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Authors	O'Mahony, Aoife M.;Doyle, D.;Darcy, Raphael;Cryan, John F.;O'Driscoll, Caitríona M.
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**University College Cork, Ireland**  
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**Characterisation of cationic amphiphilic cyclodextrins for neuronal delivery of siRNA: effect of reversing primary and secondary face modifications.**

*A.M. O'Mahony<sup>a</sup>, D. Doyle<sup>b</sup>, R. Darcy<sup>b</sup>, J.F. Cryan<sup>c</sup> and C.M. O'Driscoll<sup>a\*</sup>*

<sup>a</sup>Pharmacodelivery Group, School of Pharmacy, University College Cork, Ireland.

<sup>b</sup>Centre for Synthesis and Chemical Biology, UCD Conway Institute, University College Dublin, Ireland.

<sup>c</sup>Dept. of Anatomy and Neuroscience, University College Cork, Ireland

\*Corresponding author. Address: University College Cork, Cavanagh Pharmacy Building, College Rd, Cork, Ireland. Tel.: +353214901396; fax: +353214901656.

Email address: [caitrona.odriscoll@ucc.ie](mailto:caitrona.odriscoll@ucc.ie) (C. O'Driscoll)

## **Abstract**

Significant research is focused on the development of non-viral vectors for delivery of siRNA to neurons and the central nervous system. Cyclodextrins (CDs) have shown great promise as efficient and low toxicity gene delivery vectors in various cell types. Here, we investigate two CDs for siRNA delivery in a neuronal cell model. These CDs were substituted on opposite faces (primary and secondary) with amphiphilic and cationic groups.

Physical properties of CD.siRNA complexes, including size, charge and stability were measured. *In vitro* investigations were carried out in immortalised hypothalamic neurons. Neuronal cell uptake was measured by flow cytometry and cytotoxicity was assessed by MTT assay. Knockdown of a luciferase reporter gene was used as a measure of gene silencing efficiency.

Both CDs interacted with siRNA, yielding nanosized cationic complexes which exhibited good stability on storage. A favourable toxicity profile was demonstrated for the CD.siRNA complexes. However, only one of the two CDs mediated high levels of neuronal uptake and efficient gene silencing, equivalent to those achieved with a commercial lipid-based vector.

Despite the suitability of both CDs as siRNA delivery vectors in terms of their ability to complex siRNA and the properties of the complexes yielded, only one CD achieved good transfection efficiency. This was likely due to the differences in their chemical structures. The effective CD offers great potential as a novel non-toxic vector for neuronal siRNA delivery.

**KEYWORDS:** modified cyclodextrins, siRNA delivery, neurons, low-toxicity

## 1. Introduction

RNA interference (RNAi) holds great promise for neurodegenerative and neurological diseases, but the challenge of delivering siRNA to neurons remains (Bergen et al., 2008; Perez-Martinez et al., 2011). Among the various classes of non-viral vectors which have been investigated, cyclodextrins (CDs) have shown great potential as gene delivery vectors (Mellet et al., 2011). CDs are macrocyclic oligosaccharides which have been well characterised as pharmaceutical excipients (Davis and Brewster, 2004; Sallas and Darcy, 2008). In addition, CDs lend themselves to chemical modifications at the primary and/or secondary faces yielding a series of compounds with diverse properties, which render them useful for gene delivery. In particular, cationic and amphiphilic CDs have shown low toxicity and high transfection efficiency in various cell types (Cryan et al., 2004a; McMahon et al., 2008; Méndez-Ardoy et al., 2011).

It is somewhat surprising, therefore, that the potential of CDs as neuronal siRNA delivery vectors has not been widely realised. Indeed, neuronal siRNA delivery has relied largely on cationic lipid-based vectors (Bergen et al., 2008). Although efficient, these are associated with considerable toxicity and less toxic alternatives are required (Bergen et al., 2008). The application of modified CDs to neuronal siRNA delivery is a recent concept (O'Mahony et al., 2012). Therefore, in this paper we have investigated the efficiency of additional novel CDs.

To this end, we have assessed two modified CDs in this study and for the first time, we directly compared the transfection efficiencies of two CDs based on their structural modifications. This represents an interesting and novel development in the chemistry of CD vectors. The chemical structures and schematic representations of these CDs are shown in Figure 1. The first of these, the so-called 'inverted' SC8CDcysteamine (Fig. 1 (a, c)) was

modified with cationic groups on the primary face and lipophilic chains at the C-2 position (Byrne et al., 2009). This positioning is the inverse of previous CD based gene delivery vectors developed by our group. 'Inverted' SC8CDcysteamine was previously shown to mediate high levels of luciferase gene expression in undifferentiated and differentiated Caco2 cells (O' Neill et al., 2011). The second CD used in this study was SC8CDcysteamine, which has the reverse arrangement of cationic and lipophilic modifications (Fig. 1 (b, d)). This type of positioning is similar to that of the majority of CD-based gene delivery vectors investigated by our group (Cryan et al., 2004a; McMahon et al., 2008; McMahon et al., 2012).

Here, we investigate the ability of these CDs to act as non-viral neuronal siRNA delivery vectors, by comparing them in terms of their ability to complex siRNA and size, charge and stability of complexes formed. In other studies, we assessed the toxicity and gene silencing efficiency of the CDs in neuronal cells.

## **2. Methods**

### *2.1 Preparation of vector.siRNA complexes*

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) and pGL3 luciferase siRNA (sense sequence 5'- CUU ACG CUG AGU ACU UCG A) were obtained from Qiagen (California, USA).

CD.siRNA complexes: CD synthesis was previously reported (Byrne et al., 2009). CD complexes were prepared as previously described (Cryan et al., 2004a; McMahon et al.,

2008; O' Neill et al., 2011). Briefly, CDs were weighed and 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 deionised water (DIW) (CD concentration 1 mg/ml), followed by sonication for one hour at room temperature (RT) for 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 ( $\mu\text{g CD} : \mu\text{g siRNA}$ ).

