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Cyclodextrin-siRNA conjugates as versatile gene silencing agents

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Introduction

Oligonucleotides have great potential as a new generation of therapeutics (Kole et al., 2012; Malhotra et al., 2011). Yet the application of RNA in therapies is hindered by problems with pharmaceutical delivery (Guo et al., 2011). Attempts at overcoming the barriers to effective systemic and targeted cell-specific delivery of these polyanionic macromolecules have explored many formulations, most often using cationic lipids and polymers. The cationic lipids have included amphiphilic cationic cyclodextrins (Cryan et al., 2004; O'Mahony et al., 2013; O'Mahony et al., 2012; Mendez-Ardoy et al., 2011). Cyclodextrins, as unique monodisperse oligosaccharides, lend themselves to chemical modification with multiple charges and polar or lipid groups, giving them versatile assembly properties in aqueous conditions (Sallas et al., 2008). Modifications which are possible while maintaining the useful assembly properties of cyclodextrins are many, such as: incorporation of cationic groups to neutralise the anionic charges on RNA to promote complexation; and coating of the nanoparticles with ligands by molecular inclusion. We have used amphiphilic cationic cyclodextrins for successful delivery of siRNA to neuronal cells (O'Mahony et al., 2012; O'Mahony et al., 2012; Godinho et al., 2014; Gooding et al., 2015), to prostate cancer cells (Evans et al., 2015), and in a scaffold-based 3D prostate cancer bone metastasis model (Fitzgerald et al., 2015). These cyclodextrins have also shown successful siRNA delivery and inhibition *in vivo* in an animal model of Huntington's disease and a xenograft model of prostate cancer (Godinho et al., 2013; Guo et al., 2012). Molecular inclusion of adamantyl groups has been employed to coat nanoparticulate formulations with the anisamide targeting ligands of adamantly-PEG-anisamide (Fitzgerald et al., 2016).

Besides complexation with cationic lipids and polymers, chemical conjugation of siRNAs to active ligands has also shown promise in facilitating delivery (Gooding et al., 2016). This may, for example, involve conjugating siRNAs to small molecules such as peptides that can penetrate cellular membranes (Rozema et al., 2007) or to lipophiles such as cholesterol (Wong et al., 2012). Other studies have shown that conjugation of PEG molecules to siRNA can lead to increased stability and gene knockdown efficiency (Gunasekaran et al., 2011; Gaziova et al., 2014; Shokrzadeh et al., 2014).

Now we have conjugated β-cyclodextrin (CD) to the sense strand of siRNAs using both a bioreducible (disulfide) and a non-reducible (sulfanyl) linker, and have assessed the resulting CD-siRNA conjugates for gene knockdown efficiency in cancer cell lines. We reasoned that if this major modification to siRNA did not adversely affect its gene knockdown effectiveness, then the siRNA thus conjugated could be endowed with assembly properties by the conjugation with modified functionalised cyclodextrin which would afford new ways of formulation and delivery. Initially in this study siRNA conjugated to simple beta cyclodextrin was delivered to cancer cell lines using two different approaches: 1) simple complexation with polycationic lipids, Lipofectamine 2000 or a cationic amphiphilic cyclodextrin and, 2) by formulating inclusion complexes of adamantyl-PEG-RVG peptide and adamantyl-PEG-dianisamide with the CD-RNA conjugates, followed by charge neutralisation with chitosan polycationic polymer. The adamantyl RVG- and dianisamide-targeted derivatives were directed at the acetyl choline receptors on human brain cancer cells (U87) and sigma receptors on prostate cancer cells (PC3 and DU145), respectively. In all cases the ability of the CD-siRNA conjugate to retain gene silencing activity was assessed.

Materials and Methods

Materials

Two thiol-modified siRNAs with sequences against luciferase gene and PLK1 gene were obtained from Gene Link (NY, USA). The siRNA targeted against luciferase gene had the sequence: sense strand, 5'- GAA GUG CUC GUC CUC GUC CCC C dT dT – (Thiol-M C6-D) 3' MW 7,979 Da; antisense strand, 5' - GGG GGA CGA GGA CGA GCA CUU C dT dT – 3' MW 7,514 Da. The siRNA targeted against PLK1 had the sequence: sense strand, 5'-AGA mUCA CCC mUCC UmUA AAmU AUU dT dT – (Thiol-M C6-D) 3', antisense strand, 5'-UAU UUA AmGG AGG GUG AmUC UUU-3', with a total molecular weight of 13,558 Da. The PLK1 siRNA had 2'-O-methyl "m" modifications on its nucleotides to protect it from serum nucleases.

Synthesis of β-cyclodextrin-RNA conjugates (Scheme)

All chemicals were purchased from Aldrich and were used without further purification unless otherwise noted. Triphenylphosphine was recrystallised from ethanol and dried under high vacuum for 6 hours at 50°C before use. β-cyclodextrin was dried for 12 hours at 100°C under high vacuum. DMF was purchased in Sureseal bottles over molecular sieves and stored under nitrogen.

Chromatography: Thin-layer chromatography was performed on aluminium-backed plates of Merck Silica Kieselgel 60 F_{254}. These were developed by dipping the plates in a 5% sulfuric acid in ethanol solution and heating with a heat gun; or in caesium sulfate stain (21 g $(NH_4)_6Mo_7O_{24}$,

1 g $Ce(SO_4)_2$, 1 L H_2O, 31 mL conc. H_2SO_4) followed by charring; or in a tank containing iodine mixed with sand. Flash chromatography was carried out on Merck Kieselgel 60 0.04-0.063 mm.

NMR Spectroscopy: ^1H NMR spectra were recorded using Varian 300, 400 and 500 MHz spectrometers at 25°C unless otherwise stated. ^{13}C NMR spectra were recorded on Varian 75, 100 and 125 MHz spectrometers at 25°C unless stated otherwise.

Mass Spectrometry: MALDI-TOF analysis was performed on a Perseptive (Framingham, Mass.) Voyager STR instrument equipped with delayed extraction technology. Ions were formed by a pulsed UV laser beam (nitrogen laser, 337 nm) and accelerated through 24 kV. Samples were diluted in $CHCl_3$ and mixed 1:1 v/v with the matrix solution obtained by dissolving 2,5-dihydroxybenzoic acid (DHB) in CH_3OH-0.1% trifluoroacetic acid-CH_3CN (1:1:1 by volume) at a concentration of 30 mg/mL. A sample (1mL) of this mixture was deposited onto a stainless steel 100-sample MALDI plate and allowed to dry at room temperature (RT) before running the spectra in the positive polarity.

Synthesis of 6-Deoxy-6-(3'-(N-maleimido)-propionamido)-β-cyclodextrin (2)

6-Amino-6-deoxy-β-cyclodextrin **(1)** was synthesized according to a previously published method (Tang et al., 2008). Compound **1** (115 mg, 0.10 mmol) was dissolved in deionised water (6 mL) and 3-(N-maleimido)-propionic acid N-hydroxysuccinimide ester (54 mg, 0.20 mg) was added in DMF (1.4 mL). The solution was stirred for 3 h at RT, then the product **(2)** was precipitated with acetone and washed several times with acetone (yield 101 mg, 79%).

