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In vitro investigations of the efficacy of Cyclodextrin-siRNA complexes modified with Lipid-PEG-Octaarginine:

Towards A Formulation Strategy for non-viral neuronal siRNA Delivery

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Running Head: Neuronal siRNA Delivery by R8 modified Cyclodextrin

ABSTRACT

Purpose: Development of RNA interference based therapeutics for neurological and neurodegenerative diseases is hindered by a lack of non-viral vectors with suitable properties for systemic administration. Amphiphilic and cationic cyclodextrins (CD) offer potential for neuronal siRNA delivery. Here, we aimed to improve our CD-based siRNA formulation through incorporation of a polyethyleneglycol (PEG) shielding layer and a cell penetrating peptide, octaarginine (R8).

Methods: CD.siRNA complexes were modified by addition of an R8-PEG-lipid conjugate. Physical properties including size, charge and stability were assessed. Flow cytometry was used to determine uptake levels in a neuronal cell model. Knockdown of an exogenous gene and an endogenous housekeeping gene were used to assess gene silencing abilities.

Results: CD.siRNA complexes modified with R8-PEG-lipid exhibited a lower surface charge and greater stability to a salt-containing environment. Neuronal uptake was increased and significant reductions in the levels of two target genes were achieved with the new formulation. However, the PEG layer was not sufficient to protect against serum-induced aggregation.

Conclusions: The R8-PEG-lipid-CD.siRNA formulation displayed enhanced salt-stability due to the PEG component, while the R8 component facilitated transfection of neuronal cells and efficient gene silencing. Further improvements will be investigated in the future in order to optimise stability in serum and enhance neuronal specificity.

Keywords: siRNA, neuronal delivery, cyclodextrins, octa-arginine

ABBREVIATIONS

CD: cyclodextrin

CNS: central nervous system

DLS: dynamic light scattering

DMSO: dimethylsulphoxide

DSPE: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine

DIW: deionised water

GAPDH: glyceraldehyde phosphate dehydrogenase

MR: mass ratio

mRNA: messenger RNA

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide

PAMAM: polyamidoamine

PEG: polyethyleneglycol

PLL: poly-L-lysine

ns: non-silencing

R8: octaarginine

RLU: relative luminescence units

siRNA: small interfering RNA

shRNA: short hairpin RNA

INTRODUCTION

The potential for RNA interference-based therapeutics for diseases of the central nervous system (CNS) has received much attention (1-3). Delivery of small interfering RNAs (siRNA) to neurons and the CNS remains an obstacle and, to date, cationic lipids are the most extensively used non-viral vectors (4). Cyclodextrins (CDs) have shown great promise as gene delivery vectors in various cell types (5-13). Furthermore, in pre-clinical studies, CD-based vectors have shown favourable toxicity profiles, with no effect on body weight and liver enzymes (8, 14, 15). We have recently developed an amphiphilic cationic β-CD for neuronal siRNA delivery, which exhibited minimal toxicity in neuronal cell lines and primary hippocampal neurons, whilst achieving up to 40% reduction in expression of the target housekeeping gene (16). However, progress towards systemic administration requires substantial developments in this formulation. Of particular focus are modifications which will confer stability in a salt and serum environment, thereby reducing interaction with plasma components, whilst maintaining high levels of cellular permeation and gene silencing with improved specificity and targeting.

The 'ABCD' approach to designing nanoparticles for nucleic acid delivery can be applied when considering appropriate components of a formulation (17). 'A' denotes the nucleic acid cargo, in this case siRNA, which is complexed or encapsulated by 'B', the amphiphilic cationic CD. Here we use a CD which has been shown to mediate high intracellular siRNA levels and knockdown in neurons (16), but tends to aggregate on exposure to salt- or serum-containing medium and therefore is expected to have poor stability and short circulation time *in vivo*. Therefore, an additional component 'C', which represents a polyethyleneglycol (PEG) shielding layer, should be considered to enhance the stability of the formulation by minimising interaction with negatively charged plasma proteins and erythrocytes (18-20).

However, shielding of the surface of cationic siRNA-containing nanoparticles with a PEG layer can impair cellular uptake and reduce or even eliminate their gene silencing abilities (21-23). To overcome this, a targeting or cell penetrating ligand 'D' can be included as the final component in the formulation (19).

Octaarginine (R8) is a model peptide ligand, which can be attached via the PEG moieties to assist in cell penetration. R8 has been used itself or in combination with vectors to transfer genes and siRNA into a variety of cell types *in vitro* (18, 24, 25) and *in vivo* (26, 27). In terms of neuronal delivery, stearylated R8 achieved moderate levels of gene knockdown (~50%) in primary hippocampal neurons (28) and a polyarginine vector, modified with myristic acid, reduced target mRNA expression by 25% in primary cortical neurons (29). Furthermore, both shRNA (short-hairpin RNA) and siRNA were successfully delivered to primary cortical cultures using a cationic polyamidoamine (PAMAM) dendrimer to which R8 was grafted (30, 31).

We have previously described a co-formulation approach whereby two CDs, one neutral and the other cationic were mixed together before siRNA complexation, to achieve nanoparticles with improved stability but which lacked gene silencing efficiency in non-neuronal (21) and neuronal cells (unpublished data), which was attributable, at least in part, to a reduction in cellular uptake. Efforts to synthesise a PEGylated CD with a neuronal specific targeting ligand attached are ongoing, but have proved complex. Therefore, we investigated an alternative approach to varying the CD.siRNA formulation.

One strategy for the modification of nanoparticles at their surface is by 'post-insertion' (32). This method describes the transfer to nanoparticles of amphiphilic molecules which exist in micellar form and has been extensively reported for liposomes (33, 34) and lipid

nanocapsules (35). Post-insertion has also been recently used for the surface modification of siRNA containing cationic liposomes with PEG and transferrin (36).

An 'insertion' type approach was, therefore, investigated for the modification of pre-formed CD.siRNA complexes. R8, in the form of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)–PEG_{2000}-R8, was inserted into the surface of CD.siRNA complexes, yielding a novel modified R8-PEG-DSPE-CD.siRNA formulation, which was assessed in terms of its physical properties and its silencing efficacy. It was hypothesised that the PEG component would stabilise the complexes and the R8 would aid in transfer across neuronal cell membranes.

MATERIALS AND METHODS

siRNAs

Negative control siRNA (sense sequence 5'- UUC UCC GAA CGU GUC ACG U), fluorescein labelled siRNA (sense sequence 5'- UUC UCC GAA CGU GUC ACG U, modified with 3'-fluorescein on the sense strand), pGL3 luciferase siRNA (sense sequence 5'- CUU ACG CUG AGU ACU UCG A) and GAPDH siRNA (sense 5'- GGU CGG UGU GAA CGG AUU U) were obtained from Qiagen (California, USA).

Preparation of CD.siRNA complexes

An amphiphilic cationic CD, SC12CDclickpropylamine, was synthesized as reported (21). CD.siRNA complexes were prepared as described (16, 21). Briefly, the required amount of CD was dissolved in chloroform (1 mg/ml). The solvent was removed under a gentle stream of nitrogen. Aliquots were stored at -20 ºC until required. CDs were rehydrated with deionised water (DIW) (final concentration 1 mg/ml) and sonicated for 1 hour at room temperature, then mixed with siRNA in an equal volume of DIW and incubated for 15-20

minutes at room temperature. A cationic CD:siRNA mass ratio (MR, µg CD: µg siRNA) of 20 was chosen (16).