Lf2000.siRNA complexes: Lipofectamine<sup>TM</sup> 2000 (Lf2000) (Invitrogen, USA) 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  $\mu\text{l}$  Optimem, mixed gently and incubated at RT for 5 mins. siRNA was diluted in 50  $\mu\text{l}$  OptiMem and combined with the diluted Lf2000, then mixed gently and incubated at RT for 20 mins. 1  $\mu\text{l}$  of Lf2000 was used per 20 pmol of siRNA.

## *2.2 Gel Retardation Assay*

Agarose gel electrophoresis was used to determine the binding of siRNA (Guo et al., 2012). CD.siRNA complexes were prepared from MR 0.5 to 10 and mixed with loading buffer and DIW to a final volume of 20  $\mu\text{l}$  (containing 0.3  $\mu\text{g}$  siRNA). Samples were added to wells in a 1% agarose gel containing ethidium bromide (0.5  $\mu\text{g/ml}$ ). Electrophoresis was carried out at 90 V for 20 minutes, with a Tris-borate-EDTA buffer (Tsutsumi et al., 2008). 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.

## *2.3 Size and Charge Measurements*

Particle Z-average size and charge were measured with Malvern's Zetasizer Nano ZS, using laser-light scattering and electrophoretic mobility measurements respectively, as previously described for CD.DNA complexes (Cryan et al., 2004a; Cryan et al., 2004b; O' Neill et al.,

2011). CD.siRNA complexes were prepared, using 3 µg siRNA and the appropriate volume of CD solution with increasing MR. The resulting mixtures were 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 °) 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.

#### *2.4 Serum Stability Studies*

To evaluate protection of siRNA from serum nucleases by the CD, CD.siRNA complexes were incubated with 50% FBS for 30 seconds, 30 minutes or 4 hours at 37°C , followed by addition of excess heparin (5 µl of a 1000 i.u./ml solution, Multiparin®, CP Pharmaceuticals, UK) for 1 hour to displace the siRNA (Guo et al., 2012; Katas and Alpar, 2006). Samples were analysed by agarose gel electrophoresis (1.5% agarose gel) as described above.

#### *2.5 Cell culture*

A mouse embryonic hypothalamic cell line (*mHypoE N41*) (Belsham et al., 2004) 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 °C incubator with 5 % CO<sub>2</sub>. Cells were seeded in 24 well and 96 well plates at  $3.5 \times 10^4$  and  $1.5 \times 10^4$  cells per well respectively.

#### *2.6 MTT toxicity assay*

The MTT assay is widely used as an indicator of the toxicity caused to neurons by non-viral vectors (Cardoso et al., 2008; Wong et al., 2010). This assay measures the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) by mitochondrial dehydrogenase, in viable cells only, to yield dark-blue formazon crystals. 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 (50 nM) alone, or in CD.siRNA complexes, was diluted in OptiMEM® and added to cells in serum-containing medium for 4 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  $\pm$  SEM.

### *2.7 Cellular uptake experiments*

The level of uptake mediated by transfection complexes was assessed by flow cytometry (Kumar et al., 2007; Wong et al., 2010). Fluorescently labelled (6FAM) 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 complexes, was diluted in OptiMEM®, then added to cells in serum-containing medium for 4 hours. Following this, complexes bound to the extracellular surfaces were removed by washing with PBS and by incubation with 250 µl of CellScrub buffer (Genlantis) for 15 minutes at RT (Guo et al., 2012; Read et al., 2009). 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. These data are presented as the mean  $\pm$  SEM.

### *2.8 Knockdown of luciferase reporter gene*

Silencing of an exogenous gene was assessed by measuring knockdown of a luciferase reporter plasmid (Cardoso et al., 2008; Guo et al., 2012). The pGL3-luc plasmid contains the firefly luciferase gene under the control of an upstream SV40 promoter (Guo et al., 2012). 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, cultures were washed twice with PBS prior to siRNA transfection. pGL3-luciferase siRNA (50 nM) alone, or in CD.siRNA complexes was diluted in OptiMem® and added to the cells in serum-containing medium. CD.siRNA complexes with negative control siRNA were included as controls. After 4 hours, media was replaced and after a further 20 hours, cells were washed with PBS, lysed with 400 µl of 1x Reporter Lysis Buffer (Promega) and frozen at -80 °C. 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 (relative luminescence units, RLU) produced 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) to allow normalisation of luciferase activity. Results were expressed as % gene expression relative to ‘untreated’ control samples (which were transfected with luciferase plasmid only but no siRNA). Each experiment was carried out in triplicate. These data are presented as the mean ± SEM.

### *2.9 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  $*p < 0.05$ .

## **3. Results**

### *3.1 Properties of CD.siRNA complexes*

Naked siRNA carries a negative charge due to the presence of phosphate groups in its backbone. This negative charge represents a significant challenge to its cellular uptake. Complexation of siRNA by a cationic compound is a useful strategy for overcoming this obstacle. Inverted SC8CDcysteamine was previously shown to condense and complex pDNA

for gene delivery (O' Neill et al., 2011). Figure 2 illustrates the physical properties, including complexation ability, size and charge of the CD.siRNA complexes.

Gel electrophoresis studies showed that free siRNA was able to migrate freely through the gel, producing a fluorescent band (Fig. 2 (a) and (b)). At low MRs, complexation by CDs was incomplete as there was evidence of free siRNA in the gel. It was interesting to note that complexation was complete at MR 2 for SC8CDcysteamine and at MR 5 for inverted SC8CDcysteamine. One possible reason is that the polar groups in the former may be more accessible for electrostatic interaction with the siRNA, resulting in complexation at a lower MR. Indeed, the surface charge of SC8CDcysteamine is ~ 10 mV higher than that of inverted SC8CDcysteamine when measured prior to formulation with siRNA (Figure 2 (c) and (d)).

