A click chemistry route to 2-functionalised PEGylated and cationic β-cyclodextrins: co-formulation opportunities for siRNA delivery

*A.M. O'Mahony [1†], J.Ogier[2†], S. Desgranges[2], J.F. Cryan[3], R. Darcy[2] and C.M. O'Driscoll[1]**

[1] *School of Pharmacy, University College Cork, Cork, Ireland*

[2] *Centre for Synthesis and Chemical Biology, School of Chemistry, University College Dublin, Dublin 4, Ireland*

[3]*Dept. of Anatomy and Neuroscience, University College Cork, Cork, Ireland*

**caitriona.odriscoll@ucc.ie*

[†]These authors made an equal contribution to this work

Abstract

A new approach to the synthesis of amphiphilic β-cyclodextrins has used 'click' chemistry to selectively modify the secondary 2-hydroxyl group. The resulting extended polar groups can be either polycationic or neutral PEGylated groups and these two amphiphile classes are compatible in dual cyclodextrin formulations for delivery of siRNA. When used alone with an siRNA, a cationic cyclodextrin was shown to have good transfection properties in cell culture. Co-formulation with a PEGylated cyclodextrin altered the physicochemical properties of nanoparticles formed with siRNA. Improved particle properties included lower surface charges and reduced tendency to aggregate. However, as expected transfection efficiency of the cationic vector was lowered by co-formulation with the PEGylated cyclodextrin, requiring future surface modification of particles with targeting ligands for effective siRNA delivery.

Introduction

Small interfering RNAs (siRNAs) are capable of specific and efficient gene silencing,[1] via the intracellular RNA interference (RNAi) pathway and are particularly useful for studying gene function. They also present a promising therapeutic strategy in a wide variety of diseases. As oligonucleotides however, siRNAs show poor cell penetration and limited stability in a physiological environment,[2] therefore considerable research has focused on development of vectors for their delivery.

Cyclodextrins (CDs), cyclomalto-oligosaccharides, are naturally occurring oligosaccharides which are well established pharmaceutical exipients. They offer many advantages as cores for the synthesis of non-viral vectors and are by now well established as potential delivery agents for therapeutic oligonucleotides.[3-5] Amphiphilic cationic cyclodextrins have been particularly promising.[3, 6-10] The choice of cationic group and length of amphiphilic chain have both been shown to influence binding of pDNA, properties of the complexes formed and transfection efficiency.[4, 8, 11, 12]

Cationic vectors for siRNA delivery offer several advantages which usually enhance the overall results for transfection in cell cultures, namely lowered tendency to aggregate in formulations and good (though non-specific) adhesion to cell surfaces. However, there are drawbacks associated with such cationic molecules including cellular toxicity and limited circulation *in vivo*. One approach to overcoming these drawbacks is the modification of formulations with poly(ethylene glycol) (PEG), which has been widely reported.[13-15] The PEG provides a surface which is both neutral and non-aggregating, with reduced toxicity. In addition, PEG chains are proven as long linkers for exposition of surface ligands which can guide delivery to cell receptors.[16]

Here we have devised a new strategy for synthesis of cationic and neutral PEGylated CDs which possess similar amphiphilicity, thus enabling co-assembly and co-formulation to provide a PEGylated surface to cationic CD.siRNA nanoparticles. In each series, the polar

groups are attached by cuprous-catalysed 'click' chemistry. Previously click chemistry has been used on the primary side of β-CD gene delivery vectors,[17-20] with only limited reports of its use on the secondary side.[21] The extension of any such chemistry to the secondary side with its two sets of hydroxyl groups presents a challenge in selectivity, as options are limited for introduction of groups at the 2-positions only.[22] We previously grafted short oligo(ethylene oxide) groups at these positions by base-catalysed reaction with ethylene carbonate.[3, 23] However we found this reaction of limited use for chains longer than about two to three ethylene oxide units. This may be due to steric hindrance or the formation of ester rather than oxo links. Photochemical addition of thiols to 2-alkenylated CDs has lent itself well to the introduction of lipid groups at the 2-positions,[6] but is a less convenient option for the attachment of PEG. Here, while thioalkylation is used to provide the hydrophobic groups on the primary-OH face as before,[3, 23] copper (I)-catalysed 'click' chemistry is used to attach either cationic or PEG groups to form the polar secondary side. This strategy offers many possibilities for chemical functionalisation, along with relative ease of synthesis and high yield.

In this study we synthesised two CDs, an amphiphilic cationic CD and an amphiphilic PEGylated CD and blended these together to yield co-formulations with various degrees of PEGylation. Properties of CD.siRNA complexes formed by the cationic amphiphilic cyclodextrin alone and by the co-formulated CDs were investigated, including size, charge, aggregation, transfection efficiency in Caco2 cells and effects on cell viability.

Results and Discussion

Compound **3** was synthesised from β-cyclodextrin (**Scheme 1**) and used as a common intermediate for synthesis of the cationic and pegylated cyclodextrins, **5** and **7**. Starting

from the natural β-cyclodextrin, *tert*-butyldimethylsilyl chloride selectively introduced the TBDMS group at the 6-positions to give **1**. Reaction with propargyl bromide yielded compound **2**, which was reacted with a triphenylphosphine bromine complex to afford the bifunctional intermediate **3** with an overall yield of 55%.

