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Click-modified Cyclodextrins as Non-viral Vectors for Neuronal siRNA Delivery

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ABSTRACT: RNA interference (RNAi) holds great promise as a strategy to further our understanding of gene function in the central nervous system (CNS) and as a therapeutic approach for neurological and neurodegenerative diseases. However, the potential for its use is hampered by the lack of siRNA delivery vectors, which are both safe and highly efficient. Cyclodextrins have been shown to be efficient and low toxicity gene delivery vectors in various cell types *in vitro*. However, to date they have not been exploited for delivery of oligonucleotides to neurons.

To this end, a modified β-cyclodextrin (CD) vector was synthesised, which complexed siRNA to form cationic nanoparticles of less than 200nm in size. Furthermore, it conferred stability in serum to the siRNA cargo. The *in vitro* performance of the CD in both immortalised hypothalamic neurons and primary hippocampal neurons was evaluated. The CD facilitated high levels of intracellular delivery of labelled siRNA, whilst maintaining at least 80% cell viability. Significant gene knockdown was achieved, with a reduction in luciferase expression of up to 68% and a reduction in endogenous glyceraldehyde phosphate dehydrogenase (GAPDH) expression of up to 40%. To our knowledge, this is the first time that a modified CD has been used as a safe and efficacious vector for siRNA delivery into neuronal cells.

KEYWORDS: *nanotechnology, cyclodextrins, click chemistry, siRNA delivery, neurons, gene knockdown*

INTRODUCTION

RNA interference (RNAi) has advanced rapidly since it was first discovered in mammalian cells[1] to clinical application and most recently, the first evidence of systemic administration of a targeted non-viral siRNA delivery system to humans was reported[2]. RNAi is a naturally occurring biological process which results in specific silencing of a target gene by short sequences of RNA known as small interfering RNAs (siRNAs),[3] with a subsequent reduction in the expression of the corresponding protein. Introduction of chemically synthesised siRNA into cells to reduce expression of a specific gene can be used to determine gene function or for therapeutic purposes.

However, delivery of siRNA proves a constant challenge and there are specific issues which have to be addressed when delivering to neurons and the central nervous system (CNS).[4] Neurons are notoriously difficult to transfect for reasons that have yet to be fully understood, although it is likely to be partly related to their post-mitotic nature.[5] There are many barriers to siRNA delivery to the CNS including neuronal uptake, vesicular escape and, not least, the blood-brain barrier.[6] Appropriately designed delivery systems are therefore required in order to mediate neuronal siRNA delivery and gene silencing. Viral vectors have been extensively reported and achieve efficient transfection.[7] However, non-viral vectors are more recently the focus of research to achieve a safer means of transfection. To date, there are limited reports of successful neuronal delivery of siRNA with these. The most widely used are cationic lipid based vectors, in particular Lipofectamine™ 2000 (Invitrogen), which has been shown to mediate effective gene knockdown in primary neurons,[8-10] with up to 70% reduction in gene or protein expression achieved.[9] However, such vectors are also associated with considerable toxicity in primary cells, particularly neurons.[11-13] Therefore, there remains a need for the development of alternative less toxic vectors for siRNA and gene delivery to neurons.

Cyclodextrins (CDs) are naturally occurring oligosaccharides and are well characterised pharmaceutical excipients with a favourable toxicological profile.[14] The potential of CDs as delivery agents for therapeutic oligonucleotides is well established.[15, 16] Chemical modification of CDs has yielded monodisperse non-viral gene delivery vectors.[17-21] Modifications of the basic β-CD structure, including introduction of cationic and/or amphiphilic moieties have resulted in a group of novel vectors which can complex pDNA and efficiently deliver it to various *in vitro* cell culture models, including liver (Hep G2) cells[22, 23] and undifferentiated and differentiated intestinal epithelial (Caco2) cells.[24] A novel synthetic approach, involving the application of cuprous-catalysed click chemistry to modify the 2-hydroxyl position of β-CD with polar groups was recently reported.[25] An amphiphilic cationic β-CD with hydrocarbon chains (C_{12}) on the primary face and polar (propylamino) groups on the secondary face was synthesised in this way. This CD achieved high levels of gene silencing in Caco2 cells.[25]

Given the ease with which they can now be chemically modified and their low toxicity and efficiency in non-neuronal cells, it is perhaps surprising that the utility of such CDs for neuronal delivery remains to be exploited. Therefore, in this study we investigate the aforementioned cationic amphiphilic CD for siRNA delivery in hippocampal and hypothalamic neurons.

RESULTS AND DISCUSSION

The vast potential for RNA interference technology in terms of identifying new targets in many neurological diseases, as well as offering an alternative therapeutic strategy, warrants research into the development of improved and low toxicity vectors for delivering siRNA. Developments in the chemistry of such vectors are facilitating major biological advances and in this study, we demonstrate that a novel chemically modified CD can be used for neuronal siRNA delivery. The CD chemical structure is shown in Figure 1 (a). The synthesis of this CD was previously reported.[25] Briefly, β-CD was substituted on the primary side with lipophilic chains (C_{12}) by thioalkylation.[18, 26] Introduction of amphiphilic moieties improves the transfection efficiency of cationic CDs.[18, 22] Functionalisation at the 2-position was by means of a copper-catalysed 'click' reaction, which represents an efficient and versatile strategy for modifying the secondary side with diverse groups, including cationic groups and polyethyleneglycol (PEG) chains. Click chemistry has been previously applied to modify the primary face of CD-based gene delivery vectors,[27, 28] but the selective modification at the 2-hydroxyl presented a greater challenge.[19] A schematic representation of the CD is shown in Figure 1(b). This CD has shown promise as a non-viral siRNA delivery vector in non-neuronal cells.[25]