^1H-NMR (400 MHz, DMSO-d_6): δ 6.97 (s, 2H), 5.73 (m, 14H), 4.82 (s, 7H), 4.67 (m, 7H), 3.82-3.47 (m, 30 H).

^{13}C-NMR (101 MHz, DMSO-d_6): δ 171.1, 134.9, 102.4, 82.0, 73.5, 72.9, 72.5, 60.4.

ESI-MS (m/z): 1307.66 [M + Na]+.

Molecular weight: 1285.11894 Da; molecular formula: $C_{49}H_{76}N_2O_{37}$

Synthesis of 6-deoxy-6-(3'-(2''-pyridyldithio)-propionamido)-β-cyclodextrin (4)

6-Amino-6-deoxy-β-cyclodextrin (**1**) (45 mg, 0.04 mmol) was dissolved in deionised water (4 mL) and 3-(2'-pyridyldithio) propionic acid N-hydroxysuccinimide ester (25 mg, 0.08 mg) was added in DMF (1.2 mL). The mixture was stirred for 3 h at RT and the product **(4)** was precipitated with acetone and washed several times with acetone (46 mg, 86%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.43 (d, J = 4.7 Hz, 1H), 7.91 – 7.64 (m, 2H), 7.33 – 7.11 (m, 1H), 6.00 – 5.48 (m, 6H), 4.96 – 4.67 (m, 3H), 4.43 (s, 3H), 3.87 – 3.43 (m, 10H), 3.44 – 3.08 (m, 25H, overlap with D_2O).

^{13}C NMR (101 MHz, DMSO-d_6) δ 170.49, 159.64, 149.98, 138.25, 119.53, 102.37, 73.43, 72.85, 72.46, 60.34, 34.50.

ESI-MS (m/z): 1328.66 [M - H]-

Molecular weight: 1331.27672 Da; molecular formula: $C_{50}H_{78}N_2O_{35}S_2$

Complete reduction of thiol-modified siRNA

Thiol-modified luciferase siRNA, and thiol-modified PLK1 siRNA, was fully reduced according to the manufacturer's protocol (Gene Link, USA). Briefly, 1 mL of 3% TCEP (tris(2-carboxyethyl)phosphine) solution was freshly prepared by adding 30 µL of TCEP to 970 µL of sterile RNAse-free water. From this solution, 400µL was added to the lyophilized thiolated siRNA. The solution was left at RT for 1 hr, then 50 µL of 3M sodium acetate solution at pH 5.2

was added before again vortexing. Absolute ethanol (1.5 mL) was added to the solution which was vortexed briefly and stored at -80°C for 20 minutes. The sample was centrifuged at 13,680 g for 10 minutes, the supernatant was decanted and the pellet air dried. The pellet was dissolved in 200 µL of sterile phosphate-buffered saline (PBS), pH 7.2. Total concentration of the siRNA in 200 µL was determined by measuring absorbance at 260 nm, using a Nanodrop spectrophotometer.

Conjugation of siRNA with 6-Deoxy-6-(3'-(N-maleimido)-propionamido)-β-cyclodextrin (3)

A 100-molar excess of 6-deoxy-6-(3'-(N-maleimido)-propionamido)-β-cyclodextrin **(2)** in 50 µl of RNAse-free water was added to a solution of a fully reduced thiol-modified siRNA (luciferase siRNA or PLK1 siRNA) (100 µL in PBS, pH 7.2). The reaction solution was vortexed gently and incubated at RT overnight to obtain the CDmal-siRNA conjugate (**3**). Excess of the cyclodextrin was removed with Amicon centrifugal filters (M.W. 3000) (Millipore, Ireland) at 2,370 g and the solution was reconstituted with 100 µL of RNAse-free water. The concentration of CDmal-siRNA conjugate **(3)** was determined by measuring absorbance at 260 nm using a Nanodrop spectrophotometer.

Conjugation of siRNA with 6-deoxy-6-(3'-(2''-pyridyldithio)-propionamido)-β-cyclodextrin (5)

A 100-molar excess of 6-deoxy-6-(3'-(2''-pyridylthio)-propionamido)-β-cyclodextrin (**4**) in 100 µl of DMSO was added to a solution of fully reduced thiol-modified siRNA (100 µL in PBS buffer, pH 7.2). The pH was adjusted to 4-5 with 1M HCl and 100 mM sodium acetate. The reaction mixture was gently vortexed and incubated at 35°C overnight to obtain the CDSS-siRNA conjugate **(5)**. Excess of cyclodextrin was removed by using Amicon centrifugal columns

(M.W. 3000) (Millipore, Ireland) at 2,370 g, and the solution was reconstituted with 100 µL of RNAse-free water. CDSS-siRNA concentration was determined by measuring absorbance at 260 nm using a Nanodrop spectrophotometer.

Gel retardation assay of CD-siRNA conjugates

Agarose (1% w/v) (Sigma, MO, USA) solution was prepared with 1X Tris borate EDTA (TBE) buffer to which 6 µL of SafeView™ (NBS Biologicals Ltd, England) was added to visualize siRNA bands under UV light. The unconjugated and conjugated siRNA (36 µL) were each mixed with 10X Blue Juice gel loading buffer (4 µL) (Invitrogen, Carlsbad, California). Electrophoresis was carried out at 90 V for 45 minutes in TBE buffer. The siRNA bands were visualized by UV using DNR Bioimaging Systems MiniBis Pro and Gel capture US B2 software.

Electron-spray ionisation (ESI) mass spectroscopy of CD-siRNA conjugates

The samples for ESI were prepared at a concentration of 40-50 µM in 0.1M TEAA (triethanolamine acetate) buffer, pH 7. A C18 column was used with the following mobile phase: solvent A, 15 mM TEA with 400 mM HFIP (hexafluoro-2-propanol) (pH 7.9), measured by weight; solvent B, 50% solvent A with 50% methanol (v/v).

Cell culture

The human prostate cancer cells, DU145, were kindly donated by the Conway Research Institute, (University College Dublin, Dublin, Ireland). PC3 and PC3-Luc (over-expressing luciferase gene) were procured from European Collection of Cell Cultures (ECACC), UK and were grown in RPMI 1640 cell culture medium. Human glioblastoma cell line (U87) was a kind gift from Dr. Paul Young (University College Cork); cells were grown in DMEM. All culture media were

supplemented with 10% foetal bovine serum (FBS), 2 mM L-Glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. For passaging, 0.05% trypsin-EDTA (GIBCO, UK) was used. The cells were maintained in a humidified chamber at 37 °C with 5% CO_2.

