.06
Zeta potential (mV)	45.1±1.17	41.1±1.51
siRNA entrapment efficiency (%)	94.7±0.61	89.9±2.66

Each sample contained 40 μg siRNA in 300 μL isotonic sucrose solution. Lipids used in these formulations include DOTAP, cholesterol and PEG_{2000}-C16Ceramide with molar ratio of 45:45:10. N/P ratio of 4:1 was used for all formulations. Three batches were made for each formulation method. (n=3)

[a] Represent Z_{ave}±SD. (n=3) as measured by Malvern® Nano Zetasizer

Biodistribution

Given the aim was to develop a novel method for preparing PEGylated lipid particles for *in vivo* use, we next

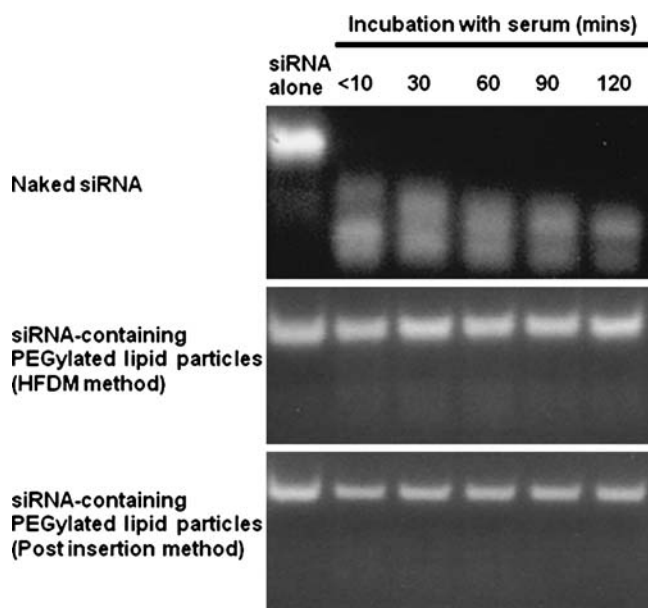

Fig. 3. Protection of siRNA from nucleases degradation by PEGylated lipid particles. Stability of naked siRNA or siRNA which was entrapped in PEGylated lipid particles were examined in fetal bovine serum (FBS). Each sample contained 2.5 μg siRNA and was incubated with 50 μL FBS at 37°C for up to 2 h prior to analysis by gel electrophoresis (Approximately 400 ng siRNA was loaded onto the gel).

wished to look at its biodistribution characteristics. For this we employed a subcutaneous tumour model system using TC-1 luciferase^{+ve} cells (28). The biodistribution of these particles, as well as their ability to accumulate in tumours following intravenous administration in mice, were examined. Particles were prepared using the HFDM or post-insertion methods and were fluorescently tagged by the lipophilic dye, DiR. For both formulations, 40 μg of siRNA in 300 μL of isotonic sucrose solution was injected into each mouse via tail vein. The location of the subcutaneous tumours was identified by injecting mice with D-luciferin and using *in vivo* luminescent imaging. The luciferase activity present in the TC-1 cells catalyses D-luciferin releasing light in the 575–620 nm range (29) (Fig. 5A). At 24 h post injection, the biodistribution of the DiR-labelled particles was determined using *in vivo* fluorescence analysis. As the D-luciferin substrate has a very short half-life, no residual luciferase activity was present to interfere. We observed particles made via both methods were able to accumulate in tumours 24 h following i.v. injection (Fig. 5B). For a more accurate measurement, major first-pass internal organs (liver, spleen and lungs), as well as tumours, were removed and analysed directly on the fluorescent imager (Fig. 5C). The majority of the particles were distributed to the lungs and liver although a similar degree of tumour deposition was achieved by particles made by either the post-insertion or HFDM methods (Fig. 5D). It was also noticed that on average, there was a 6.8 times more accumulation of particles made by the post-insertion technique in the liver compared to tumours (average fluorescent intensity of 49.1 million units *vs* 7.2 million units). This was in contrast to the formulations prepared by the HFDM method where only 2.6-fold more liver deposition was observed compared to tumours (average fluorescence intensity of 13.7 million units *vs* 5.3 million units). The difference in the degree of lung deposition between particles made by each procedure was less profound, with 2.9 times compared to 3.4 times for mice receiving post-insertion formulations verses HFDM formulations, respectively.