Other non-viral methods for siRNA delivery have been reported in recent years, including cationic lipids, polymers and cell-penetrating peptides (CPPs). Cationic lipids and polymers, although highly efficacious, exhibit considerable toxicity[29]. CPPs, on the other hand, exert minimal toxicity and have been successfully used for siRNA delivery to primary hippocampal neurons[12], primary cortical neurons[30] and the hippocampus[31] *in vivo*. However, additional non-viral delivery systems would be desirable and to this end, modified CDs offer great potential. In particular, the chemical synthesis for the CD used herein can be also applied for functionalisation of CDs with PEG groups for improved stability[25]. Moreover,

there is potential to extend this chemistry to allow for the attachment of neuronal and CNS targeting ligands. Therefore, we believe that exciting prospects exist for the application of modified CDs to neuronal RNAi**.**

To determine the suitability of the modified CD as a siRNA delivery vector, several physical properties including the ability to complex siRNA, size and charge of CD.siRNA complexes and protection of siRNA from serum degradation were assessed. Following this, *in vitro* experiments in neurons showed that the CD successfully mediated efficient uptake of siRNA and knockdown of reporter and housekeeping genes.

In the first instance, CD.siRNA complex formation was identified using agarose gel electrophoresis. Uncomplexed siRNA was used as a control and migrated freely through the gel. This was compared with CD.siRNA complexes, which were formed at increasing mass ratios (MRs; µg CD: µg siRNA). The positive charges from the primary amine group in the CD enable association with the negatively charged phosphate groups of the siRNA backbone.[32] Complete binding of siRNA appeared to occur abruptly at MR5, with no migration of siRNA seen at MR 5 or MR10 (Fig.2 (a)), demonstrating that the negatively charged siRNA had been effectively complexed by the CD at these MRs. Furthermore, there was no fluorescence apparent in the wells of the CD.siRNA lanes, indicating that complexed siRNA was not accessible to the intercalating agent ethidium bromide.

Secondly, the physicochemical properties of siRNA delivery vectors, which are well known to impact on their transfection efficiency, were examined. Key factors which can affect transfection include the size of the complexes formed with siRNA and their surface charge.[33, 34] The particle size of CD.siRNA complexes, measured using the Malvern ZetaSizer Nano particle size analyser, was dependent on MR (Fig.2 (b)). Although siRNA was shown to be fully bound at MR5, these complexes were very polydisperse and size and charge data were

therefore excluded. Complexes produced at MR10 were 218 ± 19.9 nm, whereas those complexes produced at MR20 and MR30 were 136.9 ± 21.9 nm and 130.4 ± 22.3 nm respectively. These sizes fall within the nanometre range required for cellular uptake by endocytic processes.[33]

The polydispersity index (PDI) was also noted for each size measurement, with PDI generally in the range 0.2 to 0.4, indicating some variation in the size distribution.[17] The particle sizes were stable when stored for up to six months at 4ºC, with no aggregation or change in appearance observed (data not shown).

The surface charge of CD.siRNA complexes was also measured and was similiarly dependent on MR. Complexes were cationic at all MRs, with charge (zeta potential) increasing as the MR increased from +42.2 ± 7.9 mV at MR10 to + 60.2 ± 3.3 mV at MR30, that is increasing the amount of cationic CD relative to siRNA resulted in more highly positively charged complexes. The cationic nature of the complexes formed allows association with the negatively charged proteoglycans on the surface of the cell membrane.[24]

Furthermore, stability of siRNA in serum is an important feature when considering future *in vivo* experiments. Agarose gel electrophoresis was used to characterise the protective effects of CDs on siRNA degradation by serum (Fig. 2(c)). Uncomplexed siRNA, shown in Lanes 2-5, was partially degraded after less than 30 seconds exposure to serum and completely degraded after 0.5 to 24 hours exposure. In contrast, siRNA in CD complexes (Lanes 6-11), which was released from the complexes by heparin displacement after serum exposure, remained intact for up to 4 hours which is comparable to the protection conferred by other vectors, including liposomes.[35] Even after 24 hours, the CD prevented complete degradation of siRNA.

Having confirmed that the physical properties of the CD vector were suitable for siRNA delivery, *in vitro* testing was carried out in a mouse embryonic hypothalamic neuronal cell line, mHypoE N41[36] and in primary rat embryonic (E18) hippocampal neurons.

Firstly, the cytotoxic effects of CD.siRNA complexes on neuronal cell cultures were determined by MTT assays, after cells were treated with CD.siRNA complexes for 24 hours, using 50 nM or 100 nM siRNA. Overall, a favourable toxicity profile for this vector was observed in both cultures, although the primary neurons were more susceptible to CD.siRNA mediated toxicity when compared to the immortalised N41 cells. Mitochondrial dehydrogenase activity compared to untreated neurons was used as an indicator of toxicity.

In N41 cells, CD.siRNA complexes had no effect on dehydrogenase activity at 50 nM siRNA (Fig. 3 (a)). After 24 hours treatment at 100 nM siRNA, cell viability was unaffected at MR10 and was maintained at 88 ± 1.5 % at MR20 (Fig. 3 (a)). Only at MR30 was there a significant reduction, corresponding to minor cytotoxicity (80 ± 0.9 %, $*p < 0.05$ relative to untreated controls).

Meanwhile, in primary hippocampal neuronal cultures, siRNA alone did not cause any cytotoxicity (Fig. 3 (b)) but some reduction in cell viability occurred after treatment but after treatment with CD.siRNA complexes at 50 nM siRNA, dehydrogenase activity was reduced to 88 ± 1.6 % ($*p < 0.05$) at MR10 and 83 ± 0.4 % ($*p < 0.05$) at MR20. At 100 nM siRNA, however, there was no further decrease in cell viability.

Trypan blue exclusion assays were also carried out as an alternative measure of cytotoxicity (Fig. 3 (c, d)). These data confirm the favourable cytotoxicity profile of CD vectors in both immortalised and primary neurons.