Having established that both CDs achieved siRNA binding, size and charge were investigated. These are key features which impact on siRNA delivery (Rejman et al., 2004; Resina et al., 2009). For both CDs, the particle size was smaller in the presence of siRNA compared to the CD alone. CD.siRNA complexes were generally close to or less than 200 nm, with slightly smaller sizes observed for inverted SC8CDcysteamine (Fig. 2 (c) and (d)). Polydispersity index (PDI) values were also measured during particle size analysis. PDI is a measure of the particle size distribution within a sample and gives an indication of whether aggregates are present. PDI values were 0.2 – 0.3 for inverted SC8CDcysteamine and 0.3 – 0.4 for SC8CDcysteamine, which indicates some variability in particle size distribution (Cryan et al., 2004b) and a lack of aggregation.

CD particles were cationic when measured in the absence of siRNA due to the primary amine groups in both CD structures. Surface charge of CD particles was reduced upon addition of siRNA at the lowest MR (MR 5) (Fig. 2 (c) and (d)). Increasing MR to 10 resulted in an

increase in surface charge to ~ 60 mV for inverted SC8CDcysteamine and ~ 68 mV for SC8CDcysteamine, with further increases in MR having little effect.

### *3.2 Gene Silencing by CD.siRNA complexes*

The ability of the CDs to deliver functional siRNA for RNAi was assessed by measuring the silencing of a luciferase reporter plasmid. Knockdown of luciferase is a widely reported method for assessing the efficacy of siRNA delivery vectors (Bartlett and Davis, 2007; Cardoso et al., 2008; Guo et al., 2012; Vader et al., 2012). For inverted SC8CDcysteamine, maximum gene silencing was observed at MR10 (~80%, \* $p < 0.05$  relative to untreated), with no greater knockdown achieved at MR20 (Fig. 3 (a)). On the other hand, there was no inhibition of gene expression observed for SC8CDcysteamine complexes at MR10 (Fig. 3 (b)) with only a trend towards reduction in luciferase expression at MR20 (~40%). Lf2000 was included in these studies as a positive control and achieved high levels of gene knockdown (~70%, \* $p < 0.05$  relative to untreated).

The order of gene silencing efficiency decreased as follows: Inverted SC8CDcysteamine (MR10) = Lf2000 > SC8CDcysteamine (MR20). Neither siRNA alone nor non-silencing controls had any effect on gene expression.

### *3.3 Uptake of CD.siRNA complexes*

To investigate the reason for the differences in gene silencing efficiency of the two CDs, the levels of uptake of fluorescently labelled siRNA mediated by both were measured. No significant cellular uptake of naked siRNA was detected (Fig. 4). Whilst both CDs mediated significant uptake of fluorescent siRNA relative to untreated cells (\* $p < 0.05$ ), the highest levels of uptake were achieved with inverted SC8CDcysteamine at MR10 (~45%) with no further increase at MR20 (Fig. 4 (a)). Lf2000 mediated significantly lower uptake (~22%, # $p$

< 0.05 relative to MR10 and MR20). Meanwhile, low levels of uptake were achieved for SC8CDcysteamine (~13% at MR20) (Fig. 4 (b)). This correlates with the lower levels of gene silencing achieved with this CD.

Neuronal uptake decreased in the order of Inverted SC8CDcysteamine > Lf 2000 > SC8CDcysteamine.

### *3.4 Toxicity of CD.siRNA complexes*

Toxicity is a feature associated with many cationic gene and siRNA delivery vectors, in particular in neuronal cells (Perez-Martinez et al., 2011). To this end, we investigated the toxicity of the two CDs compared to Lf2000 by means of an MTT assay. CD.siRNA complexes were applied to cells for four hours as for the transfection and uptake studies. Toxicity was determined as percentage dehydrogenase activity relative to untreated cells. Naked siRNA did not induce any toxic effects in neuronal cells (Fig.5). Moreover, neither inverted SC8CDcysteamine (Fig. 5 (a)) nor SC8CDcysteamine (Fig. 5 (b)) impaired cell viability. Finally, mHypoE N41 neuronal cells appeared to be resistant to toxicity mediated by Lf2000, which was unexpected given the high potential for toxicity associated with this cationic lipid based vector. However, previous studies have shown considerable toxicity when neuronal cells were treated with Lf2000 for up to 24 hours (O'Mahony et al., 2012). Therefore, the lack of toxicity observed here may be due to experimental conditions (i.e. the short time for which the neuronal cells were exposed to the vector (4 hours)) and the relatively low levels of uptake (~20%).

### *3.5 Stability of CD.siRNA complexes*

Several aspects of CD.siRNA complex stability were investigated, including the effects of serum and the effects of storage time.

In the first instance, extracellular and intracellular nucleases have the potential to degrade siRNA and nucleic acids and are a barrier to siRNA delivery (Bumcrot et al., 2006). Serum represents a source of nucleases and was used to assess whether CDs could protect complexed siRNA from degradation (Guo et al., 2012). CD.siRNA complexes were incubated with FBS and subsequently analysed by gel electrophoresis. Naked siRNA was completely degraded within 30 minutes of exposure to FBS (Fig. 6 (a) and (b) Lane 3), compared to siRNA which was not treated with FBS (Lane 2). The higher visible band in all of the serum-treated samples was due to components of the FBS, as previously observed (O'Mahony et al., 2012; Zhang et al., 2006). Lanes 4-7 represent CD.siRNA complexes after displacement of siRNA by heparin. Both CDs conferred a certain degree of protection to siRNA from nuclease degradation as intact siRNA was present after the incubation with serum (Lanes 5-7), although there also was some evidence of degraded siRNA present in these samples. Lane 4 contains CD.siRNA complexes which were not exposed to serum and which were incubated with an excess of heparin for competitive displacement of siRNA. However, it is clear that complete siRNA displacement did not occur, indicating that there may be some method of complexation additional to electrostatic interaction occurring between the CDs and siRNA.

Secondly, stability on storage is an important consideration for future commercialisation of any siRNA delivery vector. To this end, CD.siRNA complexes were prepared in DIW (at optimal MR for transfection) and stored at 4°C for up to 12 weeks. Gel electrophoresis was used to confirm that siRNA remained bound within stored complexes and that the siRNA remained intact (Fig. 7 (a) and (b)). Storage for 12 weeks did not affect the complexation of siRNA by either CD, as demonstrated by the lack of free siRNA migrating through the gel in Lane 3. Furthermore, after incubation with heparin, an equivalent amount of siRNA appeared

to be released from fresh (Lane 4) and stored (Lane 5) CD.siRNA complexes. There was no difference between the two CDs.