CD **3** posesses two reactive chemical functions: bromo groups for displacement by dodecyl thiolate to form the lipidic groups of the final amphiphilic cyclodextrins and azide for "click" chemical introduction of the polar groups or PEG groups on the 2-positions. Synthesis of the cationic cyclodextrin **5** from **3** started with the 'click' reaction between **3** and *tert*-butyl 3-azidopropylcarbamate**,** carried out in water with a cuprous catalyst to afford **4**. Thioalkylation of this with dodecane thiol and deprotection of the Boc-protected amine groups gave **5**. The PEGylated cyclodextrin **7** was also formed from compound **3**, starting with its cuprous-catalysed reaction with ω-O-methyl-poly(ethylene glycol) azide (**Scheme 2**). This reactive PEG was synthesised in two steps: monomethyl-PEG 500 was activated using mesyl chloride and the mesyl group substituted using sodium azide. The 1,3-dipolar cycloaddition to **3** was carried out in water-DMF to yield compound **6**, which was again thioalkyated using dodecane thiol, to obtain **7**.

The choice of SC_{12} as the lipid chain at the 6-positions was decided by previous work where we observed that self-assembly into bilayers required chains preferably greater than C_{10}, whereas above C_{12} solubility became a problem during the synthesis.[24] In deciding on the extended PEG chain, the potential for immunogenicity of chains longer than PEG 500 was kept in mind.[25]

The use of polyethylene glycol as a surface coating for nanoparticles delivering siRNA is well established with small cationic lipid and polymer vectors.[16] PEG chains have been attached by various means, such as chemical reaction with the particle surface[14] or post-formulation insertion into the outer layers of nanoparticles when the PEG is part of an amphiphile.[26] PEGylation of CD-based gene delivery vectors relied upon inclusion of adamantane-PEG into the hydrophobic cavities of surface CD groups,[27-29] rather than directly attaching the PEG to

the CD itself. The rationale is that the PEG will provide a charge-neutral and non-aggregating surface which will not interact with proteins and which has scope for receptor-ligand attachment. Here we have taken another approach, namely the co-formulation of two structurally similar vectors, where one, CD **5**, is the cationic condensing agent for the siRNA, and the other, CD **7**, bears the PEG chains. Their structural similarity made it possible to apply them as a dual formulation. This strategy enables numerous possibilities for varying the formulation by, for example, attaching different length PEG chains to the PEGylated CD, attaching cell-specific targeting ligands via the 'click' linker or by adjusting the proportions of the two CDs incorporated into the CD co-formulation. A similar approach was reported for a polyamidoamine-based polymer which was mixed with its PEGylated counterpart, before siRNA complexation to yield polyplexes with increasing extent of PEGylation.[30]

In this paper, firstly the cationic CD **5** was assessed, after complexation with siRNA at increasing mass ratios (MR, μg CD **5**: μg siRNA). Then CD **5** was co-formulated with CD **7**, at molar ratio CD **5**: CD **7** 1:0 to 1:1, yielding a series of CD formulations with varying degrees of PEGylation, which were subsequently complexed with siRNA. The properties and transfection efficiency of all formulations were assessed.

The siRNA binding efficiency of the cationic CD **5** was measured using agarose gel electrophoresis. Uncomplexed siRNA, used as a control, migrated freely through the gel. At a MR of 2, almost no migration was observed and at MR 5 and above there was complete binding (results not shown). Mixing the PEGylated CD **7** with the cationic CD up to equimolar ratio, while maintaining the same cationic CD:siRNA mass ratio, did not affect complexation. This observation is in contrast to a previous study which showed lack of pDNA condensation when a CD-based polymer was PEGylated before complexation [29] and another whereby a co-formulation consisting of cationic and neutral amphiphilic CDs exhibited poor protection of pDNA.[36]

CD:siRNA complex nanoparticles were characterised in terms of size and charge, both of which can influence transfection efficiency.[31, 32] Upon complexation with siRNA at MR 10 (equivalent to N/P ratio 6.5), the cationic CD **5** (1:0) gave nanoparticles of ~ 240 nm (Fig. 1). Smaller particles (< 150nm) were obtained at MR 20 and MR 30 (data not shown). A reduction in particle size was observed upon addition of the PEGylated CD to the formulation, in particular at molar ratio 1:1 (Fig. 1). However, the polydispersity index at this molar ratio was 0.6, indicating considerable variation in particle size.

The complexes formed between cationic CD **5** and siRNA had a positive charge (+42 mV) at MR 10 (Fig. 1). A positive surface charge contributes to interaction with cellular membrane proteoglycans, enabling uptake.[9] The co-formulations produced by mixing the PEGylated CD with the cationic CD had reduced surface charges (+19 mV at molar ratio 1:1), an expected shielding effect by the PEG groups at the particle surface. A similar modest effect on charge has previously been reported after inclusion of PEG groups into polyethylenimine and cyclodextrin-based polymers.[15] Masking the surface charge of cationic nanoparticles has been reported to reduce toxicity [13, 30] and minimise non-specific interactions with plasma components.[30, 33]

As well as reducing surface charge of the nanoparticles, incorporation of the PEGylated CD had a significant effect on aggregation in OptiMEM transfection medium (Fig. 2). Complexes formed with the cationic CD **5** and siRNA (MR10) aggregated in the high salt environment of the medium, whereas aggregation was completely prevented in the formulations containing the highest proportions of the PEGylated CD **7** (molar ratio 10:1 and 1:1). Therefore stability of the formulation was improved by incorporation of the PEGylated CD **7**.