Neuronal cells, in particular primary neuronal cells, are quite susceptible to the toxic effects of cationic vectors. For example, in primary cortical cultures, layered double hydroxide (LDH) nanoparticles caused a 20-30% reduction in viability[37] and in another study, toxicity with Oligofectamine (~80%) and with polyethyleneimine (PEI; ~76%) was reported.[38] In primary hippocampal neurons, stearylated octaarginine and artificial virus-like particles reduced cell viability to 73% and 75% respectively.[39] The data presented here showed that the modified CD did not induce significant toxicity in the hypothalamic neuronal cell line at MR10 or MR20 (Fig. 3 (a)) and caused limited impairment of viability of the primary hippocampal neurons (80%, Fig. 3(b)). Under similar experimental conditions, Lipofectamine™ 2000 was found to be considerably more toxic to primary hippocampal neurons than the CD vector, reducing viability to 61.8 ± 3.5 % (Fig. 3(b)), in the MTT assay. This was comparable to previously reported toxicity of Lipofectamine™ 2000 in primary hippocampal cultures.[12]

Secondly, cellular uptake of CD.siRNA complexes was assessed by flow cytometry. For these experiments, 6-FAM labelled siRNA was used, at a concentration of 50 nM. Fig. 4 (a) shows the percentage of 6-FAM positive cells in N41 cultures, after treatment with CD.siRNA complexes at increasing mass ratios from MR5 to MR20, for either 4 or 24 hours. No uptake was observed when cells were treated with uncomplexed siRNA at either time point. After 4 hours treatment, low levels of uptake were seen, ranging from 3.5 ± 1.2 % at MR5 to 8.8 ± 3.6 % at MR20. However, a highly significant increase in uptake was observed after 24 hours treatment, with the highest level at MR20 (54.6 ± 6.1 %, $*p < 0.05$ relative to untreated controls). One possible explanation for this delayed uptake is slow settling of the CD.siRNA complexes onto the adherent cells. The inclusion of a triazole moiety in a polycationic amphiphilic CD was previously reported to improve stability in a high salt environment.[21] Aggregation can enhance transfection *in vitro* as the aggregates are quick to

settle down onto adherent cells for association and uptake.[40, 41] Therefore, delayed uptake may result when aggregation occurs more slowly.

Although aggregation may aid cellular association *in vitro*, this is not the case *in vivo* and methods to improve formulations to prevent aggregation are required to enable prolonged circulation and prevent opsonisation.

Significant levels of uptake were also achieved when primary hippocampal neurons were treated with CD.siRNA complexes for 24 hours (Fig. 4 (b)). The percentage of 6-FAM positive cells increased from 37.7 ± 1.8 % at MR10 to 53.5 ± 2.3 % at MR20.

In both neuronal cultures, the level of uptake achieved with MR20 was significantly greater than that at MR10 with the same concentration of siRNA. Lipofectamine™ 2000 mediated similar levels of uptake in both neuronal cell models (Fig. 4).

After delivery into neurons, it is critical that siRNA is made available for RNAi by incorporation into the RISC complex in the cytoplasm, to enable downstream gene silencing. To this end, the ability of the CD to deliver luciferase siRNA into neuronal cells and reduce luciferase gene expression was investigated. Given that significantly greater uptake was achieved after 24 hours, this was chosen as the appropriate length of time for transfection experiments.

Firstly, N41 cells were transfected for two hours with the firefly luciferase reporter plasmid by cationic lipid mediated delivery (Lipofectamine™ 2000). Subsequently, cells were treated for 24 hours with CD.siRNA complexes against the luciferase gene. Non-silencing siRNA complexed to CD was used as a negative control. Luciferase expression levels decreased after treatment with MR10 and MR20 (Fig. 5), with a significant reduction achieved at MR20 (68.5 ± 7%, $*p < 0.05$ relative to untreated control). Transfection with uncomplexed siRNA did not have any effect on luciferase expression. Similarly, CD complexes with non-silencing (ns) siRNA did not reduce luciferase expression, demonstrating the specificity of knockdown.

These data confirm that effective and specific gene silencing was achieved when siRNA was delivered to neuronal cells by the CD.siRNA delivery system.

Having achieved knockdown of an exogenously transfected gene with CD.siRNA complexes, the ability of the vector to mediate knockdown of an endogenous housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed. Preliminary experiments in N41 cells showed that when siRNA was used at a concentration of 50 nM, CD.siRNA complexes had no effect on GAPDH expression. Therefore, subsequent experiments were carried out using 100 nM siRNA. GAPDH siRNA and a negative scrambled control siRNA were used. Neurons were treated for 24 hours and GAPDH mRNA levels were quantified by qRT-PCR, after extraction of total RNA from the treated cells. These data are shown in Fig. 6 (a) and (b). In N41 cells (Fig. 6 (a)), at MR20 a significant reduction in GAPDH expression was achieved (38% ± 9.3 %, $*p < 0.05$ relative to untreated control). Meanwhile, the complexes formed at MR10 did not reduce GAPDH expression under these experimental conditions. Fig 6 (b) shows a similiar level of knockdown in the primary hippocampal neurons (41% ± 5.7%, $*p < 0.05$ relative to untreated control). Neither non-silencing siRNA formulated with the CD nor uncomplexed siRNA reduced GAPDH expression, confirming specificity of gene silencing and the lack of effectiveness of naked siRNA.