Further investigation into the impact of storage time on the stability of CD.siRNA complexes in terms of the particle size and PDI was carried out. Storage at 4°C caused no changes in particle size, as shown in Figure 7 (c) and (d). PDI was also maintained within the same range over the time course studied (12 weeks).

These data imply that CD.siRNA complexes are relatively stable on storage at 4°C, both in terms of siRNA complexation and complex properties.

#### **4. Discussion**

In this study, we investigated two CDs as potential novel neuronal siRNA delivery vectors. The two CDs compared in this study, SC8CDcysteamine and inverted SC8CDcysteamine, differ in the relative positioning of the lipophilic and cationic moieties in their structures (Fig. 1). The physical properties of the CDs and their respective CD.siRNA complexes were characterised, along with their stability in various conditions including long-term storage and exposure to serum. The two CDs gave comparable results in these experiments. However, *in vitro* cell culture investigations revealed significant differences in their ability to mediate gene silencing.

Firstly, in terms of their physical properties, both CDs inhibited the electrophoretic mobility of siRNA through an agarose gel, implying complex formation (Fig. 2). The MR at which siRNA binding was complete was MR 2 for SC8CDcysteamine and MR 5 for inverted SC8CDcysteamine. Complex formation was expected, based on the primary amine groups in their structure which enables electrostatic interaction with negatively charged phosphate

groups on the siRNA backbone, as for cationic lipids (Dalby et al., 2004). Neutral CDs have previously shown inability to complex pDNA due to lack of charge interaction (Cryan et al., 2004a).

Both size (Rejman et al., 2004) and charge (Resina et al., 2009) of lipoplexes and polyplexes have been shown to influence transfection efficiency, therefore the physical properties of the CD.siRNA complexes were investigated. Particle size was generally ~150nm. This was within the typical range (100-200nm) reported for *in vitro* siRNA delivery systems (Pulford et al., 2010; Wong et al., 2010). CD.siRNA complexes were positively charged at all MRs tested. A cationic surface charge enables interaction with negatively charged heparan sulphate groups on proteoglycans on the cell surface, thus facilitating cellular uptake (Cryan et al., 2004b; Diaz-Moscoso et al., 2008; O' Neill et al., 2011).

Stability of CD.siRNA complexes was also investigated. Storage of the complexes had no effect on the particle size or polydispersity, which implies a lack of aggregation or precipitation. Furthermore, both CDs offered some degree of protection to siRNA within CD.siRNA complexes when exposed to serum. Serum degradation of naked siRNA occurs rapidly and is one reason for the lack of efficacy of naked siRNA (Bumcrot et al., 2006). Preventing siRNA digestion by serum nucleases is a key feature for non-viral delivery vectors, particularly in the context of future *in vivo* usage (Guo et al., 2010).

On the whole therefore, it was apparent that the facial positioning and chemical structures of the CDs did not affect the physical properties of the complexes formed by the respective CDs and siRNA. This is supported by data previously reported with CD.pDNA complexes (O' Neill et al., 2011).

Subsequently, *in vitro* experiments were carried out in a mouse hypothalamic neuronal cell line. mHypoE N41 cells are derived from primary mouse embryonic hypothalamic neuronal



cultures and as such, are a good model for ‘difficult to transfect’ neurons. In fact, in a recent publication, we showed that the level of knockdown of an endogenous house-keeping gene (GAPDH) was comparable in primary neuronal cultures and mHypoE N41 immortalised neurons (O’Mahony et al., 2012). Gene silencing studies identified that only one of the two CDs was capable of mediating efficient knockdown of luciferase reporter plasmid. Inverted SC8CDcysteamine achieved a significant reduction in luciferase expression at MR 10, comparable to that achieved with Lf2000, the standard commercial siRNA delivery reagent (Fig. 3) whilst SC8CDcysteamine did not mediate significant levels of knockdown (although a trend towards knockdown was observed at MR 20).

In order to investigate this further, the levels of cellular uptake mediated by the two CDs was measured by flow cytometry. These experiments revealed that inverted SC8CDcysteamine achieved high levels of intracellular FAM-siRNA, which correlates with its transfection efficiency. However, SC8CDcysteamine mediated much lower levels of uptake (<15%) (Fig. 4). Therefore, the reduced gene silencing efficiency of SC8CDcysteamine appears to be due, at least in part, to lack of intracellular uptake. The reasons for this difference in uptake are not entirely clear, given the similar physical properties of the complexes formed by both CDs with siRNA.

Interestingly, the level of uptake achieved with Lf2000 was lower than that with inverted SC8CDcysteamine, but gene knockdown efficiencies were comparable. This highlights the fact that gene silencing efficiency does not always correlate with uptake. Endosomal escape has been shown to be another rate-limiting step for silencing (Vader et al., 2011) and other possible reasons for this discrepancy include incomplete dissociation of siRNA from the CD or difference in the intracellular kinetics of the two vectors.

On cursory analysis, it appears that the main difference between the two CDs is whether the cationic and lipophilic groups are on the primary or secondary side of the molecule. However, when we look at the structures in detail, there are other differences which must also be taken into consideration. These relate to the compositions of the hydrophilic and hydrophobic components for each CD.

Firstly, the polar component in each of the CD molecules is quite different. In the inverted SC8CDcysteamine, there is a short hydrocarbon chain terminating in a primary amine as the polar component (primary side) (Fig. 1 (a)). By comparison, the polar component in the SC8CDcysteamine molecule (secondary side) consists of a longer chain of lipophilic groups terminating in the cationic primary amine (Fig. 1 (b)). This is not typical when compared to the other CD-based gene delivery vectors studied by our group (Cryan et al., 2004b; O'Mahony et al., 2012) and is likely to be influencing the hydrophilic-hydrophobic balance within this CD. Changing the hydrophilic-hydrophobic balance can result in differences in the type of self-assembled complexes formed with amphiphilic CDs (Donohue et al., 2002), which may in turn affect uptake and transfection, as has been the case for lipoplexes (Ma et al., 2007).