Cellular delivery of CD-siRNA conjugates complexed with Lipofectamine 2000

Cellular delivery of luciferase CD-siRNA conjugates was performed using Lipofectamine 2000 (Sigma, USA): 50 nM of unconjugated or conjugated siRNA was delivered using Lf2000 with final vector:siRNA ratio 1µL Lf2000 : 20 pmol siRNA, as recommended by the supplier. 10,000 PC3-Luc cells/well were seeded in a 96-well white-opaque plate and transfection was carried out after 24 hours of seeding. The cells were incubated with the treatment samples for 4 hours, after which the medium was replaced with complete growth medium. The percentage of luciferase gene knockdown (protein suppression) was analyzed at 48 hours by measuring the luminescence intensity using a multiplate reader (Perkin Elmer – Wallac Victor 2TM 1420 multilabel counter).

Cellular delivery of CD-siRNA conjugates complexed with heptakis[2-O-(N-(3''-aminopropyl)-1'H-triazole-4'-yl-methyl)-6-dodecylthio]-β-cyclodextrin (a cationic amphiphilic cyclodextrin)

The cationic amphiphilic cyclodextrin was synthesized as previously described (O'Mahony et al., 2012). CD-siRNA nanocomplexes with cationic amphiphilic cyclodextrin were prepared following a published protocol (O'Mahony et al., 2012). Briefly, the cationic amphiphilic cyclodextrin was dissolved in chloroform (1mg/ml). The chloroform was evaporated under nitrogen gas to leave a thin layer of cyclodextrin, which was rehydrated with ddH_2O, at a final concentration of 1µg/µl. The aqueous solution was sonicated for an hour at RT before forming nanocomplexes with CD-siRNA conjugates. The cationic amphiphilic cyclodextrin

nanocomplexes with CD-siRNA conjugates were formed at mass ratio (MR) 20 (Singh et al., 2010). Specifically, 60µg of the cationic amphiphilic cyclodextrin in 800µl of ddH_2O, and 3µg of either unconjugated or conjugated CD-siRNA (CDmal-siRNA and CDSS-siRNA) each in 200µl of ddH_2O were separately dissolved. The CD-siRNA conjugate (200µl) was then added dropwise (2-3 drops each time) to the cationic amphiphilic cyclodextrin solution (800µl) and vortexed at a mild speed for 10-15 seconds, to give a final volume of 1 ml. The nanocomplexes were kept at RT for 30 mins for stabilisation.

PC-3 cells (100,000 cells/well) were seeded in a 24-well plate. CD-siRNA (PLK1) conjugates (50 nM) were delivered using the cationic amphiphilic cyclodextrin vector in serum-free and antibiotic-free medium. The medium was replaced with fresh complete growth medium (10% FBS) after 24 hours transfection. The percentage gene (PLK1) knockdown was determined at 48 hours by performing RNA extraction and qRT PCR.

Preparation of targeted CD-siRNA formulations with adamantyl-PEG-ligand and chitosan, and cellular delivery

Cell-targeted inclusion complexes of CD-siRNA conjugates were prepared using adamantyl-PEG500-RVG and adamantyl-PEG5000-dianisamide (AA2), and untargeted complexes using adamantane-PEG500 and adamantane-PEG500, respectively, at molar ratio 1:1. RVG ligand targeted acetylcholine receptors on U87 (human brain cancer cells) and AA2 targeted sigma 1 receptors on DU145 (human prostate cancer cells). Equimolar ratios of targeted and untargeted adamantyl derivatives were separately added to the CD-siRNA conjugates and mixed in a thermomixer at 900 rpm for 30 mins at RT. The inclusion complexes were then added drop-wise

to a solution of chitosan polymer (0.5 mg/mL, in 1% acetic acid, pH5) to form nanoparticles at MR 1 and 10. (Conditions for this formulation with chitosan had previously been optimised: ***Supplementary information)***. The mixture was vortexed mildly for 10-15 seconds, then left at RT for 30 mins. Concentration of CDmal-siRNA conjugate used was 100 nM. The nanoparticles prepared as above were incubated with U87 cells (25,000 cells/well in 24 well plate) and DU145 cells (50,000 cells/well in 24 well plate) for 24 hrs and gene knockdown was analyzed at 48 hrs.

For competitive inhibition assay in U87 cells (25,000 cells/well in 24 well plate), inclusion complex with the adamantyl-PEG500-RVG (targeted) formulation was formed and complexed with chitosan polymer at MR 1 and 10. For DU145 cells (50,000 cells/well in 24 well plate), inclusion complex with the adamantyl-PEG5000-AA2 (targeted) formulation was formed and complexed with chitosan polymer at MR 1 and 10. The cells were either treated or untreated with the 30μM of RVG peptide (U87 cells) for 30 mins and 100μM of adamantly-PEG5000-AA2 for 1 hr (DU145 cells), followed by treatment with their respective targeted formulations for 4 hrs. The PLK 1 gene knockdown analysis was performed at 24 hrs.

Determination of gene knockdown using quantitative real-time PCR

Cells were lysed and RNA was extracted after 48 hours transfection using GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma) as per manufacturer's instructions. Concentration of the total RNA was quantified using a NanoDrop ND-1000 UV-vis Spectrophotometer and complementary DNA (cDNA) was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was assessed by real-time qPCR using the Applied Biosystem's Real Time PCR System (Model 7300). Cycling conditions were as follows: 10 min (min) at 95 °C, 40 cycles [of 15 sec at 95 °C; 1 min at 60 °C]. Assays were

performed using appropriate primer sets for PLK1 (catalogue number Hs00153444_m1) and β-actin (catalogue number Hs01060665_g1) (Taqman®, Applied Biosystems). The quantitative level of each PLK1 mRNA was measured as a fluorescent signal corrected according to the signal for β-actin mRNA. The 2-delta Ct method was used to quantify the relative changes in mRNA.

Statistical Analysis

Statistical analysis was carried out using Graphpad Prism 5 software (CA, USA). Statistical comparisons were carried out using One-way analysis of variance (ANOVA) followed by Tuckey's Post Hoc test and unpaired two-tailed student t-test. Statistical significance was set at $P < 0.05$, $P < 0.01$ and P values < 0.001 were considered highly significant.

Results and Discussion

Synthesis and characterization of CD-siRNA conjugates

CD-siRNA conjugates were prepared using biologically non-cleavable (sulfanyl) and cleavable (reducible disulphide) linkers between the cyclodextrin and siRNA. To obtain a non-cleavable link, 6-amino-β-cyclodextrin was reacted with 3-(N-maleimido)-propionic acid N-hydroxysuccinimide ester according to the synthetic **Scheme** to obtain product **2** in 79% yield. To obtain a cleavable link, 6-amino-β-cyclodextrin was reacted with 3-(2'-pyridyldithio) propionic acid N-hydroxysuccinimide ester according to the **Scheme** to obtain product **4** in 86% yield. The siRNAs used for conjugation (luciferase and PLK1) had thiol modification on the 3' end of the sense strand and were fully reduced according to the manufacturer's protocol, prior to conjugation. Reacting the reduced siRNAs with the activated cyclodextrins **2** and **4** gave the

conjugates CDmal-siRNA **3** and CDSS-siRNA **5**, where each conjugate had either luciferase or PLK1 siRNA. Successful conjugation was confirmed by gel retardation assay (**Figure 1)**, which show band shifts relative to the unconjugated siRNA. Conjugation was further confirmed by mass spectroscopic (ESI) analysis (**Figure 2**, **Table1**). The molecular weights of sense and antisense strands of luciferase siRNA as stated by the supplier (Gene Link, USA) were 7979 Da and 7514 Da, respectively. ESI showed successful conjugation of luciferase siRNA (sense strand) with CD-mal (MW 1285.11 Da) **(Figure 2B)** and CD-SS (MW 1331.27 Da) **(Figure 2C)**, resulting in increased molecular weights of 9261.63 Da and 9325.54 Da.