The level of GAPDH knockdown reported here was moderate and although there is some possibility that this may be due to incomplete dissociation of siRNA from the CD, the reduction in GAPDH gene expression achieved with Lipofectamine^{TM} 2000 was not significantly different (~ 40%, Fig. 6 (b)). Furthermore, similar levels of knockdown have been reported for other endogenous gene targets in neuronal cells. For example, a modest reduction in HMGB-1 mRNA (<40%) was achieved in primary cortical neurons using PAMAM-Arginine vector to deliver shRNA[13] and a stearylated octaarginine vector mediated

~ 50% reduction in reporter gene expression in primary hippocampal neurons[39]. Furthermore, Lipofectamine™ 2000 has been reported for siRNA mediated knockdown in N-1 hypothalamic neurons, with varying levels of knockdown depending on the target. Reductions in levels of resistin mRNA of 25%[42] to 55 – 60%[42, 43] were reported, depending on the siRNA sequence. However, other RNAi delivery methods have achieved more pronounced gene silencing effects. For example, > 80% reduction in target protein expression was reported for Penetratin-1-siRNA conjugates in primary hippocampal neurons[12] and in the hippocampus *in vivo*[31]. More substantial levels of knockdown (~90%) of another target, CCAAT enhancer binding protein α, were achieved using Lipofectamine™ 2000 in N-1 neurons.[44] In this study, similar experimental conditions to those reported in this paper were used, however, 'stealth' siRNA sequences, which are chemically modified RNA duplexes, with improved stability and transfection efficiency, were used which may explain the greater knockdown levels achieved.

It is also worth noting that a moderate reduction in target gene expression may be sufficient for a functional output in an animal model[45-47]. For example, a 30% decrease in striatal tyrosine hydroxylase was sufficient to reduce the hyperactive locomotor response to amphetamine in mice[46] and anti-depressant-like behaviours were observed in mice, after i.c.v. infusion of siRNA led to a 40% reduction in expression of the serotonin transporter[47].

In summary, an amphiphilic cationic modified β-cyclodextrin was shown to deliver siRNA to neurons for gene silencing. Efficient uptake into neuronal cells was shown, with a favourable cytotoxicity profile. Moreover, significant levels of knockdown were achieved for an exogenous gene (luciferase) and an endogenous gene (GAPDH). The physical properties of the complexes formed with siRNA and the ability of the CD to protect siRNA from serum degradation were key features in its efficiency as a delivery vector. Furthermore, future studies focused on optimising the structural modifications applied to CDs can now be carried

out having established the potential of this CD as a novel non-viral vector for neuronal siRNA delivery.

METHODS

Cell culture. *mHypoE N41*: A mouse embryonic hypothalamic cell line[36] 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.

Primary neuronal culture: Primary rat embryonic hippocampal neurons were obtained from Sprague-Dawley rats, at day 18 of gestation as previously described.[11] Briefly, the animals were sacrificed and the embryos removed. Embryonic hippocampal neurons were dissected out and plated onto poly-l-lysine coated coverslips (for immunofluoresence), or poly-l-lysine treated wells (for all other experiments), in 12 well or 96 well plates and maintained in Neurobasal medium (Gibco), supplemented with 2% B-27 (Invitrogen) and 0.5mM L-glutamine (Sigma). Cells were maintained in a humidified 37^{o}C incubator with 5% CO_2. Seeding densities were 5x 10^5 and 1.5 x 10^4 for 12- and 96- well plates respectively. Characterisation of the cultures by immunostaining for β III tubulin and GFAP confirmed the presence of 90-95% neurons in the culture.

siRNAs. siRNAs were synthesised by Qiagen. siRNA sequences are shown in Table 1.

	SENSE	**ANTISENSE**
6FAM negative control siRNA	UUCUCCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT
Negative control siRNA	UUCUCCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT
Luciferase GL3 siRNA	CUUACGCUGAGUACUUCGAdTdT	UCGAAGUACUCAGCGUAAGdTdT
Anti-mouse GAPDH siRNA	GGUCGGUGUGAACGGAUUUdTdT	AAAUCCGUUCACACCGACCdTdT
Anti-rat GAPDH siRNA	GGUCGGUGUGAACGGAUUUdTdT	AAAUCCGUUCACACCGACCdTdT

Table 1. Sequences of siRNA obtained from Qiagen.

Preparation of CD.siRNA complexes. An amphiphilic cationic CD was synthesised as previously described[25] and was dissolved in chloroform to give a 1 mg/ml solution. A stream of nitrogen was then applied to remove the solvent, leaving a CD film, which was rehydrated with water (CD concentration 1 mg/ml), followed by sonication at room temperature (RT) for one hour for vesicle size reduction. A solution of siRNA in water was then mixed with CD solution (equal volumes) and complexed for 15-20 minutes at RT (final CD concentration of 0.5 mg/ml). Different mass ratios (MR) of CD to siRNA were chosen (µg CD:µg siRNA). This 'mixing method' for CD.siRNA complex preparation was previously optimised for CD.DNA complexes.[17, 22, 24]

Preparation of Lf2000.siRNA complexes. Lipofectamine™ 2000 (Invitrogen) (Lf2000) is a cationic lipid-based transfection reagent. Lf2000.siRNA complexes were prepared as per the manufacturer's protocol. Briefly, the required volume of Lf2000 was diluted in 50 µl OptiMEM®, mixed gently and incubated at RT for 5 mins. siRNA was diluted in 50 µl OptiMEM® and combined with the diluted Lf2000, then mixed gently and incubated at RT for 20 mins. 1 µl of Lf2000 was used per 20 pmol of siRNA.

Size and Charge Measurements. Particle size and charge were measured with Malvern's Zetasizer Nano ZS, using laser-light scattering and electrophoretic mobility measurements respectively.[24, 48] CD.siRNA (3µg siRNA) complexes were prepared by the 'mixing method'. The resulting mixtures were made up to 1 ml with 0.2 µm filtered deionised water (DIW). Five readings of Z-average size (nm), polydispersity (25^{o}C, measurement angle 170^{o}) and zeta potential (mV) (25^{o}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. The data are presented as Mean ± S.D.

Gel Retardation Assay. Agarose gel electrophoresis was used to determine the binding of siRNA. CD.siRNA complexes were prepared and mixed with loading buffer and DIW to a final volume of 20µl (containing 0.3µg siRNA). Samples were added to wells in a 1% agarose gel containing ethidium bromide (0.5µg/ml). Electrophoresis was carried out at 90V for 20 minutes, with a Tris-borate-EDTA buffer.[49] 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.