Furthermore, the hydrophobic components of the two CDs are not identical. In the inverted SC8CDcysteamine structure, there are twelve lipophilic groups comprising the hydrophobic component (secondary side). Meanwhile, the corresponding group in the SC8CDcysteamine structure is comprised of ten lipophilic groups (primary side). This may also have implications for transfection. For example, greater gene expression was mediated by CDs whose lipophilic group consisted of longer rather than shorter hydrocarbon chains (Cryan et al., 2004a; McMahan et al., 2008).

Differences in CD-mediated gene transfer based on structural modifications were previously reported. For example,  $\beta$ -CD with seven cationic moieties on the primary face and fourteen hexanoyl chains on the secondary face achieved high levels of gene expression compared to the reverse arrangement (Ortega-Caballero et al., 2008). In this case, the differential gene expression was attributed to a change in the hydrophilic-hydrophobic balance within the molecule.

The results presented here point to a potential structure-activity relationship and certainly warrant further investigation. Comparison of a range of modified CDs is required to fully elucidate the nature of this relationship. In addition, however, the differences revealed here relate to transfection studies in a single cell line and therefore conclusion cannot be drawn regarding transfection in other cell types.

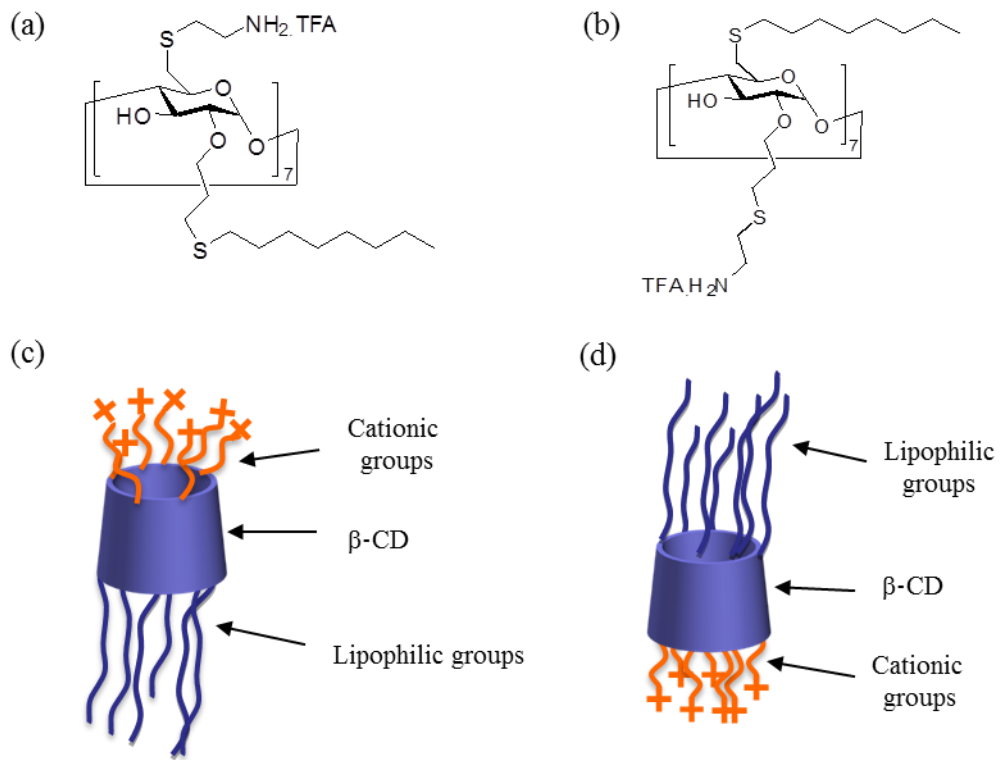
Nonetheless, we have identified a suitable CD-based neuronal siRNA delivery vector in these studies. Future work will focus on using this validated CD delivery system to target relevant therapeutic genes, including those which are implicated in the pathology of neurodegenerative diseases such as Huntington's Disease and Alzheimer's Disease.

## **5. Conclusions**

In conclusion, two modified CDs differing in the facial positioning of the amphiphilic and cationic groups showed similar physical properties and cell viability. Interestingly, only one of the two CDs, inverted SC8CDcysteamine, mediated significant cellular uptake and gene silencing. Given its low toxicity and high gene knockdown efficiency, this CD represents a promising alternative siRNA delivery vector for neuronal cells.

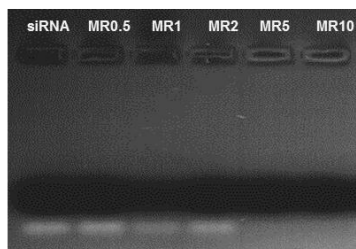
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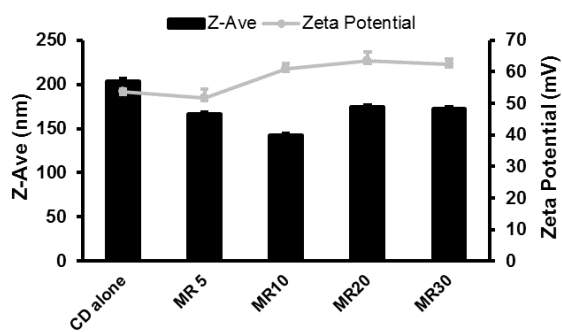


**Figure 1.** Chemical structures and schematic representations of inverted SC8CDcysteamine ((a) and (c)) and SC8CDcysteamine ((b) and (d)).