For *in vivo* experiments, 5% glucose was used in place of DIW.

Synthesis of DSPE-PEG$_{2000}$-R8

DSPE-PEG$_{2000}$-R8 was synthesized as follows. AcCysβAla(Arg)$_8$H was synthesized on a 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems, Model 433A; Framingham, MA, USA) using Fmoc solid-phase peptide synthesis. The fluorenylmethoxycarbonyl amino acids Fmoc-Arg(Pbf)-OH, Fmoc-βAla-OH and Fmoc-Cys(Trt)-OH (Merck Millipore, Germany) were coupled using 10 equivalents of *O*-Benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and Hydroxybenzotriazole (HOBt) and the Fmoc groups were deprotected using DIEA. The peptide was cleaved from the resin and the side chains were deprotected in TFA/TIS/H_2O/EDT (92.5/2.5/2.5/2.5) for 4.5 h. The product was purified by HPLC on a C18 column (Phenomenex, Cheshire, UK) to yield 55 mg of peptide.

AcCysβAla(Arg)$_8$H (55mg, 0.023mmol, as TFA salt) was dissolved in 10 mL of phosphate buffer at pH 7. 10 mmol Mal-Peg(2000)-DSPE (61 mg, 0.0207 mmol; Avanti Polar Lipids Inc., AL, USA) was diluted with 19 mL of acetonitrile. Both solutions were degassed under nitrogen prior mixing together and the biphasic mixture was stirred overnight. The solvents were evaporated and the mixture purified by HPLC on C5 column (Phenomenex, Cheshire, UK) using a column heater at 60°C to obtain the product as the TFA salt (18.5 mg, 0,00347mmol, 15% yield). The product purity was determined to be 70% by analytical HPLC.

HRMS-MALDI-TOF (*m/z*): $[M + H]^+$ calcd. for $C_{191}H_{375}N_{36}O_{67}PS$, 4311.26096; found, 4366.3800.

Preparation of R8-PEG-CD.siRNA formulation

For preparation of micelles, DSPE-PEG-R8 was hydrated with MES buffer (20 mM HEPES, 20 mM MES, pH 6.5), above the critical micelle concentration (2.3 µM) (33). This was followed by vortex mixing, heating to 38 °C for ten minutes and a second vortex mixing (36). The size range of the resulting particles was 20-30 nm.

Finally, 5 to 20 mol % (DSPE-PEG2000-R8-trifluoroacetate relative to SC12CDClickpropylamine) of R8-PEG-DSPE was added to the CD.siRNA complexes and left overnight (~ 18 hours) at 38 °C (36). The CD.siRNA MR was maintained at 20, irrespective of the amount of DSPE-PEG-R8 added to the formulation.

For pharmacokinetic studies, complexes were concentrated by ultrafiltration (37, 38). Vivaspin centrifugal concentrators (Vivascience) with a molecular weight cut off of 3000 were used. Complexes were added to the concentrator tubes and centrifuged at 2000 xg at 4 °C until concentrated to the required volume.

Gel Retardation Assay

siRNA complexation was determined by agarose gel electrophoresis (20), after modifying the formulation with R8-PEG. CD.siRNA complexes (MR20), or R8-PEG-DSPE-CD.siRNA complexes, were mixed with loading buffer and DIW to a final volume of 20 µl (containing 0.3 µg siRNA). Samples were added to wells in a 1% agarose gel containing SafeView™ (NBS Biologicals Ltd, England) (6 µl/100 mls). Electrophoresis was carried out at 90 V for 20 minutes, with a Tris-borate-EDTA buffer (39). Bands corresponding to the DNA ladder

(100 b.p.) and unbound siRNA were visualised by UV, using the DNR Bioimaging Systems MiniBis Pro and Gel Capture US B2 software.

Size and Charge Measurements

Particle Z-average size and charge were measured with Malvern's Zetasizer Nano ZS, using dynamic light scattering (DLS) and electrophoretic mobility measurements respectively. CD.siRNA and R8-PEG-DSPE-CD.siRNA complexes were prepared and made up to 1 ml with 0.2 µm filtered DIW. Five readings of Z-average size (nm), polydispersity (25°C, measurement angle 170°) and zeta potential (mV) (25°C, measurement angle 12.8^{o}) were taken. For data analysis, the viscosity (0.8872 mPa.s) and refractive index (1.33) of water were used to determine Z-average size. These data are presented as mean ± S.D.

Aggregation Studies

Stability of formulations upon exposure to salt-containing medium or serum were investigated by incubating CD.siRNA or R8-PEG-DSPE-CD.siRNA complexes in either OptiMEM® transfection medium (40, 41) or foetal bovine serum (FBS) (20) at 37 ºC for various lengths of time. Following this, size measurements were carried out by DLS as before.

Cell culture

A mouse embryonic hypothalamic cell line (*mHypoE N41)* (42) was obtained from tebu-bio (France) and was maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 10 % foetal bovine serum (FBS, Sigma) in a humidified 37 ^{o}C incubator with 5 % CO_2. Cells were seeded in 12- well, 24- well and 96- well plates at 6.6 x 10^4, 3.5 x 10^4 and 1.5 x 10^4 cells per well respectively. This cell line is a useful model for neuronal cells (16).

MTT Toxicity Assay

The MTT assay is widely used as an indicator of the toxicity caused to neurons by non-viral vectors (43, 44). This assay measures the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) by mitochondrial dehydrogenase, in viable cells only, to give a dark blue product. However, as it only measures one end-point, namely the change in mitochondrial integrity, it is not a direct measure of cell viability. Cells were seeded in 96-well plates 1 day prior to transfection. siRNA (100 nM) alone, or in CD.siRNA or R8-PEG-DSPE-CD.siRNA complexes, was diluted in OptiMEM®, then added to cells in serum-containing medium for 24 hours. Media was removed and replaced with 100 µl fresh media and MTT (20 µl of a 5 mg/ml solution) for four hours, after which the formazon crystals produced were dissolved in 100 µl DMSO. Absorbance was measured at 590 nm using a UV plate reader. Each experiment was carried out in triplicate. Results were expressed as % dehydrogenase activity compared to untreated controls. These data are presented as the mean ± SEM.

Cellular uptake experiments

The level of uptake mediated by transfection complexes was assessed by flow cytometry (20, 44). Fluorescently labelled siRNA (Qiagen) was used for these experiments. Cells were seeded in 24 well plates 1 day prior to transfection. siRNA (50 nM) alone, or in CD.siRNA or R8-PEG-DSPE-CD.siRNA complexes, was diluted in OptiMEM®, then added to cells in serum-containing medium for 24 hours. Following this, complexes bound to the extracellular surfaces were removed by washing with phosphate buffered saline (Sigma) and by incubation with 250 µl of CellScrub buffer (Genlantis) for 15 minutes at room temperature (20, 45). Cells were removed from the wells and prepared for analysis following several washing steps. The fluorescence associated with 10,000 cells was measured with a FACS Caliber

instrument (BD Biosciences) and data were analysed using Cell Quest Pro software. Each experiment was carried out in triplicate. These data are presented as the mean ± SEM.