Gene silencing by cationic CD **5** in Caco2 cells was assessed by ability of the CD:siRNA complexes to silence an exogenously transfected luciferase reporter plasmid. As shown in Fig. 3, the optimal mass ratio for gene silencing was considered to be MR 10, at which a significant reduction in luciferase gene expression (76 ± 7 %, $*p < 0.05$ compared to controls) was observed. No further improvement in knockdown was observed at MR 20 (70 ± 8 %). A

similar level of knockdown was achieved with the cationic lipid vector Lipofectamine™ 2000. Neither siRNA alone, nor a non-silencing control siRNA complexed to CD **5** (MR 10:non-silencing siRNA (MR10/ns)), mediated gene silencing (Fig. 3). Due to its high transfection efficiency, MR 10 was chosen for further experiments using the mixed CD formulation.

On addition of the PEGylated CD, no significant knockdown was observed even with low proportions incorporated into the formulation (Fig. 4). This was despite the fact that the PEGylated CD **7** had no effect on siRNA binding and did not make the co-formulated complexes totally charge-neutral. It is possible that steric effects of the PEG groups blocked charge-interaction with the cell membranes, or that the PEG groups interfered with intracellular trafficking. PEGylation of particles formed with DNA has previously been shown to reduce the transfection efficiency of polyethylenimine (PEI) and a linear cyclodextrin-containing polymer,[15] an effect attributed to either reduced cellular uptake or impaired intracellular processing. Similarly, a PEGylated lipid vector was unable to deliver pDNA or siRNA *in vitro* as compared to other polymeric or lipid vectors.[34] However, in contrast to these results, another study showed improved gene silencing *in vitro* with a PEG-modified PEI vector,[14] due to various factors including improved siRNA stability in serum and effective endosomal release. When PEG was conjugated to poly-L-lysine by a pH-sensitive linker, it was considered to be cleaved in the acidic environment of the endosome so that it did not interfere with subsequent siRNA-mediated gene silencing.[13] Therefore, the effects of PEGylation on transfection appear to depend on linker and environment. Also, reduction of transfection *in vitro* may not be seen *in vivo*, as was reported by Hatakeyama *et al*. who observed greater activity with a PEGylated lipid vector on systemic administration at a tumour site.[35]

Finally, the cytotoxicity of each of the CD vectors in Caco2 cell cultures was assessed by means of an MTT assay. Cells were incubated with increasing concentrations of either cationic CD **5** or **7** for four hours. For the cationic CD, at concentrations of up to 30 μM, at

least 85% of cells remained viable compared to untreated controls (Fig. 5). The concentration of cationic CD used for transfection experiments at CD:siRNA MR10 was approximately 1.7μM – little or no cytotoxicity was evident at this concentration. The PEGylated CD did not affect viability at any of these concentrations, as expected in view of its neutral charge.

Conclusions

Copper-catalysed 'click' chemistry can be used to modify the 2'-hydroxyl position of β-cyclodextrin in the synthesis of amphiphilic CDs having either cationic primary amine groups or lengthy PEG chains on this secondary side. The two types of amphiphile are compatible for co-formulation into nanoparticles with suitable properties for siRNA delivery.

High levels of gene silencing were achieved *in vitro* with a cationic CD alone. Nanoparticles formed with siRNA using dual formulation of the cationic and a PEGylated CD displayed improved physicochemical properties, namely reduced surface charge and lowered aggregation. As expected, the modified particle surface also led to reduced transfection efficiency compared with the unPEGylated particles, probably due to steric masking of the positive charge by the PEG groups. However, the provision of receptor cell-targeting ligands at this surface is expected to restore transfection efficiency, but with the added advantages of specificity and lowered toxicity. Meanwhile this approach to formulating PEGylated vector-siRNA complexes is a further demonstration of the advantages of cyclodextrins as core molecules for siRNA delivery.

Experimental details

Materials

Cyclodextrin (Aldrich) was dried for 12 h at 100 ◦C under vacuum. *Tert*-butyl 3-azidopropylcarbamate was from LuminoChem, and for safety this low-MW azide was stored as a refrigerated solution in dimethylformamide. Monomethoxy PEG500 was from Aldrich. Anti-luciferase siRNA (sense strand CUU ACG AGUA CU UCG AdT dT, antisense strand UCG AAG UAC UCA GCG UAA GdT dT), and negative siRNA (sense strand UUC UCC GAA CGU GUC ACG UdT dt) were from Qiagen.

Synthesis of modified cyclodextrins

Heptakis(2-O-propargyl-6-O-*tert*-butyldimethylsilyl)-β-cyclodextrin (2)

Heptakis(6-O-tert-butyldimethylsilyl)-β-cyclodextrin **1** (1g, 0.52 mmol, 1 eq.) in 100 mL of anhydrous DMF was stirred at 0 °C under nitrogen for 30 min. Sodium hydride (125 mg, 5.17 mmol, 10 eq.) was added slowly, and the solution was stirred at 0°C for 3 h and at r.t. for 12 h. The solution was then cooled to 0 °C and 550 mg (4.65 mmol, 9 eq.) of propargyl bromide (80% in toluene) was added. The resulting solution was stirred for 2 h at 0 °C and 12 h at r.t. under N_2. The solution was concentrated under reduced pressure, taken into ethyl acetate (50 mL) and washed with brine (100 mL). The residue after evaporation was purified by column chromatography over SiO_2 (cyclohexane-ethyl acetate, 3:1) to give **2** as a colourless solid (765 mg, 68%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.94 (d, *J* = 3.6 Hz, 1H), 4.66 (s, 1H), 4.50 (t, *J* = 2.4 Hz, 2H), 3.98 – 3.87 (m, 2H), 3.63 (d, *J* = 11.2 Hz, 1H), 3.52 (dd, *J* = 9.0, 4.3 Hz, 3H), 2.42 (t, *J* = 2.3 Hz, 1H), 0.84 (s, 9H), 0.00 (d, *J* = 2.7 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 101.32, 82.13, 79.69, 79.12, 75.20, 73.62, 71.92, 61.92, 59.56, 27.13, 26.13, 18.49, -4.93. MALDI-MS: 2224.48 $[M+Na]^+$; 2240.58 $[M+K]^+$.