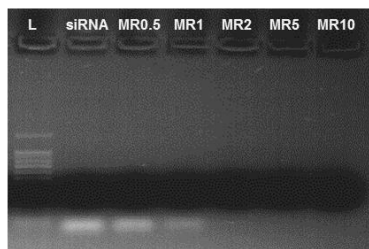
(a) Inverted SC8CDcysteamine



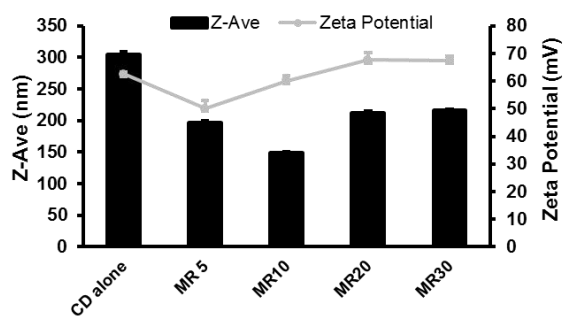
(c) Inverted SC8CDcysteamine



(b) SC8CDcysteamine

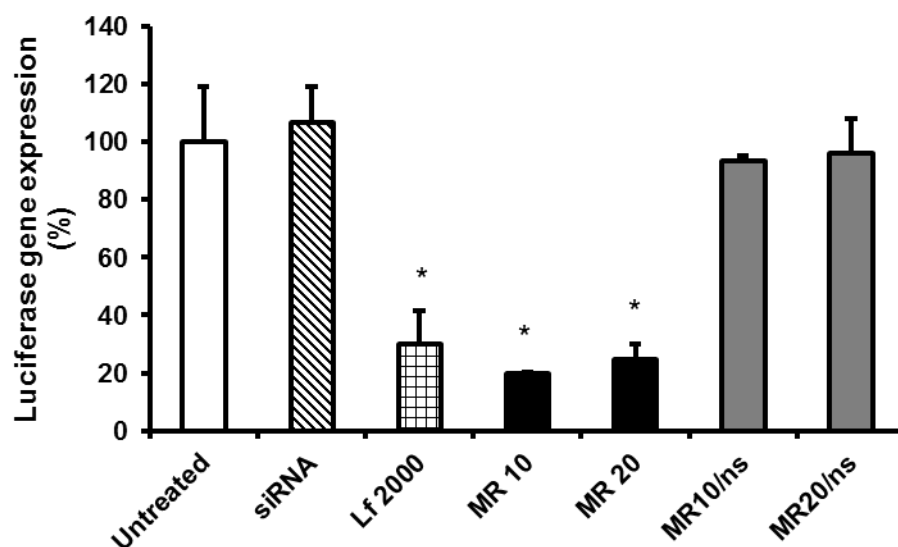


(d) SC8CDcysteamine

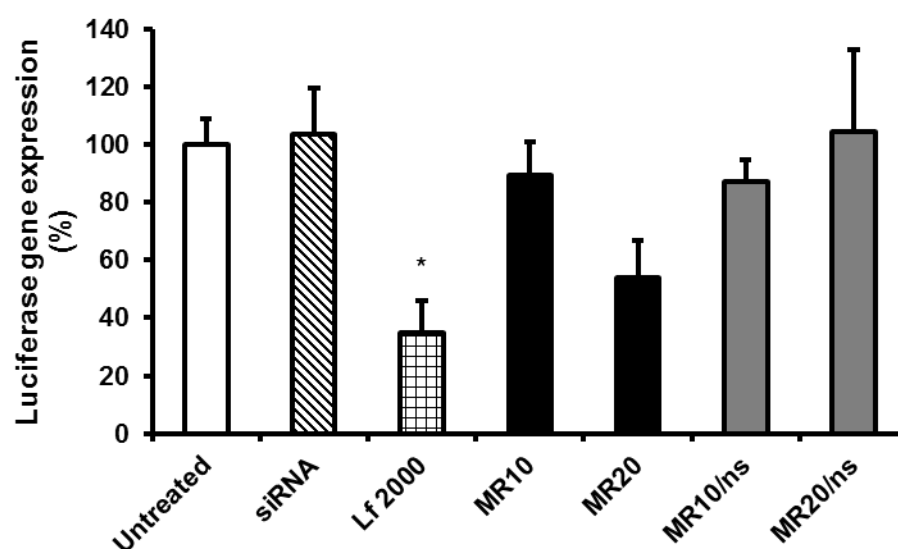


**Figure 2.** Physical properties of CD.siRNA complexes, including siRNA binding properties and size and charge of inverted SC8CDcysteamine (a) and (c) and SC8CDcysteamine (b) and (d). (MR refers to mass ratio, i.e.  $\mu\text{g CD} : \mu\text{g siRNA}$ ).