Serum Stability Studies. To evaluate protection of siRNA from serum nucleases by the CD, CD.siRNA complexes were incubated with 50% FBS for 30s, 0.5h, 4h or 24h at 37ºC, followed by addition of excess heparin to displace the siRNA.[50] Uncomplexed siRNA samples were also incubated with serum in the same way. Samples were analysed by agarose gel electrophoresis (1.5% agarose gel) as described above.

Toxicity Assays. *MTT Toxicity Assay:* The MTT assay is widely used as an indicator of the toxicity caused to neurons by non-viral vectors.[13, 37, 38, 51] 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, therefore, a direct measure of cell viability. Cells were seeded in 96-well plates for 1 day before transfection then were treated with CD.siRNA complexes for 24 hours. Medium was removed, and replaced with 100 µl fresh medium and MTT reagent (20µl of a 5mg/ml solution) for two hours, after which the formazon crystals produced were dissolved in 100µl dimethylsulfoxide. Absorbance was measured at 590nm using a UV plate reader. Each experiment was carried out in triplicate. Results were expressed as % dehydrogenase activity

compared to untreated controls. The data are presented as the Mean ± SEM. *Trypan Blue Exclusion Assay:* Cell viability was also assessed using trypan blue exclusion analysis as previously reported.[37, 52] Briefly, cells were seeded in 24-well plates for 1 day before transfection then CD.siRNA complexes (100 nM siRNA), diluted in OptiMEM®, were applied in serum-containing medium, for 24 hours. Cells were detached with trypsin-EDTA (0.25%) and stained with 0.02% trypan blue. Viable cells were counted and results were expressed as % viable cells compared to untreated controls. The data are presented as the Mean ± SEM.

Cellular uptake experiments. The level of uptake mediated by transfection complexes was assessed by flow cytometry.[37, 53, 54] Fluorescently labelled (6FAM) siRNA (Qiagen) was used for these experiments. Cells were seeded in 24-well plates for 1 day before transfection. siRNA (50nM) alone or in CD.siRNA complexes was diluted in OptiMEM® and added to cells in serum-containing medium, for 4 or 24 hours. Following this, uninternalised complexes were removed by washing cells with PBS and by incubation with 250 µl of CellScrub buffer for 15 minutes at room temperature.[48] 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 was analysed using Cell Quest Pro software. Each experiment was carried out in triplicate. The data are presented as the Mean ± SEM.

Knockdown of luciferase reporter gene. Silencing of an exogenous gene was assessed by measuring knockdown of the firefly luciferase gene[51, 55, 56] Cells were seeded in 24-well plates for 1 day before transfection. Cells were transfected with luciferase reporter plasmid, pGL3 luciferase (1 µg/well) complexed to Lipofectamine 2000® (2.5 µl/ µg pDNA), for two

hours. Following this, cells were washed twice with PBS prior to siRNA transfection. siRNA (50 nM) alone, or complexed to CD, was diluted in OptiMEM® and added to the cells in serum-containing medium. Non-silencing siRNA complexed to CD was used as a control. After 24 hours, cells were washed with PBS and lysed. Lysate (20 µl) was assayed for expression of luciferase by adding to 100 µl of luciferin (Promega) and measuring the luminesence in a Junior LB 9059 luminometer (Promega). Total protein levels in each sample were determined by the BCA Protein Assay (Thermo Scientific) to allow normalisation of luciferase activity. Results were expressed as % gene expression relative to control (no RNAi) samples. 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 GAPDH. This gene is widely used to assess knockdown efficiency in *in vitro* studies.[57, 58] Cells were seeded in 12-well plates for 1 day before transfection. GAPDH siRNA (100 nM) alone, or complexed to CD, was diluted in OptiMEM® and added to the cells in serum-containing medium. Non-silencing siRNA complexed to CD was used as a control. After 24 hours, total RNA was extracted from N41 cells using Stratagene Absolutely RNA® Miniprep Kit, according to the manufacturer's instructions and from primary hippocampal cells using Trizol reagent (Invitrogen) for primary hippocampal cultures. The concentration of RNA was measured by UV absorbance on the NanoDrop ND-1000 UV-Vis Spectrophotometer and the 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). Amplification was carried out by 40 cycles of

denaturation at 95ºC (15 sec) and annealing at 60ºC (1 min). β-actin endogenous gene was used for relative gene quantification [58]. The 2–delta Ct method was used to calculate relative changes in mRNA [59]. Each experiment was carried out in triplicate. Results were expressed as % GAPDH gene expression relative to untreated (non-transfected) controls. The data are presented as the Mean ± SEM.

Statistics. One-way analysis of variance (ANOVA) was used to compare multiple groups followed by Bonferroni's post hoc test. Statistical significance was set at $*p < 0.05$.

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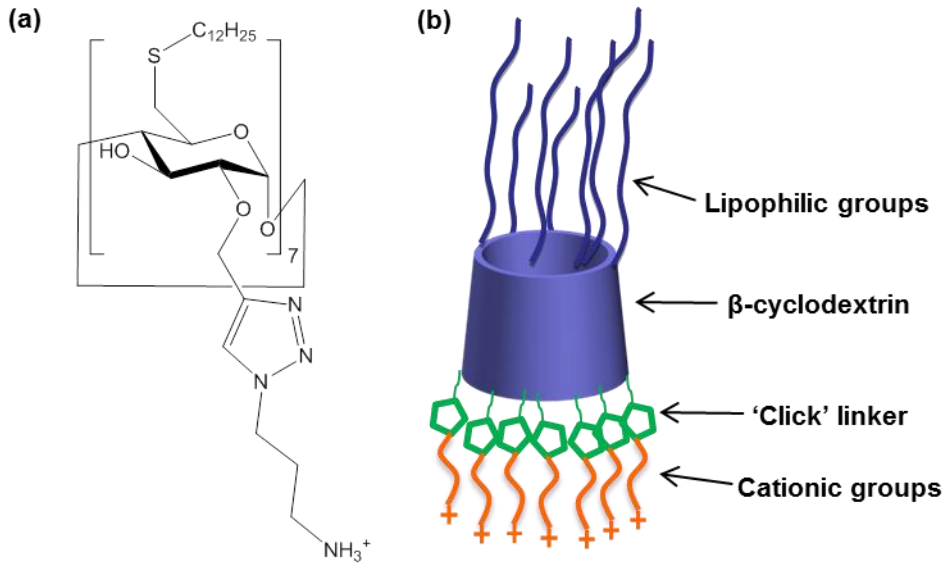


Figure 1. (a) Chemical structure of CD used for siRNA delivery and (b) schematic representation of CD structure.

