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Title Page

A novel, anisamide-targeted cyclodextrin nanoformulation for siRNA delivery to prostate cancer cells expressing the sigma-1 receptor.

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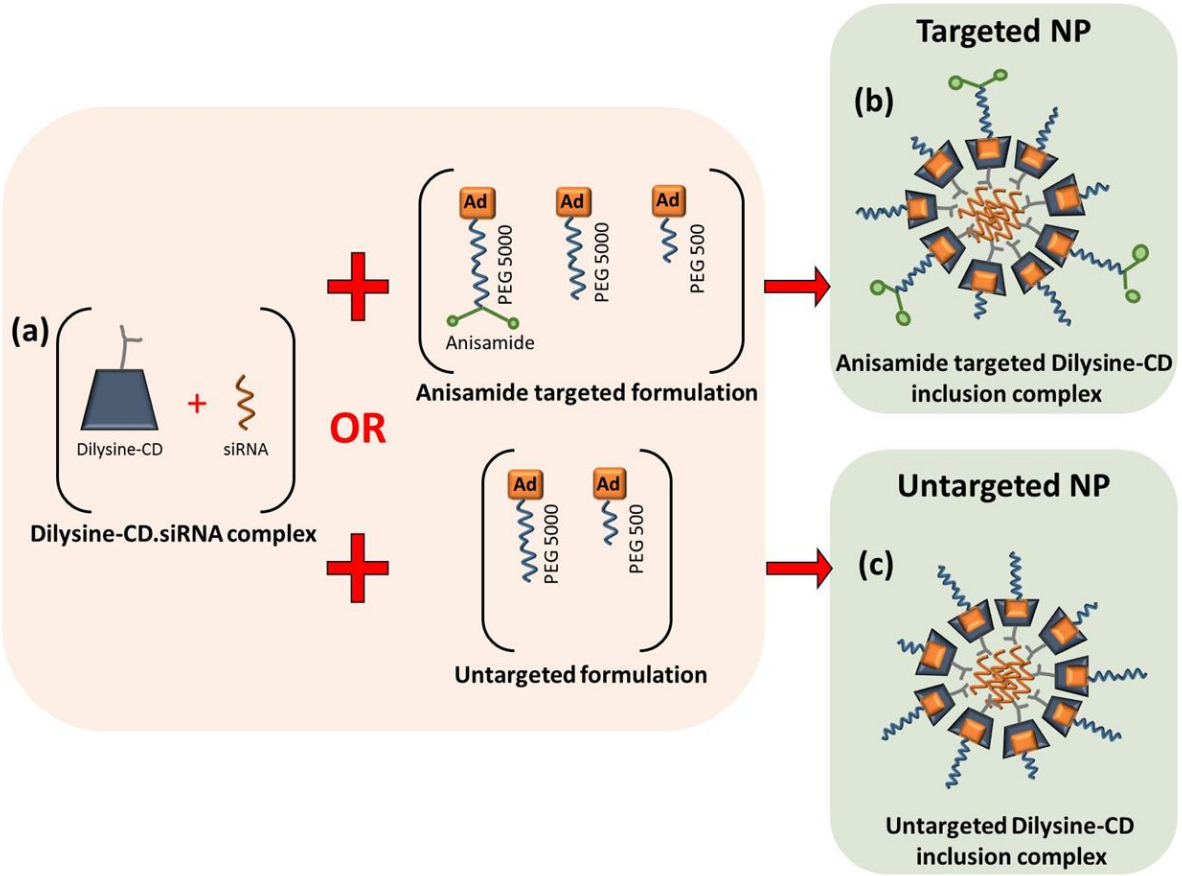
Graphical abstract:



Abstract

Prostate cancer is a leading cause of cancer-related death in men and RNA interference (RNAi) has emerged as a potential therapeutic option. However, the absence of a safe and specific delivery vector remains a major obstacle to the clinical application of RNAi. Cyclodextrin derivatives are known to be efficient delivery systems with low toxicity in a variety of cell types. In this study, a cationic cyclodextrin derivative was synthesized to complex siRNA. The nanoparticle was then further modified by exploiting the ability of the β-cyclodextrin cavity to form an inclusion complex with the hydrophobic molecule adamantane. PEGylated adamantane derivatives were synthesized with and without the anisamide-targeting ligand on the terminal end of the PEG chain. Anisamide is known to bind specifically to the sigma receptor which is overexpressed on the surface of prostate cancer cells. The resulting nanocomplexes were slightly cationic and less than 300 nm in size. They successfully protected siRNA from serum-induced nuclease degradation and were non-toxic to prostate cancer cells. In addition, the targeted nanoparticles mediated high levels of siRNA cellular uptake and corresponding PLK1 gene knockdown in prostate cancer cells *in vitro.* To our knowledge, this is the first time that the ability of cyclodextrins to form inclusion complexes with adamantane derivatives has been exploited for the targeted delivery of siRNA to prostate cancer cells via the sigma receptor.

Keywords

Gene delivery; Prostate cancer; Anisamide-targeted cyclodextrin; Targeted nanoparticle, Sigma receptor, Inclusion complex formation.

Abbreviations:

AA: anisamide

Ad-PEG500: adamantane-PEG500

Ad-PEG5000: adamantane-PEG5000

Ad-PEG5000-2xAA: adamantane-PEG5000-2xAnisamide

CD: cyclodextin

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

PEG: polyethylene glycol

siRNA: small interfering RNA

1 Introduction

Cyclodextrins are cyclic oligosaccharides of 6, 7 or 8 glucose units which are known as α, β or γ-cyclodextrins respectively (1, 2). They exist in a 3D cone-like conformation with a hydrophilic outer surface and a hydrophobic inner cavity (3). The differing reactivities of the glucose hydroxyl groups at positions C-2, C-3 and C-6 and the symmetrical structure of cyclodextrin allows for high yielding regiospecific chemical modification (4).

Of the different classes of cyclodextrin available, β-cyclodextrin is the most widely studied (5). Each β-cyclodextrin molecule contains 21 hydroxyl groups that can be easily substituted to make an unlimited number of cyclodextrin derivatives (5). β-cyclodextrins modified with amphiphilic, cationic entities have been investigated for delivery of small interfering RNA (siRNA) and have successfully mediated gene knockdown both *in vitro* (6, 7) and *in vivo* (8). However, cationic polyplexes are prone to aggregation in physiological media and further modification is necessary prior their application in systemic delivery (9, 10).

In terms of developing an optimised nanoparticle for gene delivery, Kastarelos and Miller have proposed an 'ABCD' formula outlining the key components necessary for *in vivo* gene delivery. They suggest that nanoparticles for such applications should consist of (A) a gene therapy molecule which is condensed by (B) a nanoparticle-forming material. (11). To avoid *in vivo* toxicity associated with cationic complexes, further modification with a biological stabilising group (C) is required (11). The most widely investigated stabilising group in the formulation of nanoparticles is polyethylene glycol (PEG). PEG chains alter the surface properties of nanoparticles thus reducing the tendency toward aggregation and in addition increasing circulation in the blood (12). An ideal delivery system should be active only against the cells of interest, thus avoiding any unwanted activity in healthy cells (13). It is widely reported that the incorporation of PEG into a nanoparticle can reduce cellular uptake

(14). In order to ensure targeted delivery and enhance uptake into disease cells a distal biological-targeting or cell penetrating ligand (D) must also be attached to the nanoparticle (11).

A number of formulation strategies to incorporate a PEG shielding layer following siRNA complexation with a cationic CD have previously been reported. Such methods include a 'co-formulation' approach using two different amphiphilic cyclodextrins (one cationic and one with a PEG chain) (10, 14, 15) and a 'post-insertion' method using an amphiphilic cationic cyclodextrin and a PEGylated DSPE chain (16). In addition, a prostate cancer cell-targeted cyclodextrin derivative was successfully prepared with a cationic group on the primary face (to complex siRNA) and a biologically targeted-PEG500 chain conjugated directly onto the secondary face (17).