Cellular delivery of CD-siRNA conjugates using polycationic lipids Lipofectamine 2000 and heptakis[2-O-(N-(3′′-aminopropyl)-1′H-triazole-4′-yl-methyl)-6-dodecylthio]-β-cyclodextrin

Figure 3 shows the reduction in luminescence intensity of PC3-Luc cells (overexpressing luciferase protein) on treatment with luciferase siRNA or its cyclodextrin conjugates, CDmal-siRNA and CDSS-siRNA, mediated by Lf2000 for 4 hrs. Suppression of luciferin protein in PC3-Luc cells by the conjugated siRNA was found to be comparable to that for the unconjugated siRNA ($P>0.05$). This confirmed that conjugating cyclodextrin at the 3'end of siRNA does not hinder the silencing ability of siRNA.

A similar mode of delivery used the less cytotoxic cyclodextrin vector heptakis[2-O-(N-(3′′-aminopropyl)-1′H-triazole-4′-yl-methyl)-6-dodecylthio]-β-cyclodextrin. Mass ratio MR 20 (cyclodextrin:siRNA) is known to be optimal (O'Mahony et al., 2012) and non-toxic to cells over 24 hours (Evans et al., 2015). **Figure 4** shows that PLK1 gene knockdown by CD-siRNA

conjugates, quantified by qRT-PCR, was comparable to that of the unconjugated siRNA (67% expression) ($P<0.05$). The siRNA conjugated through a cleavable (disulfide) linker showed greater knockdown (57% expression) compared to the siRNA conjugated through a non-cleavable (maleimide) linker (73% expression) ($P<0.001$). This result is in accordance with other studies (Singh et al., 2010). More importantly, siRNA conjugation with cyclodextrin does not cause the silencing inhibition observed, for example, with conjugated PEG molecules of large molecular weight (Jung et al., 2010; Iversen et al., 2013).

Cellular delivery of CD-siRNA conjugates using a targeted nanoparticle formulation

Besides delivery by polycationic lipids, which demonstrated the ability of the CD-siRNA conjugates to produce knockdown, we incorporated the conjugates into nanoparticulate formulations to demonstrate the special assembly properties conferred on the RNA by such conjugation. As outlined in **Figure 5A**, the molecular inclusion properties of the cyclodextrin moiety was first used to incorporate an adamantyl-conjugated targeting ligand (RVG or dianisamide), and the resulting polyanionic RNA assembly was charge-neutralised with (polycationic) chitosan to create ligand-directed nanoparticles. An isothermal calorimetry (ITC) analysis for CD and adamantyl-PEG compounds has shown that PEGylation of adamantane does not seriously inhibit the formation of inclusion complexes with cyclodextrins (Fitzgerald et al., 2016). Following the formation of inclusion complexes with the CD-siRNA conjugates, the complexes were mixed with chitosan polymer at MR 1 and 10. The use of chitosan polymer was first optimized with unmodified, non-conjugated siRNA (FAM-labelled scrambled) ***(Supplementary information)***. Chitosan was chosen as a charge-neutralising polymer due to its low toxicity, biodegradability and "proton sponge" effect that aids endosomal release of the

nanoparticle (Malhotra et al., 2009). This optimization ensured that cellular uptake of nanoparticles is specifically through the targeting ligands on their surface and not through the positive charge of excess chitosan.

Figure 5A illustrates the steps to form targeted nanoparticles using CD-siRNA conjugates. The optimization ***(Supplementary information)*** confirmed formation of nanoparticles with near neutral surface charge and size ~100 nm at chitosan:siRNA MR of 1 and 10. The purpose of selecting nanoparticles at MR 1 and 10 was to compare the ability of the nanocomplexes to deliver the therapeutic via receptor-mediated delivery, thereby eliminating non-specific adsorptive endocytosis. Also, it is known from literature that loose complex with less electrostatic interaction fail to deliver therapeutics as efficiently as the compactly complexed nanoparticles (Park et al., 2013). The reason is the lack of target specificity, which leads to a lack of endocytosis by the cell membrane. Thus, we tested complexes formed at MR 1 (loose nanoparticles) and MR 10 (compact nanoparticles), which had just the optimal amount of cationic chitosan polymer necessary to compact the siRNA while preventing inhibition of the ligand-guided, receptor-mediated delivery. **Figure 5B and 5C** represent relative PLK1 expression in U87 and DU145 cells analyzed using qRT-PCR. Gene knockdown of 30 - 40 % ($P<0.0001$) was obtained at MR1 and 60 - 70% ($P<0.0001$) at MR10 in U87 cells, with the RVG-targeted formulation being especially significant ($P<0.05$), when compared with the untargeted formulation or the conjugate alone. A similar trend was obtained in DU145 cells with the anisamide targeting ligand where gene knockdown levels of: 40 - 50% at MR1 ($P<0.0001$) and 75% at MR 10 ($P<0.0001$) were detected. At MR 1, however the difference was not statistically significant.

To further validate the receptor-mediated uptake of the targeted nano-formulation, a competitive inhibition assay was performed. As shown in **Figure 5D** and **5E**, while the effect detectable with MR1 was not significant, pre-treatment with the ligand significantly lowered the knockdown levels for chitosan formulations at MR10. These results demonstrate that ligand targeting can be successfully achieved with these formulations by exploiting the inclusion complex formation abilities retained in the CD-siRNA conjugates.

In summary, this is the first example of formation of CD-siRNA conjugates. It has been shown that cyclodextrin can be covalently attached to the sense strand of siRNA without adversely affecting its knockdown ability. Such conjugation can also involve the RNA in the versatile self-assembly properties of the cyclodextrin thus providing new opportunities to develop unconventional formulations for therapeutic oligonucleotides.

Conclusion

Cyclodextrin has been successfully conjugated at its 6-position, via disulfide and sulfanyl links, to the sense strands of siRNAs at the 3'-position. Retention of the gene knockdown activity of the RNAs, directed at cancer cells that over-express either luciferase gene or PLK1 gene, was assessed by delivery of the CD-RNA conjugates using two polycationic lipids: Lipofectamine 2000 and a polycationic amphiphilic cyclodextrin. Knockdown activity has been found to be comparable to that of the unconjugated RNAs as delivered by the same vectors, demonstrating that the chosen modes of conjugation to the cyclodextrin do not adversely affect its functioning as an enzyme substrate in the interference pathway. In addition, the disulphide-linked conjugates demonstrated a significantly higher level of knockdown compared to the sulfanyl-linked conjugates, probably due to disulfide biolability.