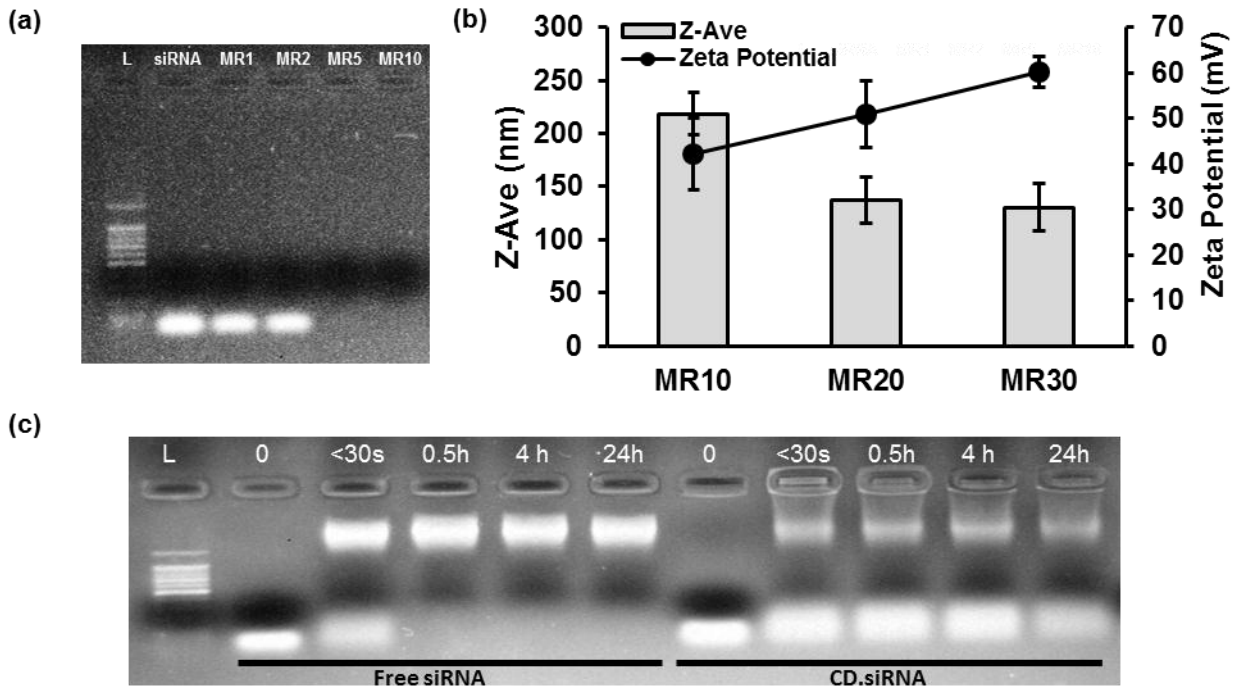


Figure 2. Properties of CD.siRNA complexes. (a) CD.siRNA binding was shown by gel electrophoresis. Fluorescent bands correspond to 100 b.p. DNA ladder (L), free siRNA and unbound siRNA. (b) Z-Ave (size, (nm)) and Zeta Potential (charge, (mV)) of CD-siRNA complexes at increasing mass ratios (MR; µg CD: µg siRNA), were measured in DIW (final siRNA concentration 3 µg/ml), using dynamic light scattering (Malvern ZetaSizer Nano). (c) Serum degradation assay, as shown by gel electrophoresis. Uncomplexed siRNA or CD.siRNA was exposed to 50% serum for up to 24hours. CD at least partially protected siRNA from degradation.