Heptakis(6-bromo-6-deoxy-2-O-propargyl)-β-cyclodextrin (3)

Triphenylphosphine (1.22 g, 4.0 mmol, 22 eq.) in 50 mL of anhydrous dichloromethane was stirred at 0 °C under nitrogen for 30 min before addition of bromine (180 µL, 3.63 mmol, 20 eq.) dropwise. The solution was stirred for 30 min at 0 °C and for 2 h at r.t. until a white precipitate appeared. The solution was then cooled to 0 °C and 400 mg (0.18 mmol, 1 eq.) of heptakis(2-O-propargyl-6-O-*tert*-butyldimethylsilyl)-β-cyclodextrin **2** was added. The solution was stirred at r.t. for 12 h, then concentrated under reduced pressure. The residue was taken into ethyl acetate (50 mL) and washed with brine (100 mL). The residue after re-evaporation was purified by column chromatography over SiO_2 (cyclohexane-ethyl acetate, 4:1 to 1:1) to give **3** as a colourless solid (296 mg, 88%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.07 (d, *J* = 3.8 Hz, 1H), 4.76 (s, 1H), 4.55 (d, *J* = 2.3 Hz, 2H), 3.97 (t, *J* = 9.2 Hz, 1H), 3.88 – 3.82 (m, 2H), 3.75 – 3.67 (m, 2H), 3.41 (t, *J* = 9.2 Hz, 1H), 2.53 (t, *J* = 2.3 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 101.74, 85.93, 79.21, 78.40, 75.93, 73.11, 70.84, 59.85, 33.12. MALDI-MS: 1863.63 $[M+Na]^+$; 1879.84 $[M+K]^+$.

Heptakis[6-bromo-2-O-(N-(N-*t*-butoxycarbonyl-3''-aminopropyl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin (4)

Compound **3** (420 mg, 0.23 mmol, 1 eq.) and *tert*-butyl 3-azidopropylcarbamate (410mg , 2.05 mmol, 9 eq.) were dissolved in 20mL of DMF and 20mL of water. Copper (II) sulfate pentahydrate (45.54mg, 0.18 mmol, 0.8 eq.) was added, then 112 mg (0.57 mmol, 2.5 eq.) of sodium ascorbate was added and the solution was stirred at 80 °C for 12 hours. The solution was concentrated under reduced pressure and purified by column chromatography over SiO_2 (dichloromethane-methanol 9:1) to give product (715 mg, 96%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 5.34 (s, 1H), 5.04 – 4.81 (m, 3H), 4.43 (t, *J* = 6.6 Hz, 2H), 3.87 (t, *J* = 9.0 Hz, 1H), 3.74 (t, *J* = 8.6 Hz, 2H), 3.64 – 3.47 (m, 2H), 3.24 (t, *J* = 8.9 Hz, 1H), 3.09 (dd, *J* = 12.0, 5.9 Hz, 2H), 2.12 – 2.00 (m, 2H), 1.37 (s, 9H). ^{13}C NMR (101

MHz, $CDCl_3$) δ 156.23, 143.83, 123.91, 101.17, 85.50, 79.27, 79.02, 72.71, 70.63, 65.35, 47.76, 37.33, 32.92, 30.73, 28.37.

Heptakis[2-O-(N-(N-*t*-butoxycarbonyl-3''-aminopropyl)-1'*H*-triazole-4'-yl-methyl)-6-dodecylthio]-β-cyclodextrin

Dodecanethiol (978.7 mg, 4.84 mmol, 22 eq.) and 116.3 mg (4.84 mmol, 22 eq.) of sodium hydride were solubilised in 50 mL of anhydrous DMF. The solution was stirred at 80 °C under N_2 for 2 hours. The solution was then cooled to room temperature and 715.1 mg (0.22 mmol, 1 eq.) of **4** in 5 mL of anhydrous DMF was added. The solution was stirred overnight at 80 °C under N_2. The solution was concentrated under reduced pressure and purified by column chromatography over SiO_2 (dichloromethane-methanol 9:1) to give product (649 mg, 72%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (s, 1H), 5.34 (s, 1H), 5.05 (d, *J* = 12.1 Hz, 1H), 4.90 (d, *J* = 11.8 Hz, 2H), 4.48 (t, *J* = 6.6 Hz, 2H), 3.89 (t, *J* = 9.1 Hz, 1H), 3.85 – 3.75 (m, 1H), 3.51 (d, *J* = 8.0 Hz, 1H), 3.43 (t, *J* = 9.0 Hz, 1H), 3.16 (dd, *J* = 12.1, 6.0 Hz, 2H), 3.00 – 2.82 (m, 2H), 2.56 (t, *J* = 7.4 Hz, 2H), 2.21 – 2.06 (m, 2H), 1.61 – 1.17 (m, 29H), 0.88 (t, *J* = 6.8 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 156.24, 144.20, 123.77, 101.09, 85.50, 79.28, 73.02, 71.05, 65.59, 47.86, 37.45, 33.74, 31.96, 30.81, 30.57 – 29.39 (m, 9C), 29.13, 28.45, 22.71, 14.12.