Knockdown of luciferase reporter gene

Silencing of an exogenous gene was assessed by measuring knockdown of a luciferase reporter plasmid as previously described (20, 21). Cells were seeded in 24 well plates 1 day prior to transfection. Cells were transfected with pGL3-luc (1 µg/well) complexed to Lipofectamine™ 2000 (2.5 µl/µg pDNA) for three hours. Following this, cells were washed twice with phosphate buffered saline (Sigma) prior to siRNA transfection. pGL3-luciferase siRNA (50 nM) alone, in CD.siRNA complexes or in R8-PEG-DSPE-CD.siRNA complexes, was diluted in OptiMEM® and added to the cells in serum-containing medium. Complexes containing negative control siRNA (ns siRNA) were included as controls. NH_2-PEG-DSPE-CD.siRNA complexes were also included, which lacked in the R8 ligand. After 24 hours, cells were washed with PBS, lysed with 400 µl of 1x Reporter Lysis Buffer (Promega) and frozen at -80 ºC. Lysate was collected and centrifuged for 5 min at 13000 rpm. A sample of the supernatant (20 µl) was assayed for expression of luciferase by adding to 100 µl of luciferin (Promega) and measuring the light produced (relative luminescence units, RLU) 10 seconds later in a Junior LB 9059 luminometer (Promega). Total protein levels in each sample were determined by the BCA Protein Assay (Thermo Scientific). Luciferase expression for each sample was calculated as RLU per microgram protein. Luciferase expression of the 'Untreated' control samples (which were transfected with luciferase plasmid only but no siRNA) was taken as 100% and for all other samples, gene expression was calculated as a % of this untreated control. Each experiment was carried out in triplicate. These data are presented as the mean ± SEM.

Knockdown of endogenous GAPDH

Silencing of an endogenous gene was assessed by measuring knockdown of the housekeeping gene, glyceraldehyde dehydrogenase phosphate (GAPDH) (46, 47). Cells were seeded in 12 well plates for 1 day before transfection. GAPDH-siRNA (100 nM) alone, or in CD.siRNA or R8-PEG-DSPE-CD.siRNA complexes, diluted in OptiMEM®, was added to the cells in serum-containing medium. Complexes containing negative control siRNA (ns siRNA) were included as controls. After 24 hours, total RNA was extracted from N41 cells using Stratagene Absolutely RNA® Miniprep Kit, according to the manufacturer's instructions. The concentration of RNA was measured by UV absorbance on the NanoDrop ND-1000 UV-Vis Spectrophotometer and RNA integrity was confirmed by analysis using the Agilent 2100 Bioanalyzer. A high-capacity cDNA reverse transcriptase kit (Applied Biosystems) was used for complementary DNA (cDNA) synthesis. Gene expression was assessed by real-time qPCR using the Applied Biosystems Real Time PCR System (Model 7300). Assays were performed using appropriate primer sets for GAPDH and β-actin (TaqMan®, Applied Biosystems). β-actin endogenous gene was used for relative gene quantification (47). The 2–delta Ct method was used to calculate relative changes in mRNA (48). Each experiment was carried out in triplicate. Results were expressed as % GAPDH gene expression relative to untreated (non-transfected) controls. These data are presented as the mean ± SEM.

Experimental animals

Male Balb/c mice of age 8-10 weeks (20-22 g) were purchased from Harlan Laboratories (UK). Animals were given at least one week to habituate in the animal facility with food and water *ad libitum*, on a 12/12 h reversed dark-light cycle with temperature 22 ± 1°C. All experiments carried out on animals were in accordance with institutional ethics guidelines and the European Community Directive (86/609/EEC).

Pharmacokinetic study

Mice (n=5 per group) were injected with FAM-labelled siRNA (40 µg), either uncomplexed or in concentrated R8-PEG-DSPE-CD.siRNA complexes (MR20, 20% PEG R8), via the tail vein. At various time points, blood (20-30 µl) was sampled from the saphenous vein and plasma was isolated. Plasma concentrations of FAM-siRNA were determined by fluoresence measurements post extraction as previously described (49). Briefly, plasma (10 µl) was incubated for 10 minutes at 65°C with lysis buffer (90 µl; 0.1% sodium dodecyl sulphate in PBS). Methanol (200 µl) was added and samples were incubated for 10 minutes at 90°C, then centrifuged at 14000 rpm for 5 minutes. Supernatant (100 µl) was added to wells in a black 96-well plate and the fluoresence was measured by plate reader (λ_{ex} 465 nm and λ_{em} 520 nm). The concentration of FAM-labelled siRNA in each sample was determined from a prepared standard curve.

The extraction efficiency for siRNA was ~99% and for R8-PEG-CD.siRNA was ~90% and sample concentrations were corrected for these extraction efficiencies.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare multiple groups followed by Bonferroni's post hoc test. A two-tailed student's *t*-test was used to compare the pharmacokinetic parameters from the two groups. Statistical significance was set at $*p < 0.05$.

RESULTS

CD.siRNA complexes, R8-PEG-DSPE-CD.siRNA complexes (5 mol% R8-PEG-DSPE) and R8-PEG-DSPE-CD.siRNA complexes (20 mol% R8-PEG-DSPE) will be referred to as MR 20, MR 20 5% R8 and MR 20 20% R8 respectively in the relevant figures. In preliminary experiments, there was little difference between CD.siRNA complexes modified with 5

mol% R8-PEG-DSPE compared to 10 mol%, therefore we eliminated the latter from further investigations.

Physical properties of R8-PEG-DSPE-CD.siRNA complexes

Firstly, we investigated whether the insertion of R8-PEG-DSPE to CD.siRNA complexes interfered with complexation, by gel electrophoresis (Fig. 1(a)). A band corresponding to free siRNA was visible in the siRNA lane only, indicating that siRNA remained fully complexed after insertion of 5 or 20 mol % R8-PEG-DSPE.

Having confirmed that siRNA remained complexed after the insertion process, size and charge measurements were carried out.

The particle sizes (Z-Ave) of CD.siRNA complexes before and after insertion of R8-PEG-DSPE were measured by dynamic light scattering. CD.siRNA complexes measured 91.5 ± 4 nm, with slight increases in sizes observed after insertion, up to 113.7 ± 4.6 nm for R8-PEG-DSPE-CD.siRNA (20 mol % R8-PEG-DSPE) (Fig. 1 (b)). Surface charge (zeta potential) of complexes was also determined. All complexes were cationic, although zeta potentials decreased from + 53.3 mV to + 38.9 mV with the inclusion of 20 mol% R8-PEG-DSPE (Fig. 1 (c)). The effect of insertion, therefore, was to slightly increase the particle size and to reduce the zeta potential.

Effects of salt-containing medium and serum on R8-PEG-DSPE-CD.siRNA complexes

Cationic particles are known to aggregate in salt-containing medium and on exposure to serum, whilst PEGylation can help to prevent these effects. Therefore, CD.siRNA complexes (MR 20) and R8-PEG-DSPE-CD.siRNA complexes (MR 20, 20% R8) were incubated in OptiMEM® or FBS for various lengths of time and the particles sizes and presence of aggregates was determined by dynamic light scattering. Incubation in a salt-environment

caused significant aggregation of CD.siRNA complexes, particularly after 12 and 72 hours (Fig. 2 (a)). On the other hand, there was no aggregation evident after incubation of R8-PEG-DSPE-CD.siRNA in OptiMEM®, indicating greater stability due to the presence of a R8-PEG-DSPE layer (Fig. 2 (a)).