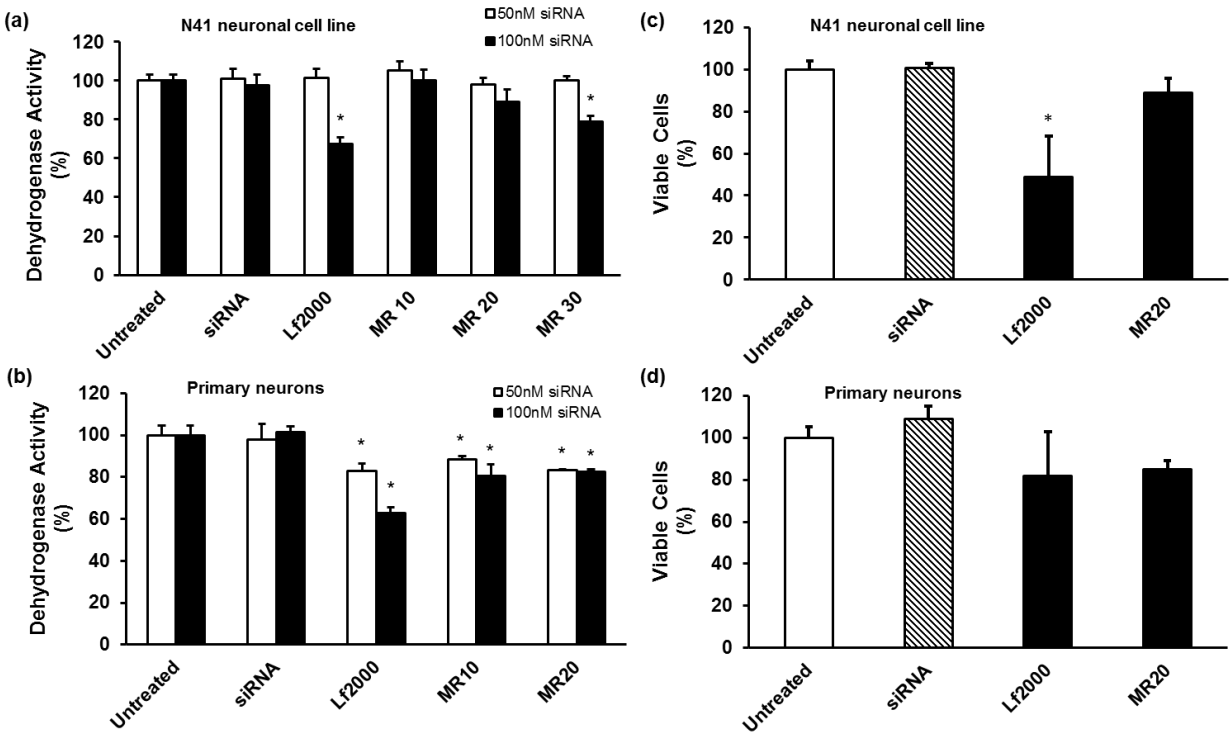


Figure 3. Toxicity was determined by MTT assay (a, b) and Trypan blue exclusion assay (c, d) after 24 hours incubation with CD.siRNA complexes (50 or 100 nM siRNA) in mHypoE N41 cells and primary rat (E18) hippocampal neurons. Data (n=3) are presented as the mean ± SEM. *$p < 0.05$ relative to untreated controls.

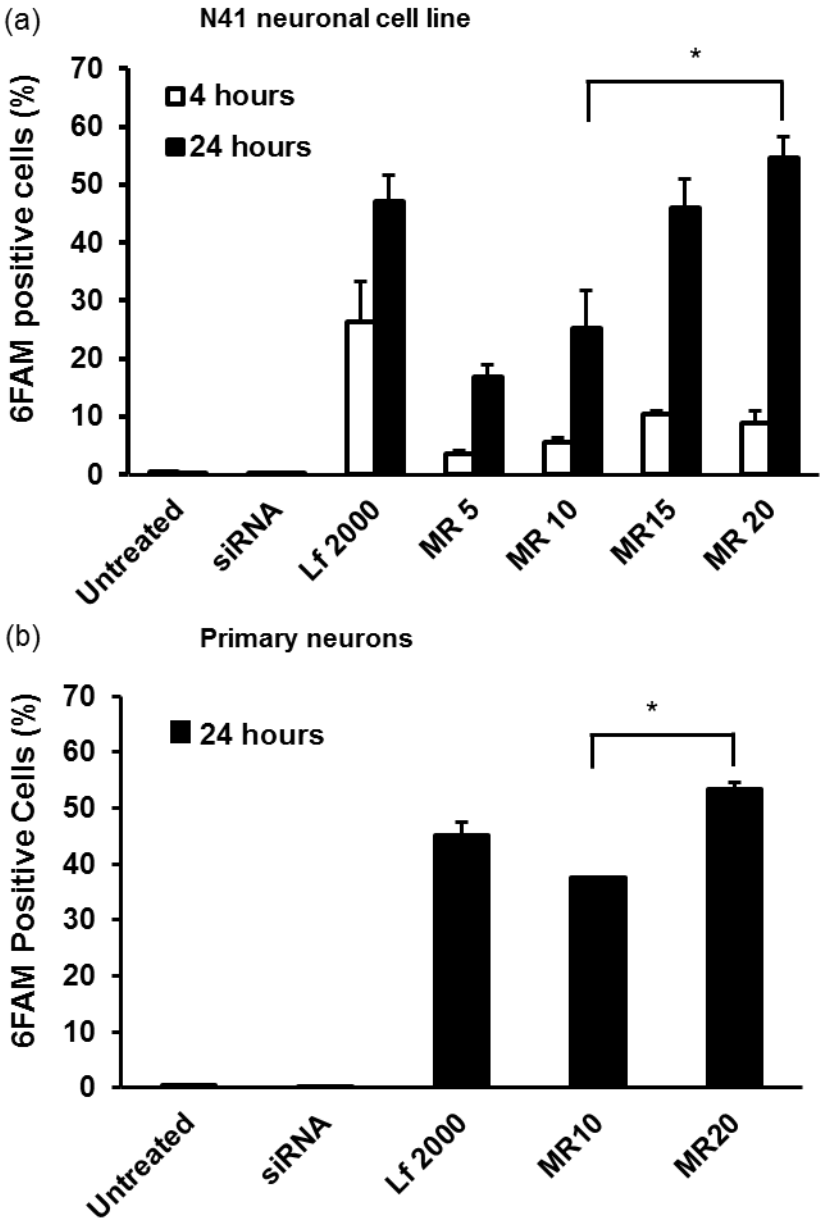


Figure 4. Internalisation of CD.siRNA complexes by (a) mHypoE N41 cells and (b) Primary rat (E18) hippocampal cells. FAM-siRNA uptake, after incubation with CD.siRNA complexes (siRNA 50 nM), was measured by flow cytometry. Data (n=3) are expressed as the mean ± SEM. *$p < 0.05$ relative to untreated controls.