Following confirmation of the silencing activity of CD-siRNA conjugates, an example of performance of the cyclodextrin moiety in enabling a new mode of formulating RNA was assessed. This involved formation of a molecular inclusion complex by the conjugated cyclodextrin with adamantyl-PEG-ligand to exploit ligand targeting, and charge neutralisation by chitosan. Superior knockdown was obtained with these targeted formulations, demonstrating that CD-siRNA conjugates can enable formation of targeted charge-neutral formulations for receptor-mediated endocytosis. Further studies will involve evaluation of RNA conjugates with modified functionalised cyclodextrins.

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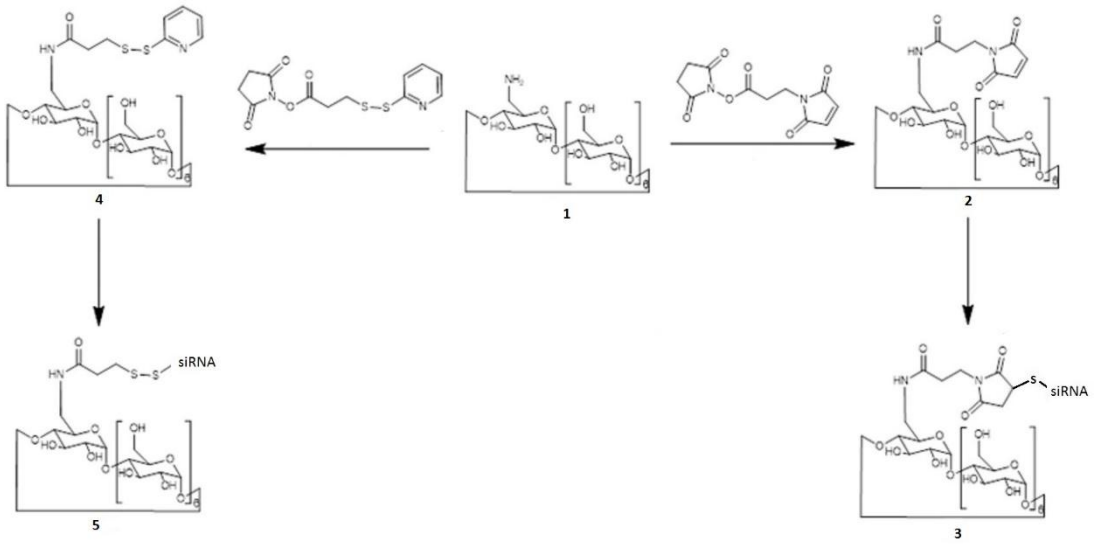
Figures:



Scheme: Synthesis of CD-siRNA conjugates from mono-6-amino-6-deoxy-β-cyclodextrin **(1)**.