Heptakis[2-O-(N-(3''-aminopropyl)-1'*H*-triazole-4'-yl-methyl)-6-dodecylthio]-β-cyclodextrin trifluoroacetate (5)

Heptakis[2-O-(N-(N-*t*-butoxycarbonyl-3"-aminopropyl)-1'*H*-triazole-4'-yl-methyl)-6-dodecylthio]-β-cyclodextrin (649 mg, 0.16 mmol) was dissolved in 20 mL of DCM and 20 mL of TFA. The solution was stirred at r.t. under N_2 for 6 h. The solution was then

concentrated under reduced pressure and dried under high vacuum to give product (628 mg, 95%).

^{1}H NMR (400 MHz, CD_3OD / $CDCl_3$) δ 7.98 (s, 1H), 5.05 (s, 1H), 4.92 (dd, *J* = 54.1, 11.7 Hz, 2H), 4.56 (t, *J* = 6.6 Hz, 2H), 3.83 (t, *J* = 8.8 Hz, 2H), 3.59 – 3.45 (m, 2H), 3.08 – 2.86 (m, 4H), 2.61 (t, *J* = 7.0 Hz, 2H), 2.39 – 2.28 (m, 2H), 1.64 – 1.53 (m, 2H), 1.43 – 1.20 (m, 18H), 0.89 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD/ $CDCl_3$) δ 144.47, 124.85, 101.13, 85.75, 80.63, 73.25, 71.57, 65.60, 47.62, 37.11, 34.11, 33.90, 32.31, 30.63 – 29.19 (m, 9C), 28.15, 23.03, 14.33. MALDI-MS: 3394.55 $[M+H]^+$; 3416.28 $[M+Na]^+$. λ_{max} 220 nm. FT-IR (cm^{-1}): 3414, 2923, 2853, 1133, 1048.

1-O-Mesyl-ω-methoxy-PEG$_{500}$

Monomethyl-PEG 500 (1.42 g, 2.57 mmol, 1 eq.) in 80 mL of DCM and 3.2 mL (18.02 mmol, 7 eq.) of diisopropylethylamine was cooled to 0 °C and 1 mL (12.87 mmol, 5 eq.) of mesyl chloride was added. The solution was stirred for 12 h at r.t. under N2. The solution was concentrated under reduced pressure and the residue was purified by column chromatography over SiO_2 (CH_2Cl_2/methanol, 95:5) to give product (1.52g, 94%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.27 – 4.22 (m, 2H), 3.66 – 3.62 (m, 2H), 3.62 – 3.45 (m, 40H), 3.43 – 3.40 (m, 2H), 3.24 (s, 3H), 2.96 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 71.70, 70.53 – 69.85 (m), 69.19, 68.77, 58.72, 37.45. MS (ESI^-/TOF) m/z: 506.73 (16%) $[M-H]^-$; 551.16 (57%) $[M-H]^-$; 595.28 (73%) $[M-H]^-$; 639.13 (81%) $[M-H]^-$; 683.30 (100%) $[M-H]^-$; 727.29 (96%) $[M-H]^-$; 771.21 (65%) $[M-H]^-$; 859.40 (23%) $[M-H]^-$; 903.41 (12%) $[M-H]^-$.

1-Azido-1-deoxy-ω-methoxy-PEG$_{500}$

1-O-Mesyl- ω -methoxy-PEG_{500} (1.52 g, 2.38 mmol, 1 eq.) was dissolved in 30 mL of DMF. 620 mg (9.55 mmol, 4 eq.) of sodium azide was added. The solution was heated at 80 °C

overnight. The solution was concentrated under reduced pressure and the residue was purified by column chromatography over SiO_2 (CH_2Cl_2/methanol, 95:5) to give product (1.21g, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70 – 3.50 (m, 48H), 3.50 – 3.44 (m, 2H), 3.34 – 3.28 (m, 5H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 71.85, 70.91 – 70.18 (m), 69.95, 58.93, 50.60. MS (ESI^+/TOF) m/z: 476.15 (15%) $[M+Na]^+$; 520.14 (51%) $[M+Na]^+$; 564.14 (100%) $[M+Na]^+$; 608.19 (93%) $[M+Na]^+$; 652.18 (62%) $[M+Na]^+$; 696.24 (26%) $[M+Na]^+$; 740.29 (15%) $[M+Na]^+$.

Heptakis[6-bromo-2-O-(N-(1''-deoxy-ω–methoxy-PEG$_{500}$- 1''-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin (6)

Compound **3** (100 mg, 0.054 mmol, 1 eq.) and 1-azido-1-deoxy- ω -methoxy-PEG_{500} (317 mg, 0.54 mmol, 10 eq.) were dissolved in 6 mL of DMF and 6 mL of water. 13.5 mg (0.054 mmol, 1 eq.) of copper (II) sulfate pentahydrate was added. 32 mg (0.16 mmol, 3 eq.) of sodium ascorbate was added and the solution was stirred at 80 °C for 12 h. The solution was concentrated under reduced pressure and purified by size exclusion column chromatography over SEPHADEX LH20 (methanol 100%) to give product (282 mg, 88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (s, 1H), 5.07 – 4.89 (m, 3H), 4.84 (s, 1H), 4.69 – 4.47 (m, 1H), 4.01 – 3.23 (m, 52H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.62, 124.66, 101.35, 85.54, 78.41, 73.15 – 69.03 (m, 23), 64.90, 58.97, 50.25, 33.01.