An alternative strategy for developing PEGylated cyclodextrins exploits the non-polar inner cavity of β-cyclodextrin which permits the formation of 'inclusion complexes' with hydrophobic guest molecules. This strategy has been exploited in gene delivery where a β-cyclodextrin was attached to a cationic polymer (to bind siRNA) and a targeting ligand (Adamantane-PEG-Transferrin) was then incorporated via formation of an inclusion complex into the CD cavity (18). Adamantane is a highly hydrophobic molecule which demonstrates strong binding to β-cyclodextrin with an association constant in the order 10^4-10^5 M^{-1} (19). This strategy led to the first targeted delivery of siRNA in humans for the treatment of solid tumors in 2008 (18). The sole function of adamantane in these formulations is to facilitate inclusion complex formation as a means of functionalising cyclodextrins with different moieties, which are chemically conjugated to adamantane.

Prostate cancer is a leading cause of cancer-related death in men (20) and treatment using siRNA presents great potential (17, 21, 22). Our group previously synthesized an anisamide-

targeted cyclodextrin vector which facilitated receptor-mediated uptake in prostate cancer cells which overexpress the sigma receptor (17). In the current study, a cationic CD (Dilysine-CD, **Figure 1 (a)**) was synthesized and used to complex siRNA. This complex was modified by exploiting inclusion complex formation with three different PEGylated adamantane derivatives: Adamantane-PEG500 (Ad-PEG500, **Figure 1 (b)**) and Adamantane-PEG5000 (Ad-PEG5000, **Figure 1 (c)**) to mask the positive charge, and an Adamantane-PEG5000-anisamide derivative (Ad-PEG5000-2xAA, **Figure 1 (d)**) to ensure cell-specific uptake. A schematic outlining the nanoparticle structure is given in **Figure 2.** The physicochemical properties of the nanoparticles were assessed and the ability of the CD.Ad-PEG5000-2xAA nanoformulation to specifically target the sigma receptor and elicit therapeutic gene silencing was quantified.

2 Materials and Methods

2.1 Reagents

All reagents were purchased from Sigma unless otherwise stated. Penicillin-Streptomycin (Gibco) was purchased from Biosciences (Dublin, Ireland). PLK1 siRNA (sense strand sequence 5'-AGAmUCACCCmUCCUmUAAAmUAUU-3') was procured from Genepharma (Shanghai, China), where "m" represents 2' O methylated nucleotide. Negative control non-silencing siRNA (sense strand sequence 5'-UUC UCC GAA CGU GUC ACG UdT dT-3') was purchased from Sigma (Wicklow, Ireland). A similar sequence modified with 6FAM on the 3' end of the sense strand was used for fluorescent experiments (Sigma, Wicklow, Ireland).

Reactions were monitored by TLC on precoated aluminium plates of silica gel (Merck $60F_{254}$ 0.25 mm). Carbohydrates were visualised by dipping in 5 % sulfuric acid by volume in ethanol and charring. Amines were visualised by dipping in ninhydrin solution (1 g in 30 ml of ethanol) and charring. Other compounds were visualised under a UV lamp (254 nm). Column chromatography was performed on silica gel (Davosil LC60A, 40-63 µm).

^{1}H NMR and ^{13}C NMR were recorded at 25 °C with Varian spectrometers at 400 and 100, or 500 and 125 MHz. Electrospray ionisation mass spectra (ESI-MS) were recorded on a Quattro Micro LC/MS/MS system (Waters Corporation, USA). MALDI spectra were recorded on a MALDI Q-Tof Premier mass spectrometer (Waters Corporation, USA) using a matrix of either trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) or α-cyano-4-hydroxycinnamic acid (CHCA).