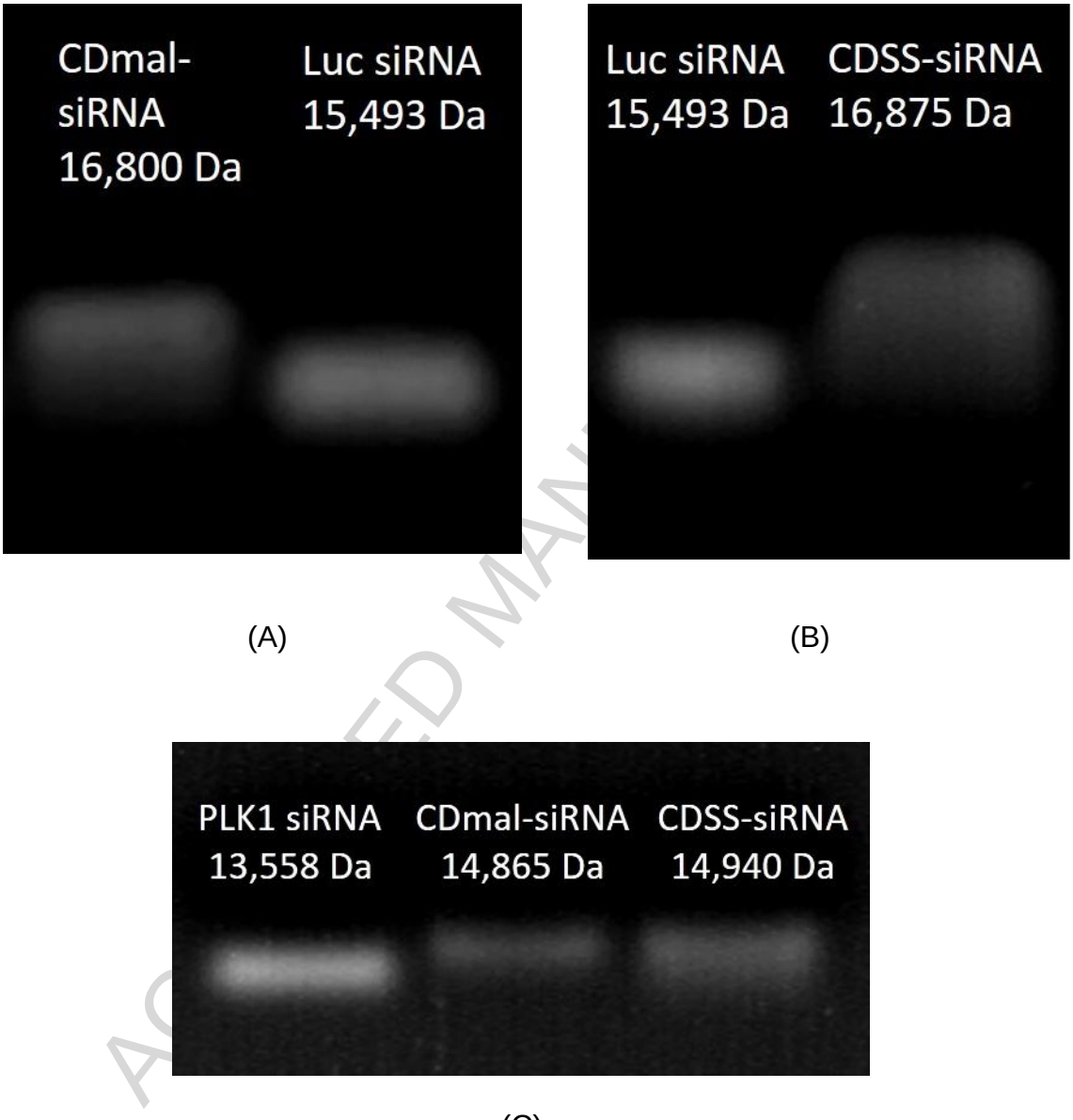
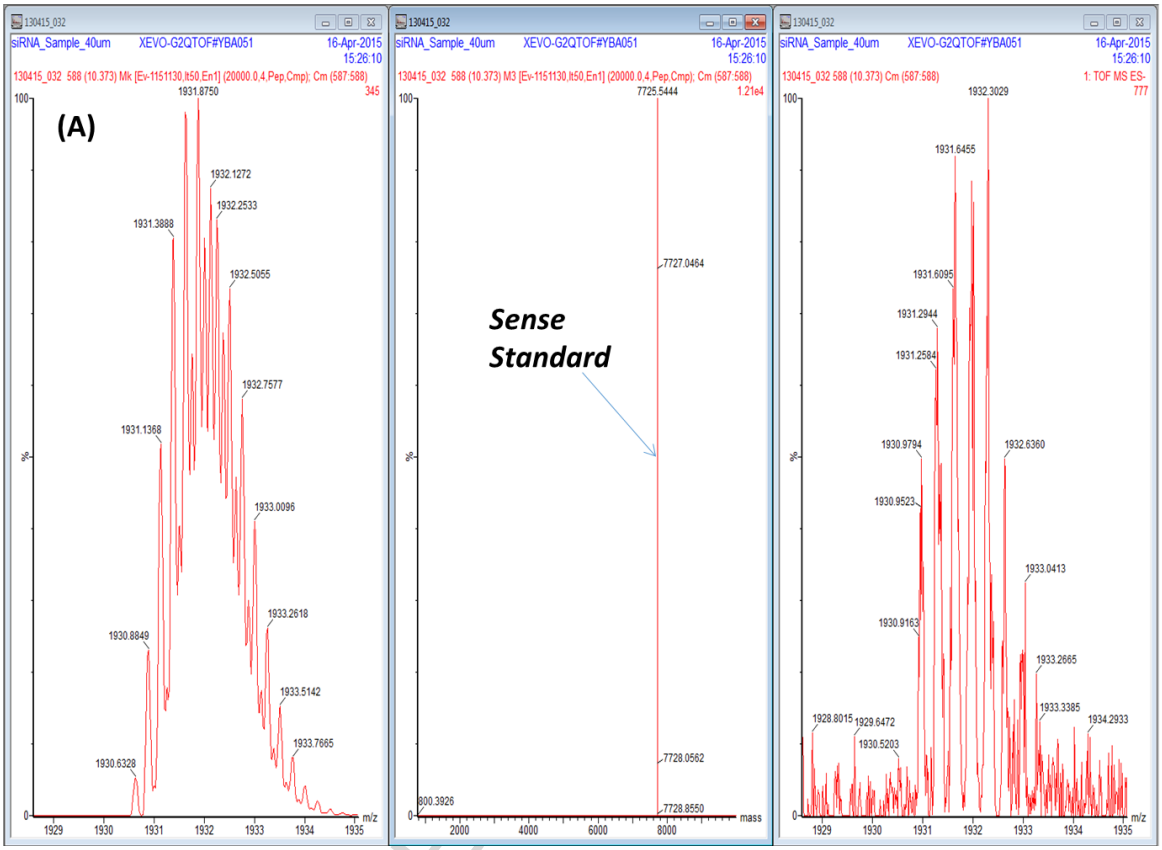
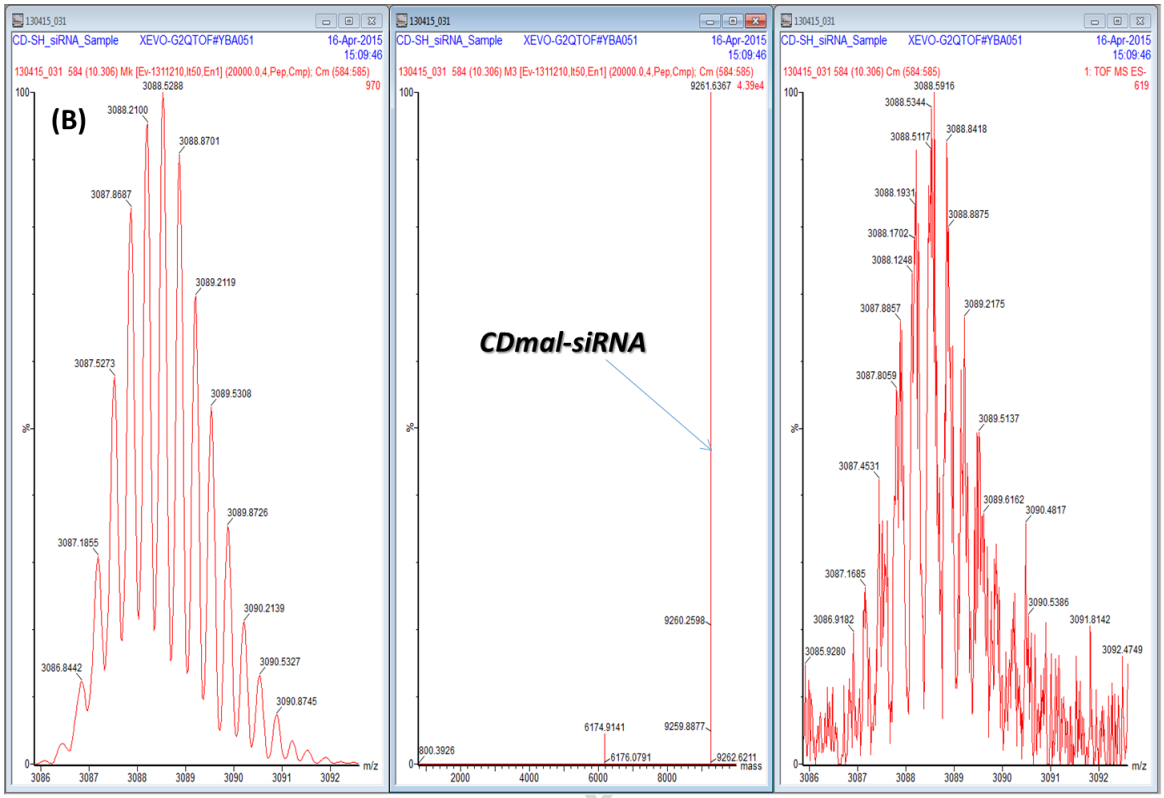


Figure 1: Gel retardation assays of siRNAs and their cyclodextrin conjugates: (a) CDmal-siRNA (luciferase); (b) CDSS-siRNA (luciferase); (c) CDmal-siRNA and CDSS-siRNA (PLK1).