Formulation Stability

Finally the stability of the particles made by the post-insertion and HFDM methods was investigated up to 1 month post manufacture. Following formulation, the particles were stored at either 4°C or room temperature (RT). It can be seen that the particle size and zeta potential remained unchanged for both types of formulations irrespective of storage time frames or storage conditions (Table III). However, siRNA entrapment efficiencies for both methods were observed to have decreased slightly over time from around 94% to 84% for the HFDM method and 90% to 83% for the post-insertion method. Surprisingly, the gene-silencing efficiency of the post-insertion samples was almost completely abolished following 1-month of storage at either 4°C or room temperature (Fig. 6). This was in contrast to particles prepared by the HFDM method where 100% of the gene-silencing efficiency was retained following storage at either 4°C or room temperature for a month.

DISCUSSION

While siRNA has great potential in the clinic, the ability to systemically deliver these molecules *in vivo* has, to date,

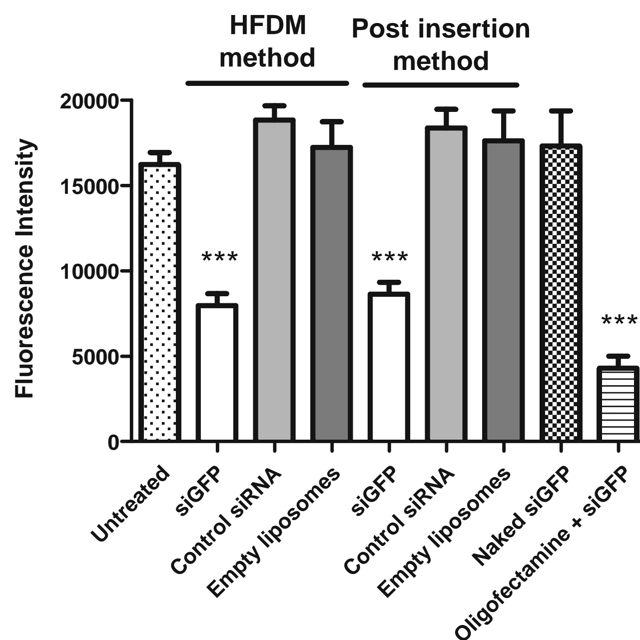

Fig. 4. GFP knockdown by siGFP delivered using PEGylated lipid particles formulated using either HFDM or post-insertion method. HeLa PLL3.7 GFP^{+ve} cells were treated with 40 nM siRNA for 3 days and were fixed with 2% paraformaldehyde prior to analysis by fluorescence activated cell sorting (FACS). The experiment was performed in Dulbecco's Modified Eagle Media (DMEM) apart from oligofectamine-siGFP sample where OptiMem™ was used as per manufacturer's instruction. Each bar and error bar represents the mean value (n=3) and the corresponding SD. ***p<0.001, significantly different compared with the untreated control.