The effects of serum incubation were also examined. After 24 hours incubation in FBS, both CD.siRNA and R8-PEG-DSPE-CD.siRNA complexes showed some degree of aggregation, as illustrated by a shift to the right in the size intensity profile of the FBS treated samples (Fig. 2 (b, c)). A time course of the aggregation of the complexes in serum was also carried out (Supplementary Fig. 1). This illustrated that both CD.siRNA and R8-PEG-DSPE-CD.siRNA complexes showed a similar extent of aggregation at each time point tested. The extent to which the complexes aggregated was greater for the longest incubation period (72 hours).

In designing vectors for siRNA delivery, it is also important to consider the stability of siRNA on exposure to serum nucleases (19). Indeed, in our recently published work we showed that SC12CDClickpropylamine confers protection to and prevents degradation of complexed siRNA for at least four hours, with some intact siRNA recovered after 24 hours, compared to naked siRNA which was degraded within minutes (16).

Viability of neuronal cultures after treatment with R8-PEG-DSPE-CD.siRNA complexes

Modification of the CD.siRNA formulation with R8-PEG-DSPE led to greater impairment of neuronal cell viability. CD.siRNA complexes (MR20 0% R8) caused minimal toxicity (Fig. 3 (a)), whereas complexes modified with 20 mol% of R8-PEG-DSPE reduced cell viability to ~74%. Toxicity was most pronounced with 50 mol % R8-PEG-DSPE (60.8 ± 2.9 %, $*p <$

0.05 relative to MR20 0% R8) and this formulation was, therefore, excluded from further *in vitro* investigations.

Uptake of R8-PEG-DSPE-CD.siRNA complexes

Modification of the surfaces of cationic vectors with a PEG shielding layer has been shown to reduce uptake of the nucleic acid cargo, likely due to impairing non-specific interactions with the cell membrane (50). One approach to overcoming this challenge is the linking of a ligand via the PEG groups, which can improve association with the cell membrane, whilst maintaining the stabilising effects of the PEG (51-53).

To investigate the intracellular delivery of R8-PEG-DSPE-CD.siRNA complexes, FAM-labelled siRNA was used in complexes and uptake measured by flow cytometry. High levels of uptake were achieved with the CD.siRNA formulation (MR20; 50 ± 6%). Insertion of R8-PEG-DSPE led to even higher levels of uptake, with up to 69.3 ± 0.2% of neuronal cells positive for FAM-siRNA after treatment with the MR20 20% R8 formulation (Fig. 3 (b)). Low levels of autofluoresence were equivalent for all formulations (data not shown), indicating that the increase in uptake was not due to toxicity.

Gene knockdown mediated by R8-PEG-DSPE-CD.siRNA complexes

The transfection efficiencies of PEGylated siRNA vectors are variable, with different reports depending on the cell type, nature of the vector and the type of linker to the PEG groups (20, 22, 23, 54). Therefore, we investigated the ability of R8-PEG-DSPE-CD.siRNA complexes to reduce expression of two target genes, luciferase reporter plasmid and GAPDH housekeeping gene.

A specific and significant reduction in luciferase expression was achieved with both the unmodified CD.siRNA (MR20) and the R8-PEG-DSPE-CD.siRNA (MR20 20% R8)

formulations (Fig. 4 (a)). In fact, almost 80% knockdown was achieved with the MR20 20% R8 formulation (*$p < 0.05$ relative to untreated cells). A NH_2-PEG-DSPE-CD.siRNA formulation was also included in these experiments and this did not have a significant effect on gene expression (Fig. 4(a)).In this formulation, the PEG terminated in a free amine, rather than the R8. This demonstrates that the PEG moiety in the formulation reduced gene silencing efficiency, as previously shown (21, 23).

Knockdown experiments targeting a highly expressed endogenous gene, GAPDH, were also carried out. Both unmodified CD.siRNA complexes (MR20) and MR20 20% R8 mediated a modest but significant reduction in GAPDH expression (~ 40%, *$p < 0.05$ relative to untreated controls) (Fig. 4 (b)).

In both of these studies, neither free siRNA nor non-silencing siRNA controls had any effects on the target gene expression.

These data demonstrate that the modified R8-PEG-DSPE-CD.siRNA complexes retain the gene silencing capabilities of unmodified CD.siRNA complexes, despite the presence of a steric shielding PEG layer.

Pharmacokinetic studies

Pharmacokinetic studies were carried out comparing the R8-PEG-DSPE-CD.siRNA formulation (MR20 20% R8) with free siRNA.

Mice were administered a tail vein injection of either FAM-labelled siRNA alone or complexed in a MR20 20% R8 formulation. Blood was sampled from the saphenous vein at various time points and the plasma was isolated. Plasma concentration of FAM-siRNA was determined by fluorescence. After 20 minutes, the concentration of free FAM-siRNA was below the detection limit (49), indicating that it had been rapidly eliminated from the blood

(Fig. 5). This represents a characteristic plasma concentration profile for free siRNA (49, 55). Differences were observed in the profile obtained with R8-PEG-DSPE-CD.siRNA (MR 20 20% R8), with plasma levels of formulated siRNA three-fold higher than naked siRNA at ten minutes post administration. However, the prolonged circulation observed here is modest relative to that observed with other PEGylated formulations. For example, while here we report a significant increase in half-life for formulated siRNA compared to free siRNA (Table 1, 15.7 ± 4.4 mins compared to 2.3 ± 0.9 mins), the half-life of siRNA in non-targeted neutral nanoparticles was extended to 20 hours (49). Furthermore, in a study comparing PEGylated poly-lysine-based (PLL) siRNA vectors, a formulation containing high molecular weight PEG (10,000 Da) achieved a significant prolongation of circulation time and slower clearance than the other formulations, whereas the PEG2000-PLL formulation was eliminated rapidly (56). The cationic nature of our formulation is likely to have influenced the relatively rapid clearance. For example, cationic liposomal and polymer vectors for siRNA and pDNA exhibited similar profiles to free nucleic acids after i.v. administration, due to their positive charge and subsequent opsonisation (57).

Other pharmacokinetic parameters were also calculated based on a one compartment model and these data are shown in Table I. The volume of distribution (V_d) was significantly reduced in the free siRNA group. Indeed, the difference in distribution of the two formulations is obvious from the plasma concentration profile, whereby the plasma levels differ significantly after 2 minutes (Fig. 5). The clearance (Cl) was also significantly higher for free siRNA. The parameters reported here with our CD-based formulation relative to free siRNA represent a similar trend to those reported for siRNA in non-targeted and targeted neutral nanoparticles, although the effect of our formulation is certainly less dramatic (49).

Table I. Comparison of the pharmacokinetic parameters of fluorescently-labelled siRNA alone or in an R8-PEG-DSPE-CD.siRNA formulation (MR 20, 20% R8).