(a) Inverted SC8CDcysteamine

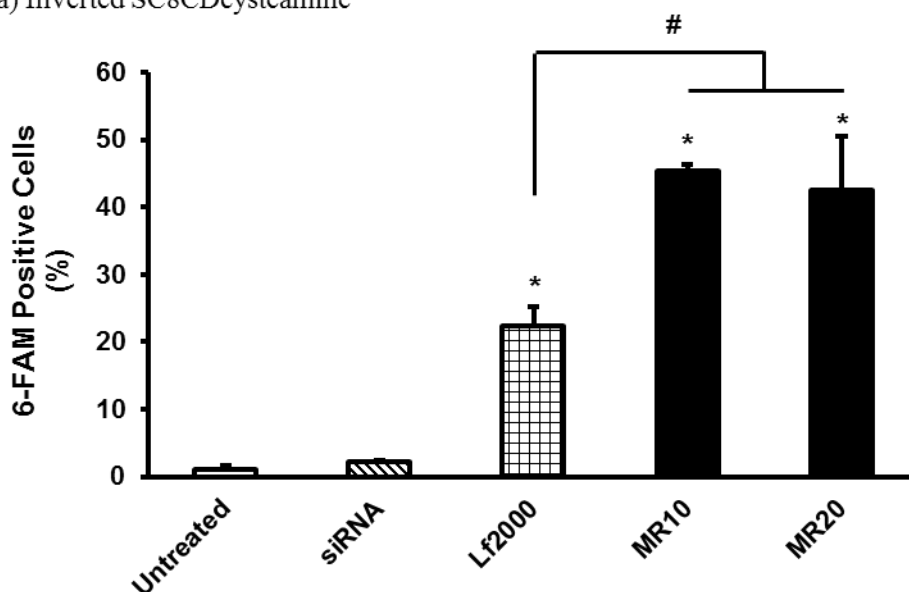


(b) SC8CDcysteamine

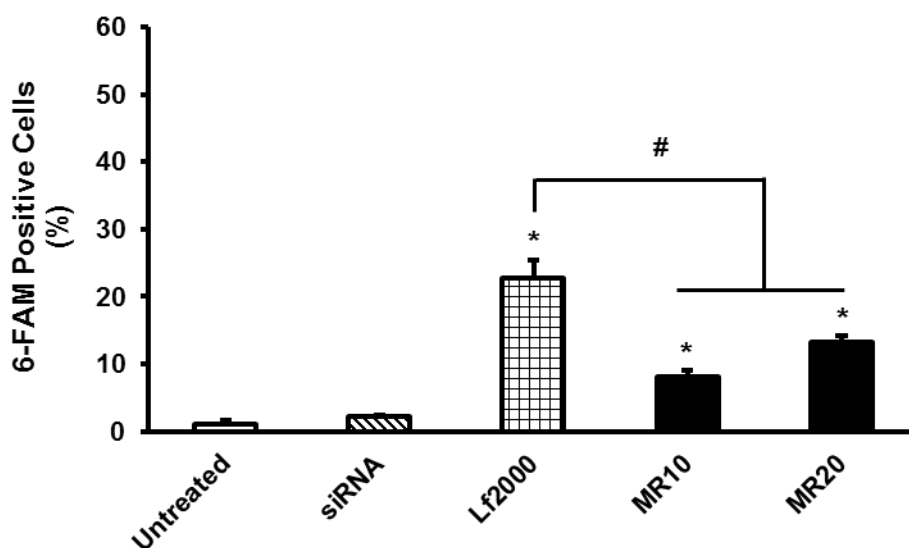


**Figure 3.** Knockdown of luciferase reporter gene in mHypoE N41 neuronal cells by CD.siRNA complexes with (a) Inverted SC8CDcysteamine and (b) SC8CDcysteamine. Cells were incubated pGL3-luc complexed to Lf2000 for 3 hours, followed by washing steps. CD.siRNA complexes (50 nM siRNA) were applied for 4 hours, then media was replaced and cells incubated for a further 20 hours before assessing for luciferase gene expression. Luciferase expression was calculated as a percentage of RNAi-untreated cells. Data are represented as mean  $\pm$  SEM. \*  $p < 0.05$  relative to untreated cells. (ns refers to non-silencing siRNA; MR refers to mass ratio, i.e.  $\mu\text{g CD} : \mu\text{g siRNA}$ ).

(a) Inverted SC8CDcysteamine



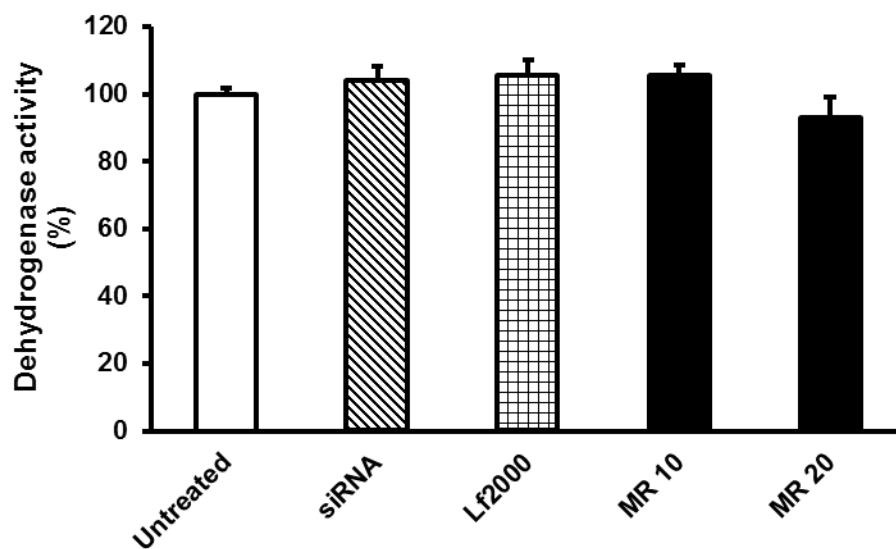
(b) SC8CDcysteamine



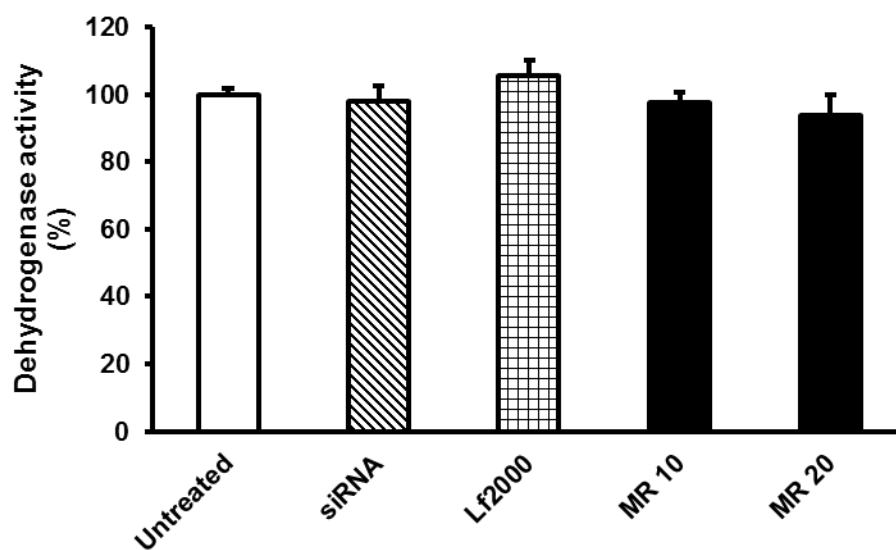
**Figure 4.** Uptake of (a) Inverted SC8CDcysteamine and (b) SC8CDcysteamine CD.siRNA complexes in mHypoE N41 neuronal cells. CD.siRNA complexes (50nM FAM-labelled siRNA) were applied for 4 hours before measuring uptake by flow cytometry. Uptake was expressed as percentage of fluorescent siRNA positive cells. Data are represented as mean  $\pm$  SEM. \* $p < 0.05$  relative to untreated controls, # $p < 0.05$  relative to Lf2000. (MR refers to mass ratio, i.e.  $\mu\text{g CD} : \mu\text{g siRNA}$ )