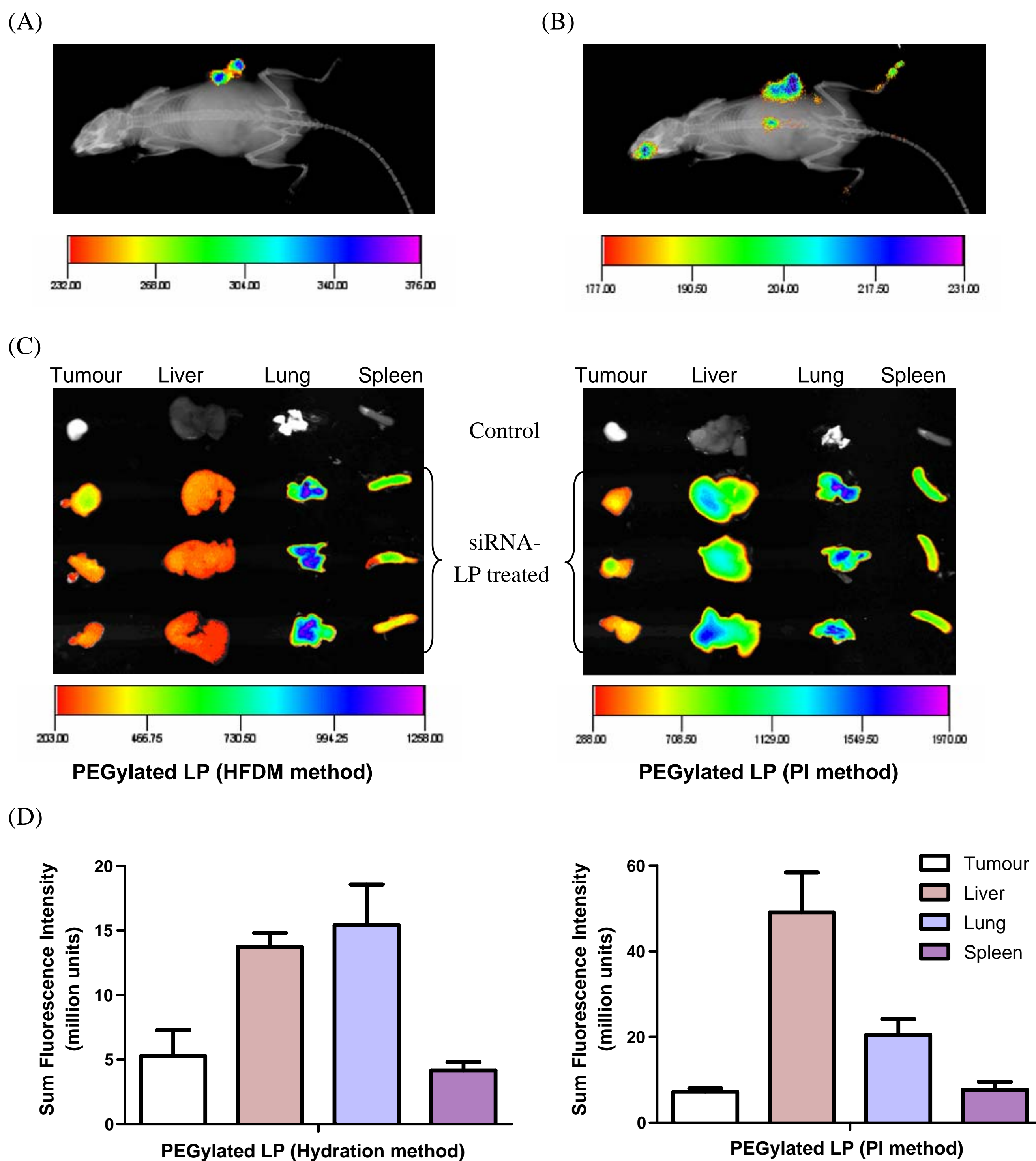

Fig. 5. Biodistribution of siRNA-loaded PEGylated lipid particles after intravenous (i.v.) injection in mice. **A** Location of the subcutaneous tumour was determined by the luciferase activity using Kodak® In Vivo Imager. **B** DiR-labelled PEGylated lipoplexes (LP) made by HFDM method were found in tumours 24 h post i.v. administration. (λ_{ex}=720 nm; λ_{em}=790 nm). **C** For a more accurate measurement of the biodistribution, major first-pass internal organs (liver, spleen and lungs), as well as tumours, were removed and analysed directly on the fluorescent imager. PEGylated LPs were formulated using either HFDM or post-insertion (PI) methods. **D** Quantitative analysis of the distribution of DiR-labelled LP in four major organs in mice at 24 h post treatment from **C**. Results were obtained using Kodak® molecular imaging software. Each bar and error bar represents the mean of the sum fluorescence intensity (n=3) and the corresponding SD.