2.2 Synthesis of Dilysine Cyclodextrin (D)

2.2.1 Synthesis of N,N',N''-tri-Boc-di-l-lysine (A)

Dilysine dihydrochloride (100 mg, 0.28 mmol) and diisopropylethylamine (372 mg, 500 µL, 2.88 mmol) were dissolved in dimethylformamide (10 ml) and water (5 ml) and stirred for 15 min at 0 °C. Di-tert-butyl dicarbonate (314 mg, 1.44 mmol) was added and the mixture warmed to room temperature and stirred for 17 h, concentrated, and purified by column chromatography over silica gel (DCM/MeOH: 9/1) to yield the product **A** (115 mg, 72%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05 (s, 1H), 5.47 (s, 2H), 4.85 (s, 2H), 4.52 (s, 2H), 4.12 (s, 2H), 3.06 (t, *J* = 11.9 Hz, 4H), 1.99 – 1.54 (m, 5H), 1.38 (s, 31H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.37 (s), 162.75 (s), 156.23 (s), 79.97 – 79.77 (m), 79.10 (s), 54.26 (s), 40.18 (s), 36.55 (s), 32.04 (s), 31.49 (s), 29.52 (s), 28.34 (d, *J* = 9.9 Hz), 22.45 (s).

MS (ESI^+-TOF) *m/z*: $[M + Na]^+$ calcd for $C_{27}H_{50}N_4O_9Na$ 597.3475; found 597.3477.

2.2.2 Synthesis of N,N',N''-tri-Boc-di-l-lysine hydroxysuccinimide ester (B)

A (102 mg, 0.18 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (38 mg, 0.2 mmol) were dissolved in anhydrous dichloromethane (10 ml) and stirred for 30 min at room temperature. *N*-hydroxysuccinimide (23 mg, 0.2 mmol) was added and the mixture was stirred for 17 h at room temperature under a nitrogen atmosphere. The reaction was diluted with DCM (25 ml), washed with water (3 x 20 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography over silica gel (DCM/MeOH: 9.5/0.5) to yield the product **B** (52 mg, 43%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (s, 1H), 4.98 – 4.48 (m, 2H), 4.08 (s, 1H), 3.20 – 2.95 (m, 2H), 2.86 – 2.74 (m, 2H), 2.10 – 1.10 (m, 21H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.19, 168.59, 167.71, 156.12, 80.02, 79.05, 50.18, 39.85, 31.58, 29.53, 29.12, 28.41, 28.27, 25.55, 22.41.

2.2.3 Synthesis of Heptakis-[2-aminoethylthio]-β-cyclodextrin trifluoroacetate (C)

2-(Boc-amino) ethanethiol (605 µL, 3.58 mmol) was dissolved in anhydrous DMF (20 ml) and sodium hydride (82 mg, 3.41 mmol) was added. The mixture was heated to 60 °C and stirred under nitrogen for 2 h. The mixture was then cooled to room temperature and heptakis-6-bromo-6-deoxy-β-cyclodextrin (250 mg, 0.16 mmol) was added in anhydrous DMF (5 ml). The mixture was heated to 60 °C and stirred under nitrogen overnight. The solvent was removed *in vacuo* and the residue washed several times with cyclohexane and water. The product was dissolved in DCM/TFA (1:1, 10 ml) and stirred for 6 h under nitrogen. The mixture was concentrated and the product precipitated from diethyl ether. The product **C** was purified on Sephadex LH20 (MeOH: 100%) (159 mg, 42%).

^{1}H NMR (400 MHz, DMSO-d6) δ 7.97 (s, 3H), 5.91 (m, 2H), 4.87 (s, 1H), 3.81-3.22 (m, 10H), 3.07-2.89 (m, 2H), 2.89-2.65 (m, 2H).

^{13}C NMR (101 MHz, DMSO-d6) δ 102.4, 84.8, 72.9, 72.5, 72.2, 65.2, 33.1, 30.3.

MS (MALDI-TOF) *m/z*: $[M + Na]^+$ 1575.6176

2.2.4 Synthesis of Heptakis-6-[dilysine-ethylthio]-β-cyclodextrin (D)

C (155 mg, 0.023 mmol) and DIPEA (112 µL, 0.64 mmol) were dissolved in anhydrous DMF (5 ml) and stirred for 15 min. *N,N',N''*-tri-Boc-di-L-lysine hydroxysuccinimide ester (151 mg, 0.23 mmol) was added and the solution was stirred at room temperature overnight.

The mixture was concentrated *in vacuo* and purified on Sephadex LH20 (MeOH: 100%). The compound was dissolved in TFA/DCM (1/1, 10 ml) and stirred for 6 h. The product **D** was precipitated and washed several times with diethyl ether (141 mg, 100%).