	$t_{1/2}$ (min)	AUC (min.ng/µl)	V_d (ml)	Cl (ml/min)
siRNA	*2.3 ± 0.9	56.1 ± 20.3	*11.87 ± 0.97	*3.97 ± 1.17
R8-PEG-DSPE-CD.siRNA	15.7 ± 4.4	79.9 ± 19.4	31.03 ± 6.29	1.43 ± 0.37

Abbreviations: $t_{1/2}$ (half-life), AUC (area under the curve), V_d (volume of distribution), Cl (clearance). The data from the plasma concentration profiles were fitted to a one compartment model and key pharmacokinetic parameters were calculated. *$p < 0.05$ compared to R8-PEG-DSPE-CD.siRNA treated animals.

DISCUSSION

The optimisation of formulation strategies for facilitating effective siRNA-mediated gene knockdown in neurons is a crucial step in the development of RNAi-based CNS therapeutics (58). Here we have identified a novel approach for countering some of the barriers towards effective vector development. These data build on previous studies whereby a cationic amphiphilic CD, SC12CDclickpropylamine, was shown to complex siRNA, protect it from degradation on exposure to serum nucleases and mediate high levels of neuronal uptake and specific and efficient gene silencing (16). However, in accordance with its cationic nature, this CD has a high tendency to aggregate, particularly in a high salt and serum containing environment which is more representative of the *in vivo* situation. Moreover, the aforementioned delivery system lacks components for addition of targeting ligands and specificity, which could improve transfection and performance on systemic administration.

Previously, we developed a co-formulation of a PEGylated and cationic CD which displayed some improvements in stability but was not effective at mediating RNAi (21). Therefore, as an alternative approach, we now report post-modification of preformed CD.siRNA complexes with an R8-PEG-lipid conjugate. In this way, we modified the complexes with a

‘C’ shielding layer (PEG_{2000}) and a ‘D’ layer for neuronal uptake (R8) as per the ABCD nomenclature described by Kostarelos and Miller (17).

A PEG molecular weight of 2000 was selected, based on previous reports that PEG molecular weight (MW) of at least 2000 to 5000 was required to confer good stability to nanoparticles (54, 59). Our previous work showed that PEG500 was not sufficient to confer serum stability to a PEG/cationic CD co-formulation (21). Therefore, the commercially available DSPE-PEG2000 was chosen as a starting material for the synthesis of the R8 derivative. It is worth noting that even further increases in PEG MW may be required to achieve optimal stability.

As outlined in the introduction, R8 is a cell penetrating peptide which has shown effectiveness in mediating gene and siRNA delivery to neurons. Liposomes containing PEG and R8 components have been used previously for siRNA delivery (18, 60). Finally, this peptide serves as a model ligand for this type of CD–based formulation and there may be potential for other neuron-specific peptides to be used in its place.

The ‘post-modification’ method described here was based on a post-insertion approach previously optimised for liposomes (36). Insertion of R8-PEG-DSPE to CD.siRNA altered the properties of the complexes, with a slight reduction in charge and a slight increase in size (Fig. 1), indicating that some degree of transfer had taken place. A method for quantitatively assaying the amount of material which has inserted into the surface of CD.siRNA complexes remains elusive to us, due to the difficulty in separating free R8-PEG-DSPE micelles from those which have been transferred. Indeed, it has been acknowledged that determining the yield of successfully modified nanoparticles after post-insertion poses great difficulties and requires further consideration (32). With regards to the current study, the alterations in physical properties and stability of post-inserted complexes imply that the complex surfaces were to some extent modified with the R8-PEG-DSPE.

A more pronounced reduction in zeta potential was anticipated, due to the shielding effects of the high MW PEG component. In fact, a previous study has shown that incorporation of DSPE-PEG into liposomal formulations significantly reduced the surface charge (61). However, our R8-PEG-DSPE-CD.siRNA complexes remained positively charged, which may be attributed to the cationic nature of the attached R8 (zeta potential of R8-PEG-DSPE alone was ~ 20mV, data not shown). In spite of their positive charge, the post-inserted complexes (MR20 20% R8) were resistant to aggregation in a salt-containing medium. However, there was an increase in complex sizes after incubation in serum. A further increase in PEG MW (up to 5000 Da) may be required to prevent serum-induced aggregation (54).

Inclusion of a PEG component has often been associated with a drop in transfection efficiency (22, 23). Indeed, in our previous studies, inclusion of a PEGylated CD in the CD.siRNA formulation eliminated gene silencing capabilities (21). This was due to a reduction in cellular uptake. However, in the current study there was a small but significant increase in uptake with 20% R8 in the R8-PEG-DSPE-CD.siRNA formulation. This occurred despite a reduction in surface charge, indicating that the R8 component facilitated transfer across the cell membrane, which counter-balances the shielding effect of PEG. For this reason, CD.siRNA complexes with 20% R8-PEG-DSPE and unmodified CD.siRNA complexes achieved equivalent gene silencing. The levels of gene knockdown were similar to those achieved with a commercial vector, Lipofectamine™ 2000 (16).

Finally, in a preliminary pharmacokinetic investigation we assessed the plasma concentration profiles of free siRNA compared to siRNA in the MR20 20% R8 formulation. Typically, following i.v. administration free siRNA is rapidly eliminated and has a short half-life, ranging from seconds to minutes (49, 62), as observed in our study. Although the MR20 20% R8 formulation altered the plasma concentration profile of siRNA and increased its half-life by several minutes, these were limited effects. Taken together with the tendency of the

formulation to aggregate on exposure to serum, these data indicate that the PEG component did not confer sufficient steric stability to the formulation. Neutral PEGylated vectors achieve a significant prolongation of siRNA circulation and ultimately represent a more desirable approach (49). It is worth noting that a comprehensive biodistribution analysis is also necessary to fully assess the utility of targeted gene delivery vectors and such studies, coupled with *in vivo* toxicity studies, will be required for further *in vivo* development of the formulation.

The approach described herein offers itself as proof of principle for attaching other PEG-targeting ligands to neutral CD.siRNA complexes. Indeed, such a system offers great prospects for application to the CNS, with many favourable properties including the small sized complexes and the high levels of transfection efficiency demonstrated in neurons. Although the R8 peptide may not be the most feasible ligand for CNS delivery, using this approach, either transferrin or the rabies virus glycoprotein peptide, which enhance neuronal siRNA delivery and transfer across the blood brain barrier, could be considered as alternative ligands for future development (63-65).

CONCLUSIONS

In summary, we successfully modified the CD.siRNA formulation, with the addition of R8-PEG-DSPE by to preformed complexes, resulting in a suitable formulation for neuronal delivery. Properties of the new formulation were improved, including a reduction in surface charge and significant resistance to salt-induced aggregation. High levels of neuronal uptake *in vitro* and subsequent silencing of two target genes were achieved. Cell viability was maintained at greater than 70% after treatment with the new formulation. Limitations of the current formulation include its cationic nature, tendency to aggregate in serum and lack of

specificity, rendering it less useful for long-term *in vivo* use. Indeed, the development of a neutral PEGylated formulation with a targeting ligand is the ultimate goal for siRNA-based therapeutics.