Heptakis[2-O-(N-(1''-deoxy-ω–methoxy-PEG$_{500}$- 1''-yl)-1'*H*-triazole-4'-yl-methyl)-6-dodecylthio]-β-cyclodextrin (7)

Dodecanethiol (62.24 mg, 0.308 mmol, 15 eq.) and sodium hydride (8 g, 0.35 mmol, 17 eq.) were dissolved in 5 mL of anhydrous DMF. The solution was stirred at 80 °C under N_2 for 2 h. The solution was then cooled to room temperature and 114 mg (0.021 mmol, 1 eq.) of **6** in

5 mL of anhydrous DMF was added. The solution was stirred overnight at 80 °C under N_2. The solution was concentrated under reduced pressure and purified by size exclusion column chromatography over SEPHADEX LH20 (methanol 100%) to give product (130 mg, quant.).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81 (s, 1H), 5.07 – 4.83 (m, 2H), 4.78 (s, 1H), 4.63 – 4.44 (m, 2H), 4.04 – 3.22 (m, 50H), 3.06 – 2.68 (m, 2H), 2.68 – 2.38 (m, 2H), 1.64 – 1.40 (m, 2H), 1.40 – 1.11 (m, 20H), 0.84 (t, *J* = 6.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 143.95, 124.49, 101.20, 85.50, 79.05, 74.13–68.27 (m, 24C), 65.04, 58.98, 50.14, 33.64 (2C), 31.90, 30.89–28.25 (m, 8C), 22.65, 14.07. MALDI –MS: M_{avg}= 6350 g/mol. λ_{max} 210 nm. FT-IR (cm^{-1}): 3422, 2922, 2855, 1107, 1047.

Preparation and characterisation of CD.siRNA complexes

Cyclodextrins, dissolved in chloroform (1mg/ml), were mixed to give required molar ratios. The solvent was removed with a gentle stream of nitrogen. Aliquots were stored at -20 ºC until required. For preparation of CD:siRNA complexes, CDs were rehydrated with deionised water (final concentration 1µg/µl) and sonicated for 1 hour at room temperature (RT), then mixed with siRNA in an equal volume of water and used after 20-30 min. For experiments involving the cationic and pegylated CD mixes, a fixed cationic CD:siRNA mass ratio of 10 was chosen.

Particle size (Z-Ave) and surface charge (ζ-potential) of the CD:siRNA complexes were measured by dynamic light scattering, using the Malvern Zetasizer Nano. Samples were measured in deionised water and OptiMEM (Invitrogen) (final volume 1ml containing 3µg siRNA), in two independent experiments, each with five measurements.

For measurement of cyclodextrin-siRNA binding by gel electrophoresis, the cyclodextrin and siRNA were mixed with loading buffer and deionised water 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 min,with a Tris-

borate-EDTA buffer. 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.

Cell culture and transfection

Caco2 (human colon carcinoma) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and L-Glutamine (all from Sigma-Aldrich), in a 37 ºC, 5% CO_2 environment. For transfection, cells were seeded at 1 x 10^5 cells/well in 24-well plates, or 3.5 x 10^4 cells in 96-well plates. Transfection was carried out when cultures were ~70% confluent.

For gene silencing, Caco2 cell cultures were transfected with luciferase reporter plasmid pGL3 luciferase (1µg/well) complexed with Lipofectamine™ 2000 (Invitrogen) (2.5µl/µg pDNA), for 2 hrs. Following this, cultures were washed twice with phosphate-buffered saline (PBS, Sigma-Aldrich). siRNA (50nM) alone, or complexed with Lipofectamine™ 2000 or CD(s), in OptiMEM (Invitrogen) was added to the cultures in serum-containing medium. At 4 h media were replaced, and a further 20 h later cells were washed with PBS, then lysed with 400 µl of 1x Reporter Lysis Buffer (Promega Corp.) and frozen at -80 ºC . Lysate was collected and centrifuged for 5 min at 13,000 rpm. A sample of the supernatant (20 µl) was assayed for expression of luciferase by adding to 100 µl of luciferin (Promega), and measuring immediately in a luminometer. Luciferase expression is reported in relative luminescence units (RLU) per mg protein, as determined by BCA Protein Assay (Pierce). Results are expressed as Mean ± S.E.M. of triplicate values.

Cell viability in presence of cyclodextrins was determined by the MTT assay, which measures the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenase in viable cells. Cell cultures were treated with CD **5** or **7** for 4h. Media was replaced with 100 µl fresh media and MTT (20µl of a 5mg/ml solution) for four

hours, after which the formazon crystals produced were dissolved in 100µl DMSO. Absorbance was measured at 590 nm using a UV plate reader. Cell viability was calculated as a percentage of untreated control cells. Results are expressed as Mean ± S.D. of triplicate values.