been poor. One of the limiting factors is the lack of an efficient formulation procedure for preparing siRNA-loaded particles that are suitable for *in vivo* applications. In this study, we have developed a novel hydration of freeze-dried matrix (HFDM) method to formulate these particles such that the process is relatively simple and produces particles with favourable characteristics for *in vivo* delivery. Moreover, the final products are also suitable for long term storage as

Table III. Effect of Storage Conditions on Particle Size, Zeta Potential and siRNA Entrapment Efficiencies of siRNA-Loaded PEGylated Lipid Particles Formulated Using HFDM Method (A) or Post-insertion Method (B)

		Storage condition			
		4°C		Room temperature	
	Freshly prepared	2 weeks	4 weeks	2 weeks	4 weeks
(A) HFDM method					
Particle size (nm)	189.5±3.76	193.7±12.5	199.8±10.7	187.6±15.8	192.0±3.63
Zeta potential (mV)	49.8±4.04	48.1±3.84	49.1±3.20	52.7±2.57	52.7±0.95
Entrapment %	94.7±0.61	88.1±2.04	84.4±4.00	88.8±1.34	84.2±4.56
(B) Post-insertion (PI) method					
Particle size (nm)	163.7±4.99	174.5±2.84	172.6±8.47	172.1±1.97	171.0±1.28
Zeta potential (mV)	43.6±5.18	43.9±9.21	47.5±7.62	53.2±3.87	51.3±1.36
Entrapment %	89.9±2.66	87.4±0.76	82.7±2.96	86.4±2.60	83.1±1.00

Formulations were stored at either 4°C or room temperature and three batches were made for each condition. (n=3) Tests were performed at 2 and 4 weeks post manufacture. Results are presented as mean±SD

it is prepared in the solid state and can be hydrated prior to use.

This novel HFDM method involves a two-step process which comprises of freeze-drying a solution of lipid and siRNA in a *t*-butanol/water co-solvent system followed by rehydration of the freeze-dried matrix. This procedure is significantly less labour intensive compared to other established methods, such as ethanol dialysis (20), post-insertion (16) or reverse-phase evaporation (17). Moreover, it does not require the use of specialised equipment, such as extruder, which is not available in most laboratories. This HFDM method therefore presents as an attractive alternative procedure to prepare siRNA-loaded lipid particles.

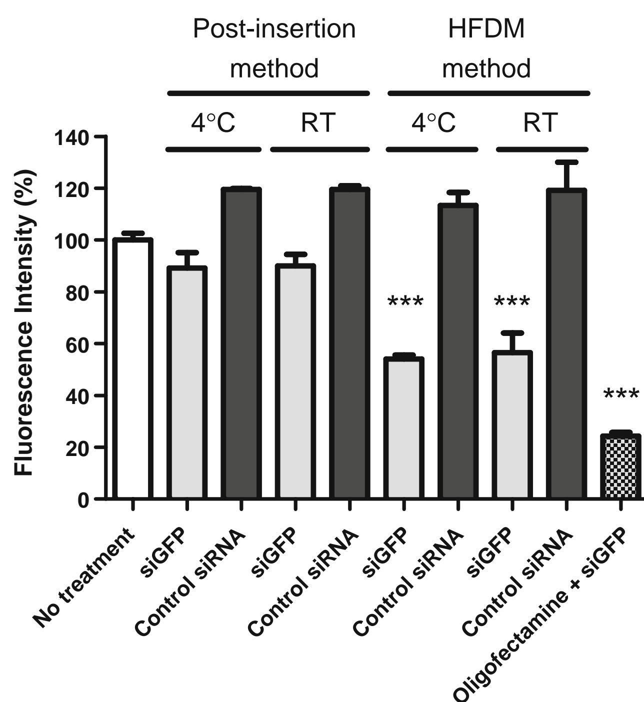

Fig. 6. Minimal knockdown of GFP was observed for the samples prepared by post-insertion technique after being stored at either 4°C or room temperature (RT) for 4 weeks. In contrast, efficient gene silencing was achieved for the 1-month-old siRNA-loaded PEGylated lipid formulations prepared by HFDM method. Experiment was performed using HeLa PLL3.7 GFP^{+ve} cells and cells were treated with 40 nM siRNA for 3 days before being fixed in 2% paraformaldehyde prior to analysis by FACS. Each bar and error bar represents the mean value (n=3) and the corresponding SD. ***p<0.001, significantly different compared with the untreated control.