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Figures

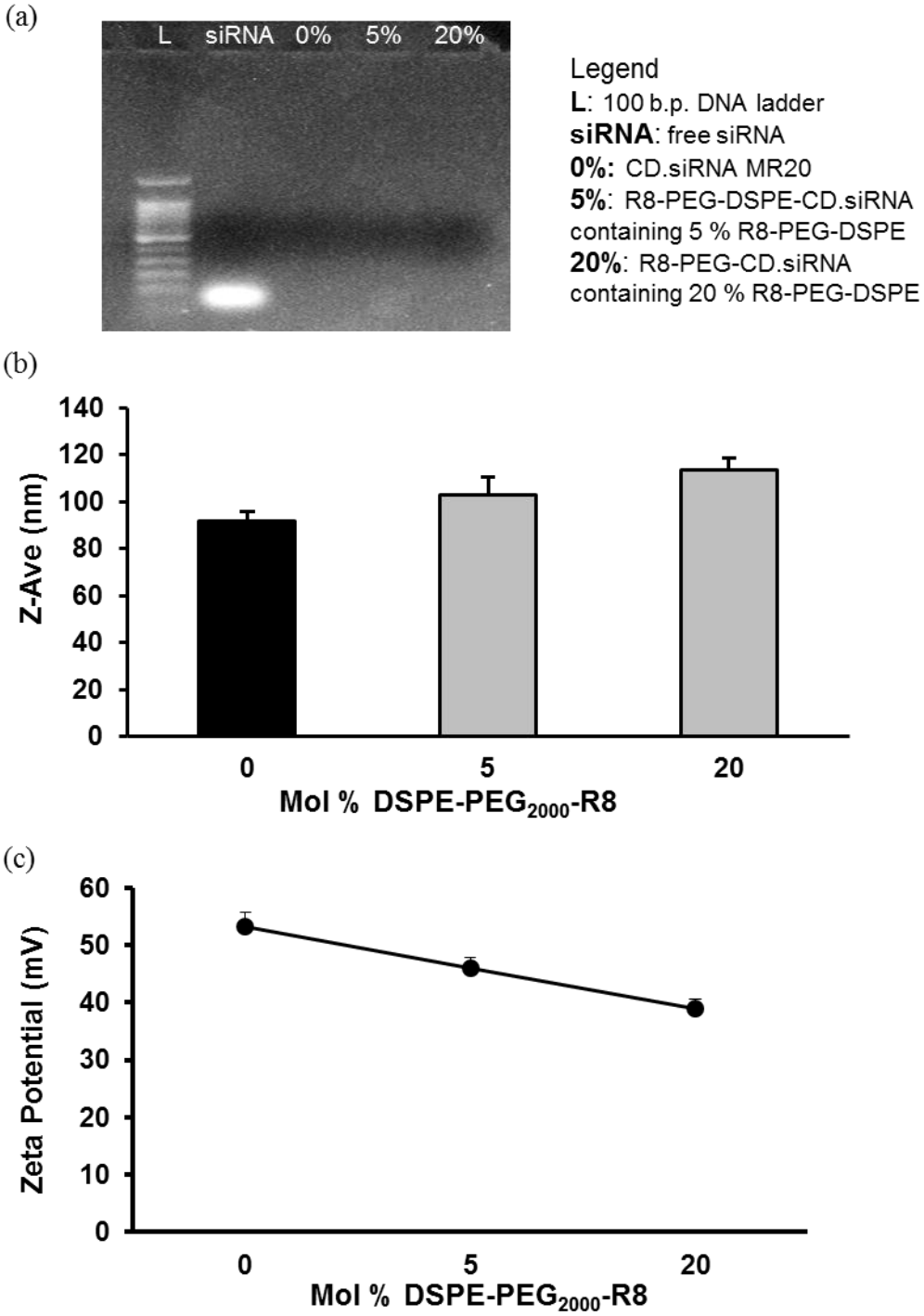


Figure 1. Physical properties of R8-PEG-DSPE-CD.siRNA complexes. (a) Gel retardation assay examining siRNA binding properties, (b) Size (Z-Ave (nm)) and (c) charge (zeta potential (mV)) of CD.siRNA and R8-PEG-DSPE-CD.siRNA complexes in DIW. Data are presented as the mean ± S.D (n = 3).

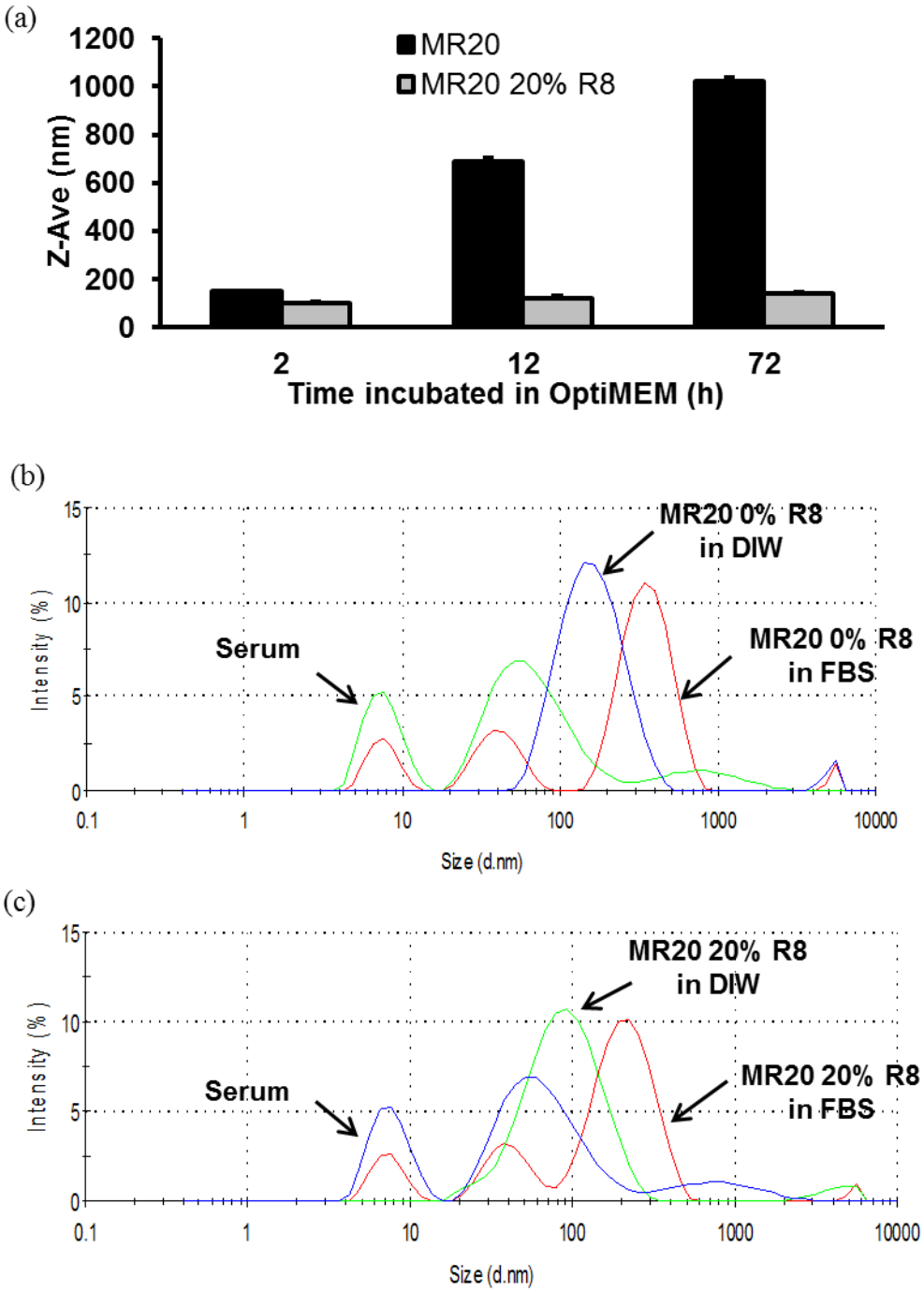


Figure 2. Stability of CD.siRNA (MR20) or R8-PEG-DSPE-CD.siRNA (MR20 20% R8) complexes after incubation in (a) OptiMEM® for 2, 12 or 72 hours or (b) and (c) 90% FBS for 24 hours.