Statistics

GraphPad Prism (San Diego, CA, USA, version 5.0) was used for statistical calculations. 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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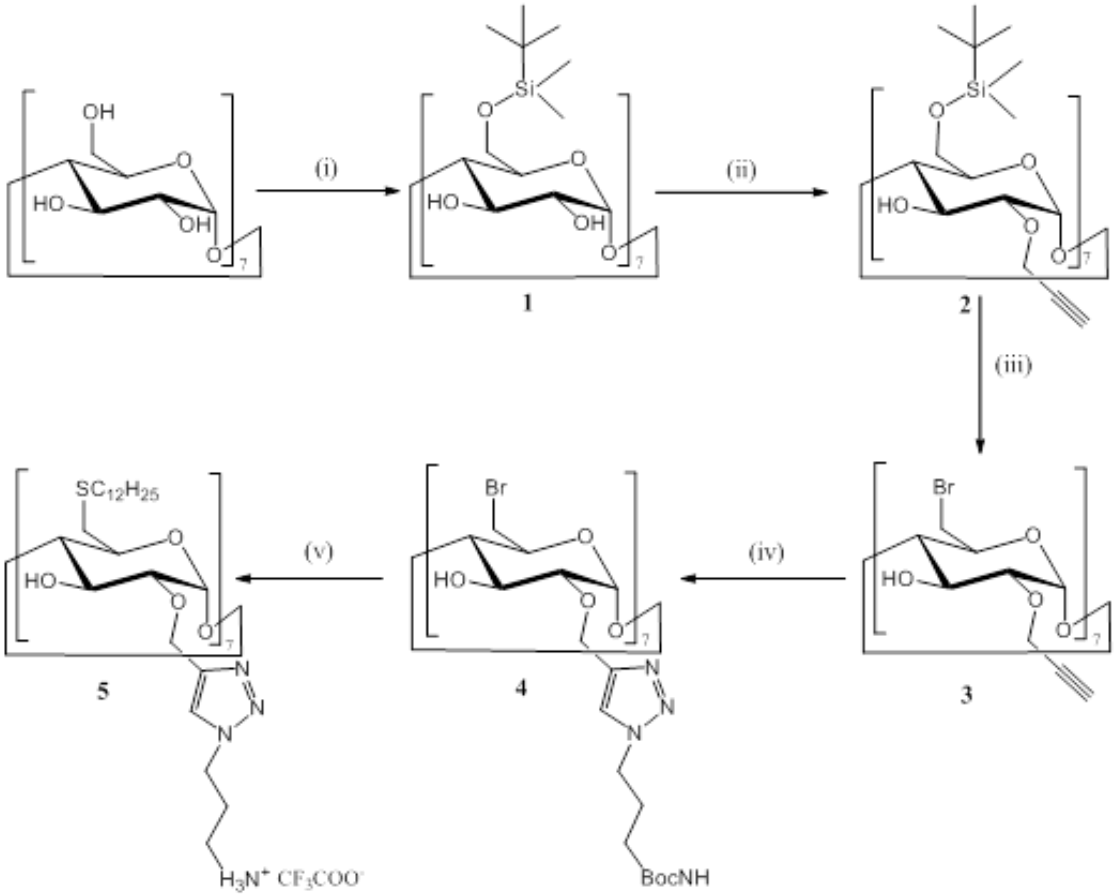
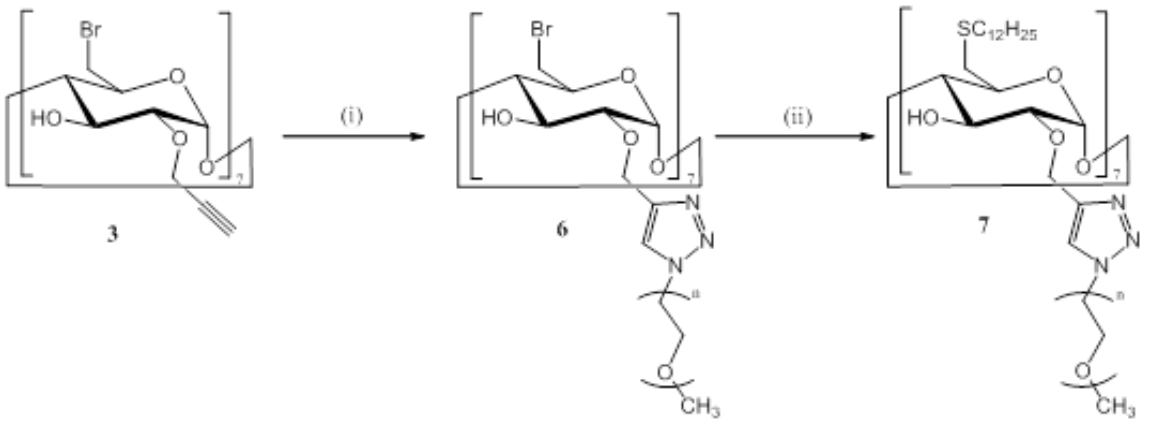


Scheme 1 Synthesis of the cationic cyclodextrin **5**

(i) TBDMSCl, pyridine, 88% (ii) propargyl bromide, NaH, DMF (iii) PPh_3, Br_2, CH_2Cl_2, (iv) *tert*-butyl 3-azidopropylcarbamate, $CuSO_4.5\ H_2O$, sodium ascorbate, DMF-H_2O, 80 °C (v) a) dodecanethiol, NaH, 80 °C, b)TFA, CH_2Cl_2



Scheme 2 Synthesis of the pegylated cyclodextrin **7**

(i) 1-Azido-1-deoxy-ω-methoxy-PEG_{500} , $CuSO_4$, 5 H_2O, sodium ascorbate, DMF-H_2O, 80 °C (ii) dodecanethiol, NaH, 80 °C.