In the development of this method, we studied the particle size distribution, the efficiency of the particles to protect siRNA from nucleases degradation, their gene-silencing efficiency *in vitro* as well as their biodistribution *in vivo*. Our results indicated that concentrated siRNA-lipid formulation can be prepared using the HFDM method as the particle size distribution is unaffected by the final hydration volume as shown in Fig. 2A. Since high dose of siRNA is usually required for *in vivo* applications, this is advantageous as no further volume reduction procedure is required before its use *in vivo*. We found that the particle size can, however, be altered by varying the amount of sucrose present in the formulations, with higher sucrose levels giving rise to formation of smaller particles (Fig. 2B). This observation was not surprising as it has been previously reported that the presence of sucrose may prevent the fusion of various lipid domains during the drying process thereby permitting the formation of smaller particles upon hydration (22). However, while our observation was consistent with that reported by Li and Deng (22), the amount of sucrose we required here to obtain particle size of around 200 nm was considerably higher, with a sucrose/lipid weight ratio of around 20 compared to 2.5 reported by Li *et al.* (22). This discrepancy is likely due to the difference in the types of lipids (cationic *vs.* neutral) and bioactives (polynucleotides *vs.* ketoprofen) present in the formulations between our study and the study by Li and Deng. In addition to the sucrose level, it was also observed that PEG played an important role in controlling the particle size distribution as shown in Fig. 2C and D. It is believed that PEG, being a hydrophilic polymer, can hinder the interaction between cationic lipids and anionic polynucleotides thereby permitting the formation of small particles (30). We have shown that in the presence of 10% PEG-C16Ceramide, small (<200 nm) monodispersed siRNA-containing particles can be successfully formulated (Table II). This size distribution will permit the use of these particles in treatment of diseases

where the target sites have leaky vascular architecture, such as tumours (31). Overall, these results suggested that HFDM method is suitable for formulating polynucleotide-containing lipid particles given that sufficient amount of sucrose and PEG are included in the formulations.

We further compared the siRNA-loaded PEGylated lipid particles prepared by HFDM method with the ones formulated by an established post-insertion technique (16). We noticed that the mean particle size was comparable for these two procedures although particles formulated using HFDM method displayed slightly higher polydispersity due to the absence of the extrusion step. However, it is believed that these particles still possess suitable characteristics for them to be used directly *in vivo* without further size reduction. Like particle size distribution, siRNA entrapment efficiency was also found to be similar between these two formulation methods (Table II). It was therefore not surprising to find that particles made by either method can protect siRNA against nuclease degradation to a similar level as shown in Fig. 3. More importantly, the gene-silencing data showed no difference in the siRNA delivery efficiency *in vitro* between these two types of formulations (Fig. 4), which suggests that the HFDM method is a feasible and convenient method for formulating functional siRNA-containing PEGylated lipid particles.