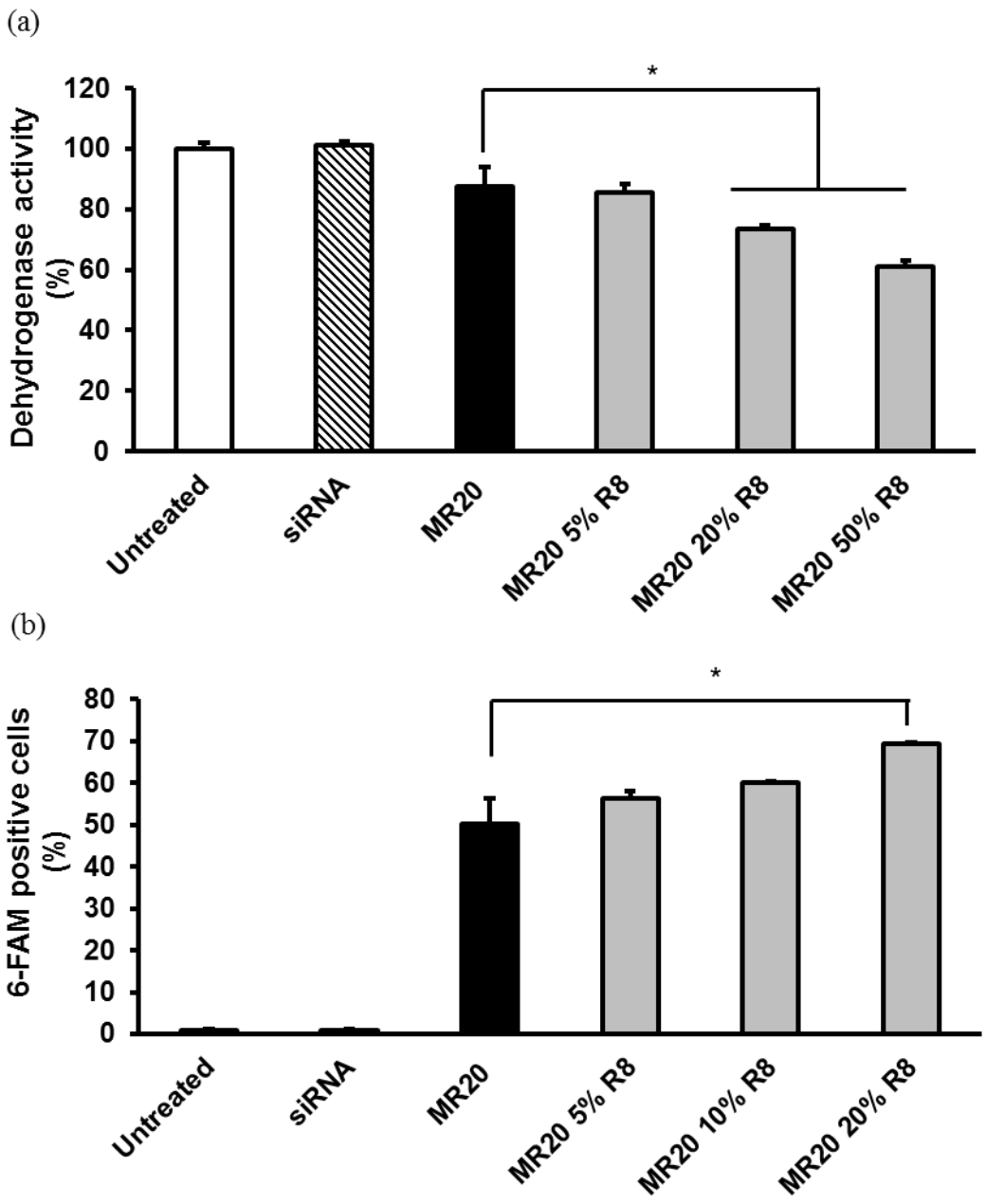


Figure 3. (a) Toxicity in mHypoE N41 neuronal cells was determined by MTT assay after treatment with CD.siRNA (MR 20) or R8-PEG-DSPE-CD.siRNA (MR 20 20% R8) complexes (100 nM siRNA) for 24 hours. (b) Uptake of CD.siRNA (MR 20) or R8-PEG-DSPE-CD.siRNA (MR 20 20% R8) complexes (50 nM siRNA) in mHypoE N41 neurons. Cells were treated for 24 hours before measuring uptake by flow cytometry. Uptake was expressed as percentage of fluorescent siRNA positive cells. Data are expressed as mean ± SEM (n = 3). *$p < 0.05$ relative to unmodified CD.siRNA complexes (MR 20).