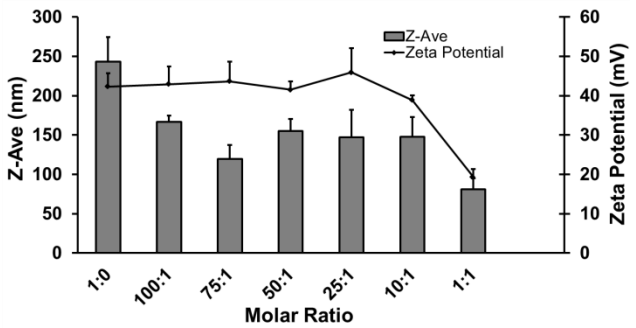


Fig. 1 Effects of molar ratio of cationic CD **5** to pegylated CD **7** on the size (Z-Ave) and charge (zeta potential) of CD:siRNA complexes (mass ratio cationic CD **5**:siRNA=10) in water. Results are expressed as Mean ± S.D. (n=5).

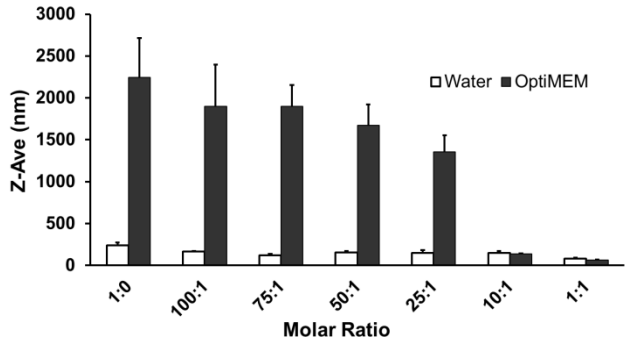


Fig. 2 Effect of molar ratio of cationic CD **5** to pegylated CD **7** on size (Z-Ave) of CD:siRNA complexes in deionised water and OptiMEM (mass ratio cationic CD **5**:siRNA=10). Results are expressed as Mean ± S.D. (n=5).

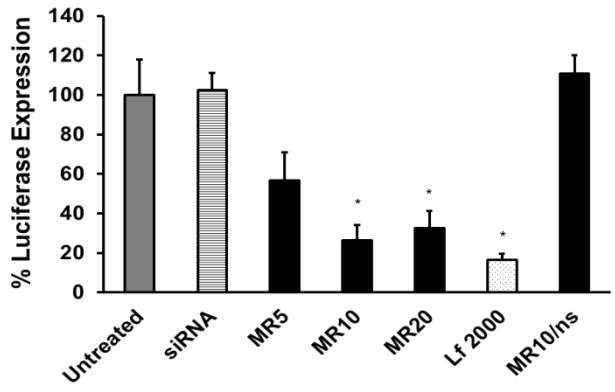


Fig. 3 Silencing of luciferase reporter gene expression in Caco2 cells with cationic CD **5** at increasing mass ratios (MR, cationic CD **5**:siRNA). Lipofectamine 2000 (Lf 2000) was included as a positive control. Results are expressed as Mean ± S.E.M. (n=3). * $p < 0.05$ relative to untreated cells

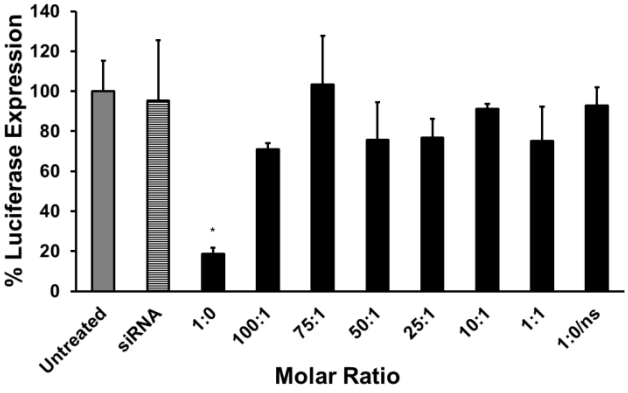


Fig. 4 Effects of molar ratio of cationic CD **5** to pegylated CD **7** on silencing of luciferase reporter gene expression in Caco2 cells at mass ratio cationic CD **5**:siRNA=10. Results are expressed as Mean ± S.E.M. (n=3). * $p < 0.05$ relative to untreated cells.

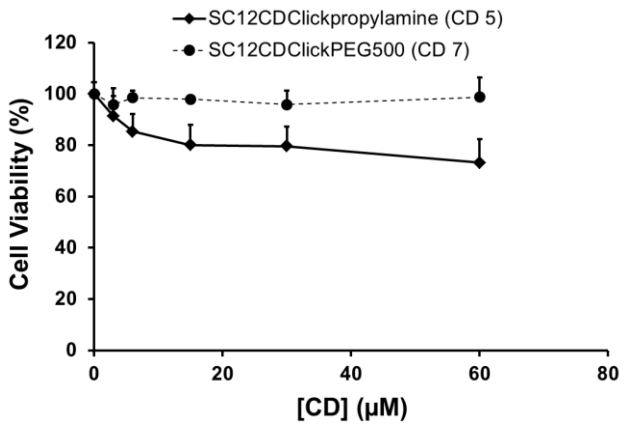


Fig. 5 Effect of cationic CD **5** (solid line) and pegylated CD **7** (dotted line) on viability of Caco2 cells over concentration range 0 to 60 µM. Cells were treated for 4 hours and cell viability was assessed by an MTT assay. Results are expressed as Mean ± S.D. (n=3).

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