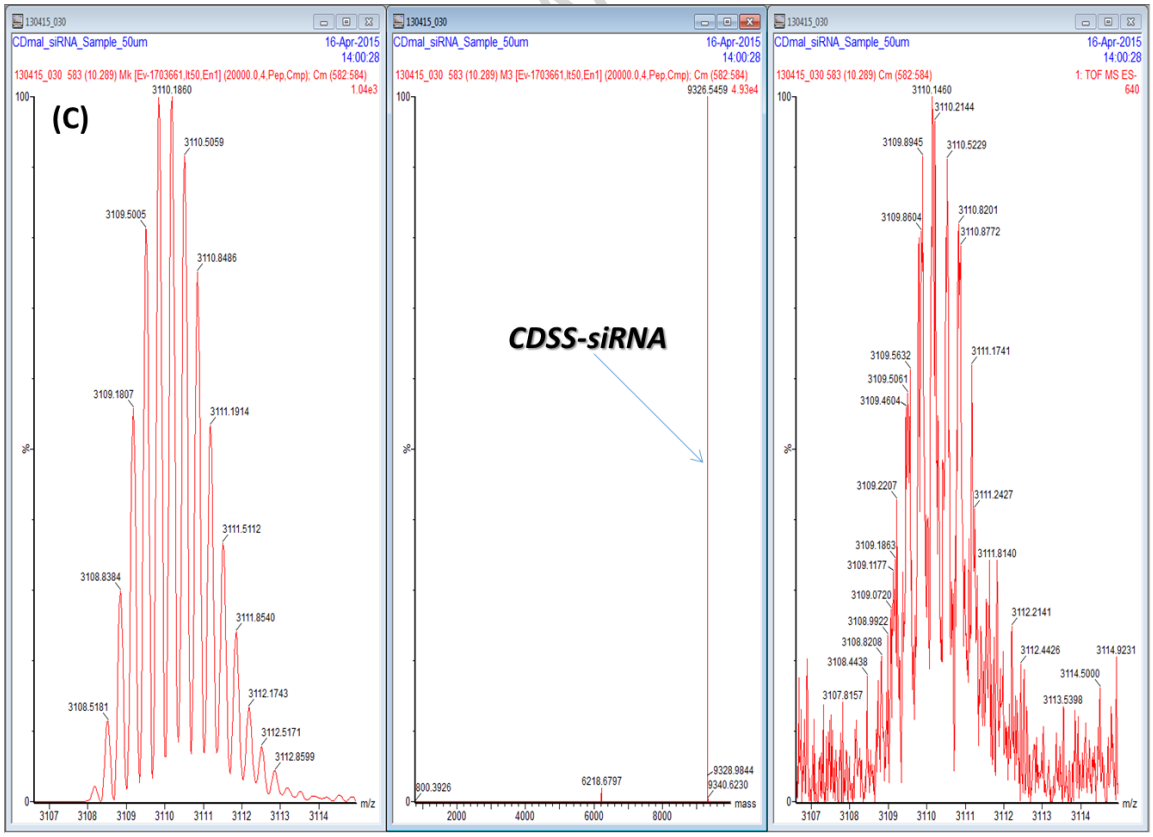


Figure 2: ESI measurement of **(A)** unconjugated sense strand of Luciferase siRNA (M.W. 7725.54), **(B)** conjugated sense strand of siRNA with CDmal (M.W. 9261.63) and **(C)** conjugated sense strand of siRNA with CDSS (9325.54).

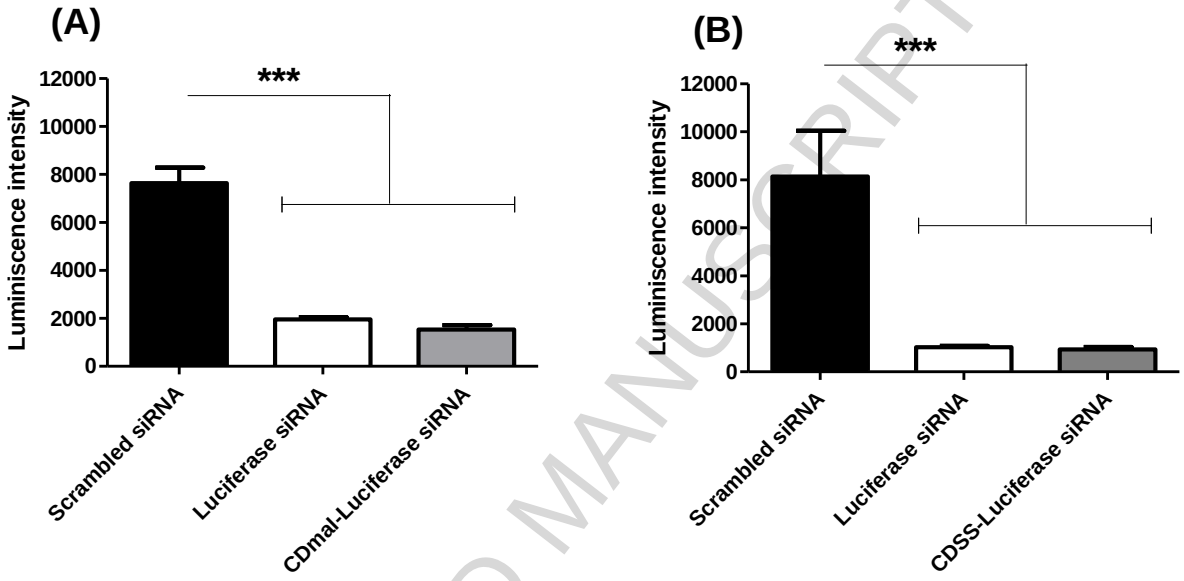


Figure 3: Reduction in luminescence intensity of PC3-Luc (luciferase) cells on treatment with CD-siRNA (luciferase) conjugates delivered by Lipofectamine 2000: **(A)** CDmal-siRNA, **(B)** CDSS-siRNA. The graph shows a representative result of (n = 6) mean ± S.D. ***P < 0.001 was considered highly significant based on Tuckey's posthoc analysis.

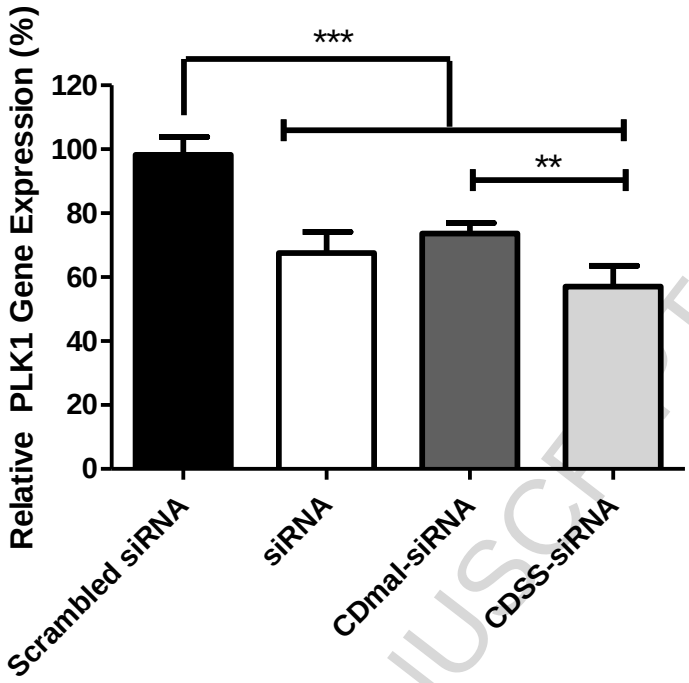
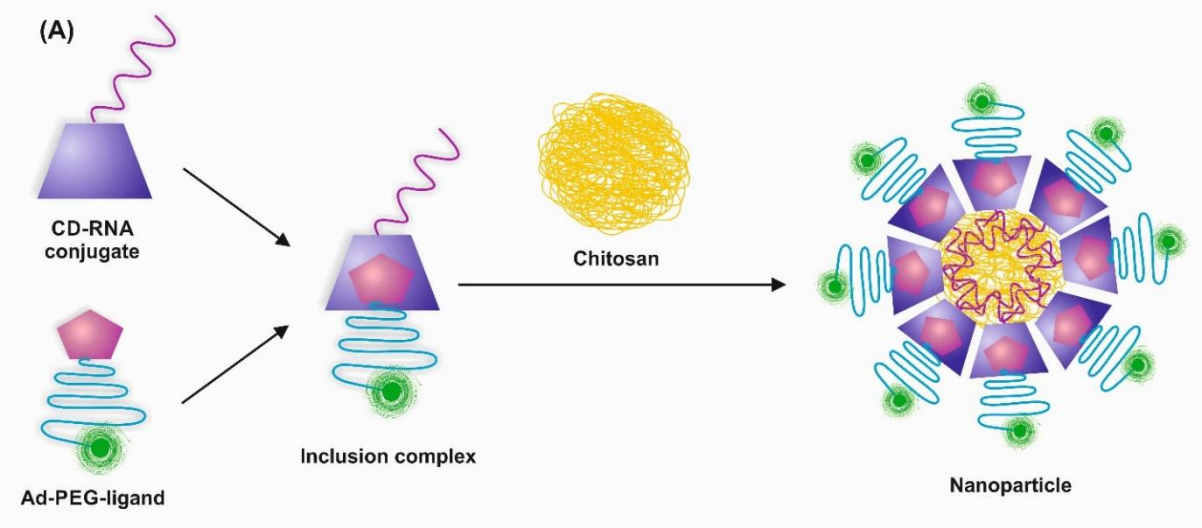