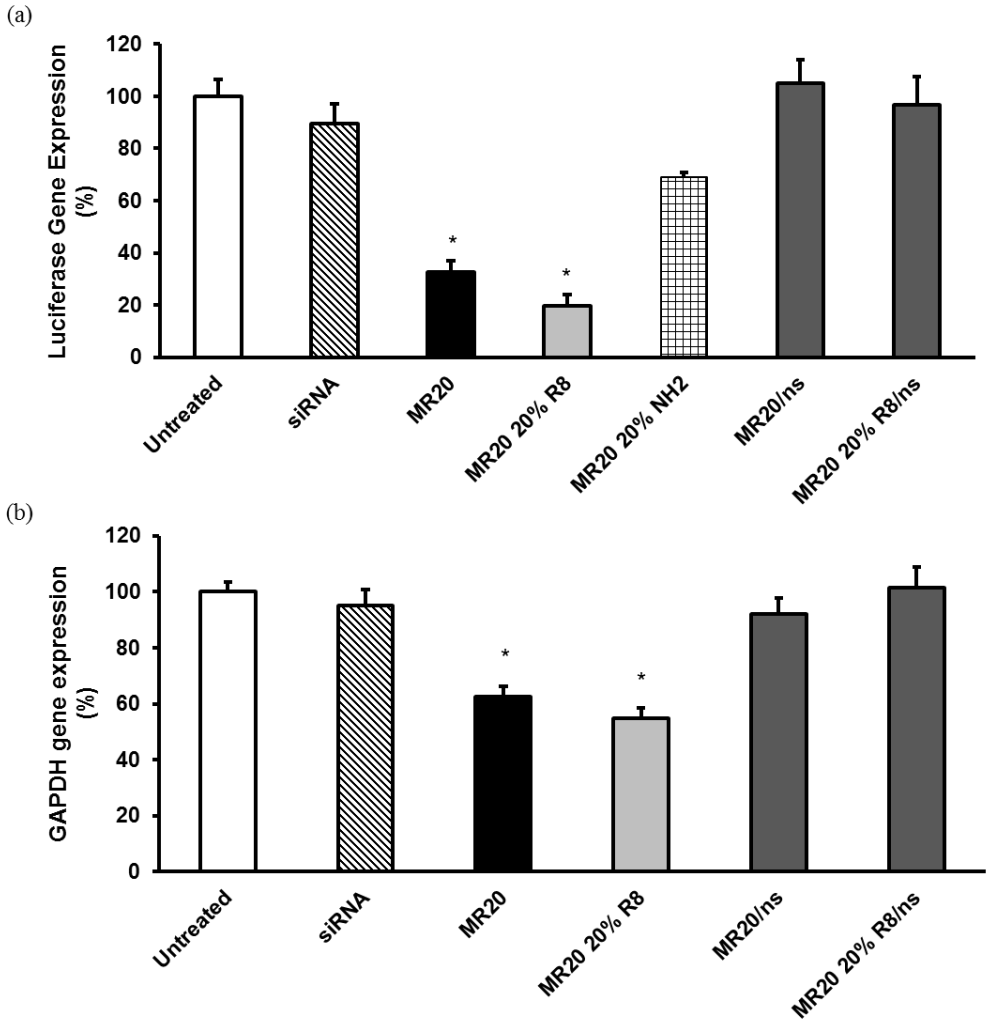


Figure 4. (a) Knockdown of luciferase reporter gene in mHypoE N41 neurons by CD.siRNA (MR 20) or R8-PEG-DSPE-CD.siRNA (MR 20 20% R8) complexes (50 nM siRNA). Luciferase expression was calculated as a percentage of RNAi- untreated cells. NH_2-PEG-DSPE-CD.siRNA (MR 20 20% NH_2) complexes were included as a control lacking in R8. (b) Knockdown of endogenous GAPDH in mHypoE N41 neurons by CD.siRNA or R8-PEG-CD-DSPE.siRNA complexes (100 nM siRNA). GAPDH expression was calculated as a percentage of RNAi-untreated cells. Data are expressed as the mean ± SEM (n = 3). $*p < 0.05$ relative to untreated controls.

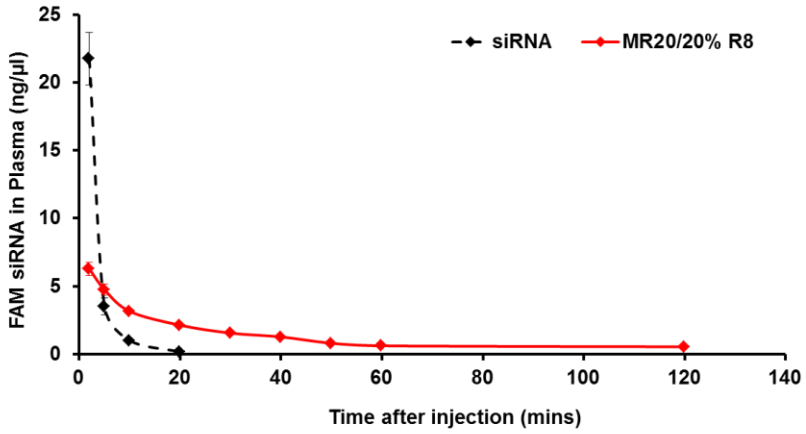
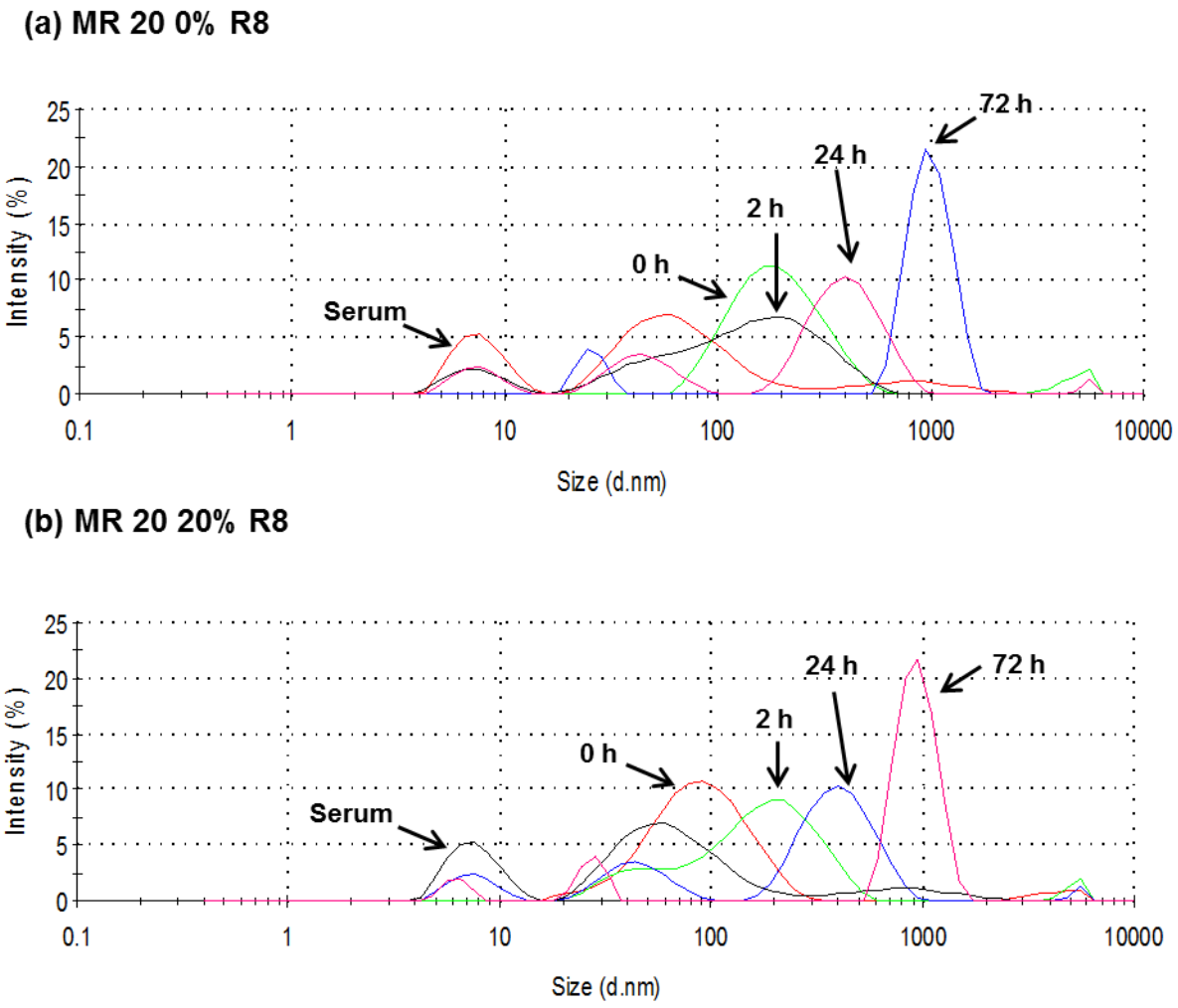


Figure 5. Plasma concentration profiles of FAM-labelled siRNA alone, or formulated with R8-PEG-DSPE-CD.siRNA (MR20 20% R8). Data are presented as mean ± S.E.M (n= 3-5).



Supplementary Figure 1. Size intensity profiles of (a) MR 20 0% R8 and (b) MR 20 20% R8 after incubation in FBS for 0, 2, 24 and 72 hours.

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