^{1}H NMR (400 MHz, CD_3OD) δ 5.00 (m, overlaps with D_2O, H1), 4.37-4.25 (m, 1H, Lysα), 4.07-3.73 (m, 2H, H3, H5), 3.70-3.31 (m, 4H, H2, H4, CH_2NH), 3.02-2.69 (m, 4H, Lysε, SCH_2), 2.45-2.33 (m, 1H, NH), 2.02-1.61 (m, 4H, Lysβ, Lysδ), 1.59-1.37 (m, 2H, Lysγ).

^{13}C NMR (101 MHz, CD_3OD) δ 172.2, 168.7, 159.9 (q), 102.24 (m), 78.1, 77.8, 77.5, 72.8, 53.6, 52.5, 39.3-38.5 (m), 33.0, 32.4, 31.3, 30.5, 28.1-26.2 (m), 22.5.

MS (MALDI-TOF) *m/z:* $[M + Na]^+$ 3365.8286.

2.3 Synthesis of Adamantane Derivatives

2.3.1 Ad-PEG500 and Ad-PEG5000

1 g of PEG monomethyl ester (PEG5000: 0.2 mmol; PEG500: 2.1 mmol) was dissolved in anhydrous DCM (PEG5000: 25 ml; PEG500: 150 ml) and cooled to 0 °C. Adamantane carbonyl chloride (5 eq), DIPEA (10 eq) and DMAP (1% cat.) were added and the mixture warmed to room temperature and stirred overnight under nitrogen. The mixture was transferred to a separating funnel and washed with 5 % citric acid (50 ml), saturated $NaHCO_3$ (50 ml), and brine (50 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Ad-PEG500 was purified by column chromatography over silica gel (DCM/MeOH: 9/1) to yield the product **E** as a yellow oil (1.1 g, 83%). Ad-PEG5000 was dissolved in a minimum amount of DCM and precipitated from diethyl ether to yield the product **F** as a white solid (805 mg, 78 %).

Ad-PEG500 (E):

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.24 – 4.15 (m, 2H), 3.75 – 3.59 (m, 30H), 3.59 – 3.51 (m, 2H), 3.38 (s, 2H), 2.09 – 1.95 (m, 5H), 1.95 – 1.80 (m, 8H), 1.80 – 1.63 (m, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 177.55, 71.84, 70.41, 69.14, 63.22, 58.92, 40.60, 39.37, 38.71, 36.85, 36.41, 28.42, 27.85.

MS (ESI-TOF) *m/z*: $[M + Na]^+$ 657.63.

Ad-PEG5000 (F):

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.22 – 4.18 (m, 2H), 3.84 – 3.79 (m, 2H), 3.64 (s, 271H), 3.57 – 3.53 (m, 2H), 3.49 – 3.44 (m, 2H), 3.38 (s, 2H), 2.05 – 1.97 (m, 6H), 1.92 – 1.87 (m, 4H), 1.77 – 1.65 (m, 6H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 71.9, 70.6, 63.2, 38.8, 36.5, 27.9.

MALDI-MS: M_{avg} = 5193.0122 g mol^{-1}

2.3.2 Ad-PEG5000-2xAA (L)

2.3.2.1 Synthesis of Fmoc- Lys(Boc)-anisamide (G)

To a solution of Fmoc-Lys(Boc)-COOH (1.5 eq, 1 g) in dry DCM (50ml) was added EDC.HCl (1.6 eq, 436 mg), TEA (1.6eq, 317 µl) and anisidine (175 mg). The reaction mixture was stirred at room temperature overnight then diluted with DCM and extracted. The organic phase was washed with 5 % citric $acid_{aq}$ and brine then dried ($MgSO_4$) and evaporated. The residue was purified on silica gel column chromatography (From DCM 100 % to DCM 99:MeOH 01) to afford compound **G** (495 mg, 61 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.22 (s, 1H, NH), 7.76 (d, *J* = 7.5 Hz, 2H, CH, Ph), 7.59– 7.27 (m, CH, Fmoc), 6.84 (d, *J* = 8.9 Hz, 2H, CH, Ph), 5.64 (s, 1H, NH), 4.65 (s, 1H, NH), 4.44 (m, 2H, CH_2, Fmoc), 4.29 (m, 1H, CH, Fmoc), 4.21 (t, *J* = 6.9 Hz, 1H, CHα, Lys), 3.79 (s, 3H, OMe), 3.16 – 3.08 (m, 2H, CH_2ε, Lys), 1.97 and 1.71 (s, 1H each, CH_2β, Lys), 1.54 (m, 2H, CH_2δ, Lys), 1.44 (s, 10H, *t*-Bu; CH_2γ Lys).