Figure 4: Gene knockdown in PC3 cells with CD-siRNA (PLK1) conjugates (50 nM) delivered by cationic amphiphilic cyclodextrin at MR 20. The graph shows a representative result of (n = 4) mean ± S.D. **P < 0.01 and ***P < 0.001 were considered highly significant based on Tuckey's posthoc analysis.



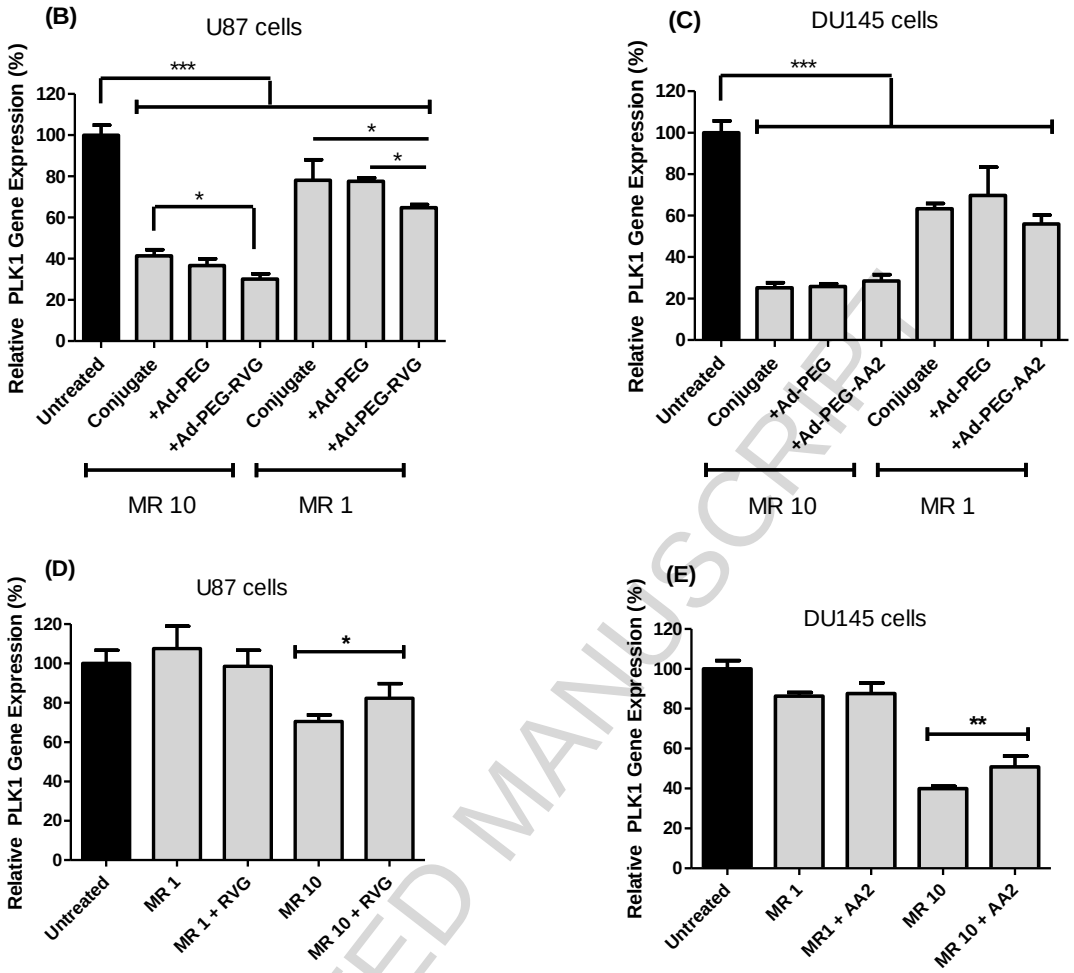


Figure 5: (A) Impression of complexation of CD-siRNA with adamantyl-PEG-ligands and chitosan to form targeted nanoparticles. **Gene knockdown** in **(B)** U87 and **(C)** DU145 cells by CDmal-siRNA (PLK1) conjugate (100 nM), complexed with adamantyl-PEG (Ad-$PEG_{500/5000}$) or –PEG_{500}-RVG or –PEG_{5000}-AA2, and chitosan at MR (chitosan:CD-siRNA conjugate) 1 and 10. **Competitive inhibition assay** in **(D)** U87 cells and **(E)** DU145 cells with CDmal-siRNA (PLK1) conjugate (100 nM), complexed with adamantyl-PEG_{500}-RVG and adamantyl-PEG_{5000}-AA2, respectively and chitosan at MR (chitosan:CD-siRNA conjugate) 1 and 10. The graph shows a representative result of (n = 4) mean ± S.D. Graphs (B) and (C) were analyzed by One-way Anova where *P < 0.05 and ***P < 0.001 were considered highly significant based on Tuckey's posthoc analysis. Graphs (D) and (E) were analysed by unpaired, two-tailed student t-test where *P < 0.05 and **P<0.01 were considered significant.

Sample	Calculated mass of sense strand (Da)	Theoretical mass of sense strand (Da)
Luciferase siRNA	7725.54	7979
CD-mal-siRNA	9261.63	9264.11
CD-SH-siRNA	9325.54	9310.27

Table 1: ESI mass of CDmal (M.W. 1285.11 Da) and CDSS (M.W. 1331.27 Da), conjugated with the sense strand of (Luciferase) siRNA.

Graphical abstract