The question we then wish to address is whether siRNA-containing PEGylated lipid particles prepared by HFDM method can reach the target sites after systemic administration in mice. In our study, we used mice bearing subcutaneous tumours as the animal model to assess the use of these siRNA-containing lipid particles in cancer treatments. The observed preferential accumulation of these particles in first-pass organs was not surprising given the presence of cationic lipids in the formulations (32) (Fig. 5). Nevertheless, the degree of tumour accumulation was found to be comparable for particles formulated using both HFDM and post-insertion methods. Although the particles which have been excreted through urine or the ones remaining in the bloodstream were unaccounted for in our experiment, out of the four major organs where lipoplexes normally accumulate after systemic administration (33), more than 13% of the siRNA-loaded lipid particles prepared by HFDM method were found in tumours 24 h post i.v. injection. Using detergent-dialysis method, MacLachlan *et al.* have also reported 5% to 10% accumulation of polynucleotide-loaded PEGylated lipid particles in subcutaneous tumours after i.v. administrations in mice (34). In contrast to these results, however, Huang's laboratory has recently reported 70% accumulation of siRNA-containing lipid particles in subcutaneous tumours after i.v. administration in mice, in which the same post-insertion technique was used to prepare those lipid particles as the one employed in our study (35). This discrepancy is likely due to the difference in the tumour size at the time of injection, imagining time-point, lipid/siRNA ratio, as well as lipid compositions used between these two studies. More interestingly, while investigating the biodistribution of particles after intravenous injections in our study, we found that the particles formulated using post-insertion technique accumulated more in the liver relative to the tumours compared to particles formulated using HFDM method. The exact mechanism behind this biodistribution difference is yet to be investigated. Nevertheless, these results provide evidence that HFDM is a feasible method to formulate siRNA-containing PEGylated cationic lipid particles for *in vivo* applications. However, as it has been previously established that neutral lipid particles have superior biodistribution characteristics compared to the cationic ones (36,37), the adaptability of this hydration procedure in formulating PEGylated siRNA-loaded neutral lipid particles remains to be evaluated. It is anticipated, however, that lower entrapment efficiency of siRNA may occur when neutral lipids are utilized in the formulation due to the lack of electrostatic interactions between lipids and siRNA. pH-sensitive lipids, such as 1,2-Dioleoyloxy-3-(dimethylamino)propane (DODAP), may therefore be of value in this application as the charge of these lipids can be altered by varying the pH value.

The HFDM method for preparing siRNA-loaded lipid particles also offers the advantage of product stability. Although numerous reports have been published previously on the stability of PEGylated lipid particles containing DNA plasmids (11,23,38,39), this is the first report on the stability of such lipid formulations containing siRNA. In our study, we found that the size of siRNA-loaded lipid particles remained unchanged after 1 month of storage at either 4°C or room temperature, irrespective of the method of preparation. In addition, siRNA entrapment efficiency was found to have decreased slightly over time for formulations prepared by both methods and this was consistent with the finding reported by Zhang *et al.* (40). This decrease in siRNA entrapment, however, did not influence the gene-silencing efficiency of the particles formulated using HFDM method, which retained 100% of their gene-silencing efficiency after 1 month of storage at either 4°C or room temperature. In contrast, 80% decrease in gene-silencing efficiency was observed for the aqueous formulations prepared by post-insertion procedures after 1 month of storage. Compared to previous reports on DNA plasmid-containing lipid preparations (11,39), these findings suggest that siRNA is much more unstable than the DNA plasmids in an aqueous environment. Lyophilisation is therefore absolutely essential for the long term storage of such siRNA-containing preparations. Although Yadava and colleagues have recently reported the application of freeze-drying siRNA-containing lipoplexes (41), their procedure, however, still requires the time-consuming pre-formulation steps. The HFDM method offers one-pot formulation of PEGylated siRNA-lipid particles in the lyophilised powder form, which only requires rehydration immediately prior to use. This not only offers the convenience of formulation preparation but also significantly enhances the stability of final products.

CONCLUSION

This is the first report describing the formulation procedure for preparing PEGylated siRNA-containing cationic lipid particles where the end product is in the solid state. This hydration of freeze-dried matrix method requires minimal preparation time compared to all other existing formulation procedures. We have demonstrated that particles prepared by this hydration method can protect siRNA from nucleases degradation in serum and effectively facilitate the delivery of siRNA *in vitro*. These particles were also able to accumulate in subcutaneous tumours after systemic administration, which highlights their potential use in cancer therapies. Finally, the formulations prepared by hydration method also showed

superior stability over those formulated using post-insertion method. Overall, the development of this new method will not only benefit the optimisation process for *in vivo* siRNA delivery systems but also offers the advantage of product stability which will be vital for the translation of siRNA from the laboratory to clinic.

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