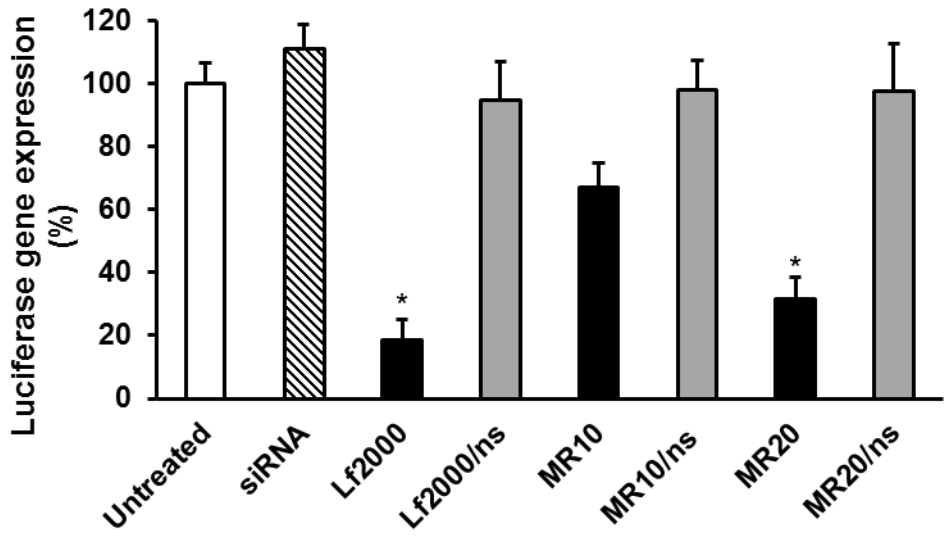


Figure 5. Knockdown of luciferase reporter gene in mHypoE N41 cells by CD.siRNA complexes. Cells were incubated with luciferase reporter plasmid (1μg/well). Four hours later, after washing and changing media, luciferase siRNA (50 nM), either uncomplexed, or complexed to CD, was added. After 24 hours, reduction in gene expression was measured by luciferase assay. Data (n=3) are expressed as the mean ± SEM. *$p < 0.05$ relative to untreated controls.

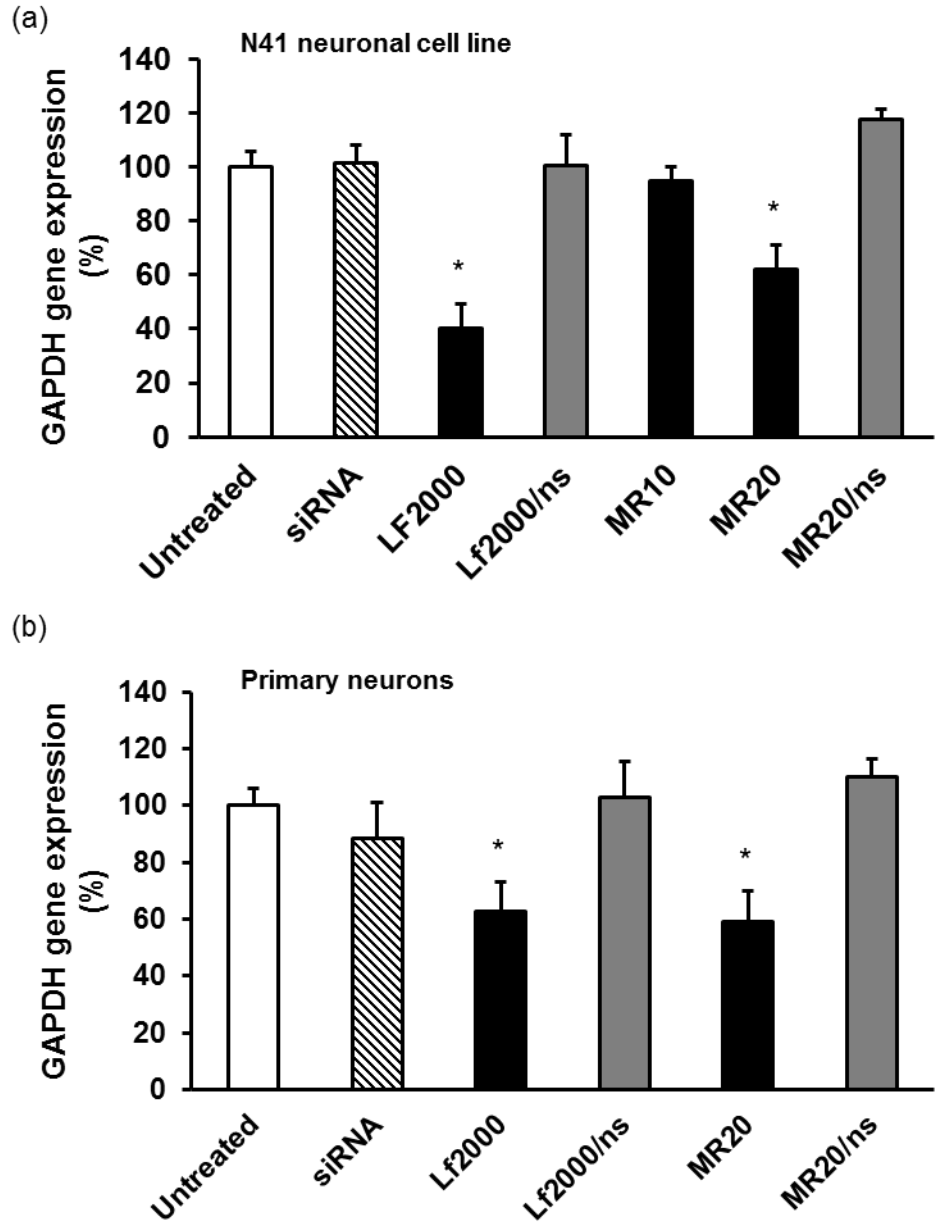


Figure 6. Knockdown of endogenous GAPDH in (a) mHypoE N41 cells and (b) Primary rat (E18) hippocampal cells by CD.siRNA complexes. Cells were incubated GAPDH siRNA (100 nM), either uncomplexed, or complexed to CD, for 24 hours. Total RNA was extracted and reverse transcribed to cDNA for qRT-PCR, to determine the reduction in GAPDH mRNA expression. Data (n=3) are expressed as the mean ± SEM. *$p < 0.05$ relative to untreated controls.

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