(a) Inverted SC8CDcysteamine

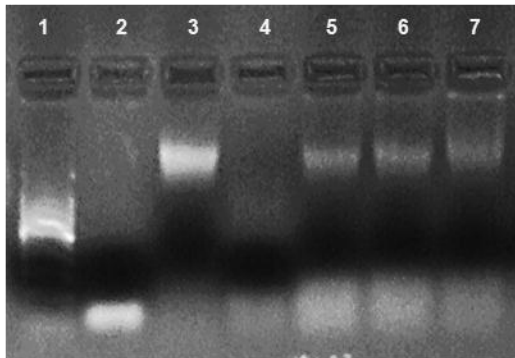


(b) SC8CDcysteamine



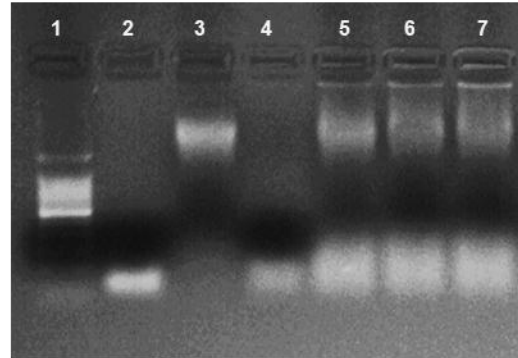
**Figure 5.** Toxicity was determined by MTT assay in mHypoE N41 neuronal cells after treatment with (a) Inverted SC8CDcysteamine and (b) SC8CDcysteamine CD.siRNA complexes. CD.siRNA complexes (50 nM siRNA) were applied for 4 hours, then fresh media and MTT reagent were added for a further 4 hours. Data are expressed as mean  $\pm$  SEM. (MR refers to mass ratio, i.e.  $\mu\text{g}$  CD:  $\mu\text{g}$  siRNA)

(a) Inverted SC8CDcysteamine



1: 100b.p DNA ladder  
2: siRNA  
3. siRNA & serum (30mins)

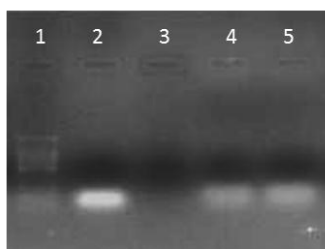
(b) SC8CDcysteamine



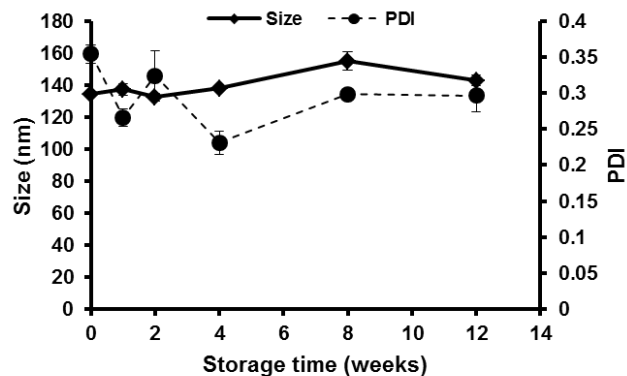
4: CD.siRNA  
5: CD.siRNA & serum (< 30 seconds)  
6: CD.siRNA & serum (30 mins)  
7: CD.siRNA & serum (4hours)

**Figure 6.** Effect of serum on CD-complexed siRNA stability. (a) Inverted SC8CDcysteamine and (b) SC8CDcysteamine CD.siRNA complexes (at optimal MR for transfection) were exposed to 50% serum for <30 seconds, 30 minutes or 4 hours. Heparin (5  $\mu$ l of 1000 i.u./ml solution) was added to liberate siRNA from the complexes and samples were then assessed by gel electrophoresis.

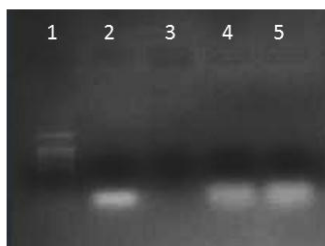
(a) Inverted SC8CDcysteamine



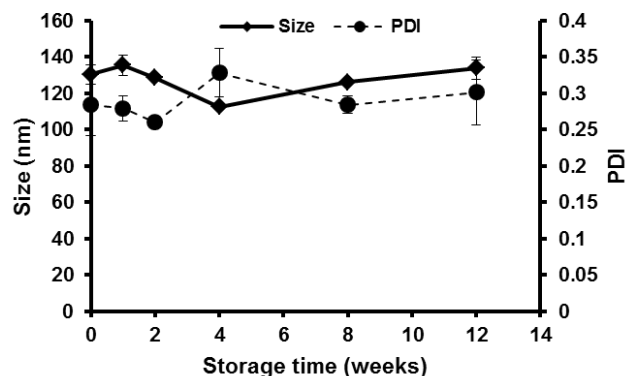
(c) Inverted SC8CDcysteamine



(b) SC8CDcysteamine



(d) SC8CDcysteamine



**Figure 7.** Effect of storage on siRNA complexation and stability of inverted SC8CDcysteamine ((a) and (c)) and SC8CDcysteamine ((b) and (d)) CD.siRNA complexes. CD.siRNA complexes were stored at 4°C for 12 weeks. CD.siRNA complexation and stability of siRNA within complexes was assessed by gel electrophoresis and size measurements. (Legend for gel images (a) and (b) **1:** 100b.p DNA ladder, **2:** siRNA, **3.** CD.siRNA (12 weeks), **4:** CD.siRNA (0 weeks) & heparin, **5:** CD.siRNA (12 weeks) & heparin).

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