2.3.2.2 Synthesis of Fmoc-Lys-Di-anisamide (I)

A solution of **G** (380mg) in dioxane (20ml) was cooled to 0 ºC. A solution of HCl 4N in dioxane (10 ml) was added and the reaction mixture was stirred at 0 ºC for 2h then at room temperature for another 2 h. The solvents were evaporated to dryness and the residue dissolved in a minimum of MeOH. The free amino intermediate (**H**) was precipitated by addition of Et_2O. The precipitate was filtered and washed with Et_2O and used without further purification (268 mg, 79 %).To a solution of the crude amine **H** (265 mg) in dry DCM (100 ml) was added EDC.HCl (1.7 eq, 170 mg) followed by TEA (10 eq, 725 µL) and anisic acid (1.5 eq, 119 mg). The reaction mixture was stirred at room temperature overnight then diluted with DCM and extracted. The organic phase was washed with 5 % citric $acid_{aq}$, water and brine then dried ($MgSO_4$) and evaporated. The residue was purified on silica gel column chromatography (DCM 97:MeOH 03) to afford compound **I** (147mg, 47%).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.27 (t, 3H, 3 x NH), 7.85 (d, *J* = 7.6 Hz, 2H, CH, Phε), 7.77 (d, *J* = 8.5 Hz, 2H, CH, Phα), 7.71– 7.27 (m, CH, Fmoc), 6.92 (d, *J* = 8.3 Hz, 2H, CH, Phε), 6.84 (d, *J* = 8.6 Hz, 2H, CH, Phα), 4.25-4.03 (m, 4H, CH_2 Fmoc; CH Fmoc; CHα Lys), 3.76 and 3.68 (2x s, 2x3H, 2xOMe), 3.21 (m, 2H, CH_2ε, Lys), 1.65 (m, 2H, CH_2β, Lys), 1.50 (m, 2H, CH_2δ, Lys), 1.34 (m, 2H, CH_2γ Lys).

^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 169.81 , 156.55 , 143.69 , 141.29 , 127.74 , 127.09, 125.01 , 121.82 , 119.97 , 114.12 , 67.18 , 55.47 , 47.15 , 39.64 , 31.82 , 29.53 , 28.43 , 22.50.

2.3.2.3 Fmoc-PEG5000-Lys (Anisamide)$_2$ (K)

To a solution of **I** (147mg) in DMF (5 ml) was added piperidine (500 µL). The reaction mixture was stirred at room temperature for 2 h. DMF was evaporated under reduced pressure. The residue was purified on silica gel column chromatography (DCM 97:MeOH 03:Et_3N:0.35) to afford compound **J** (92 mg, Q.Y.). The crude amine was used without further purification.

To a solution of Fmoc-PEG_{5000}-OSu (294mg) in dry DCM (10ml) was added amine **J** (4 eq, 92 mg) and DIPEA (7 eq, 73 µL). The reaction mixture was stirred at room temperature for 3 days then diluted with DCM and extracted. The organic phase was washed with saturated ammonium chloride and brine then dried ($MgSO_4$) and evaporated. The residue was taken up in a minimum amount of DCM and precipitated by addition of Et_2O. The precipitate was filtered and washed with Et_2O then loaded on a LH-20 gel filtration chromatography (MeOH 100 %) to afford **K** (224 mg, 71 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.67 (t, 3H, 3 x NH), 7.77 – 7.26 (m, CH, Fmoc; CH, Phε; CH, Phα), 6.87 (d, *J* = 8.8 Hz, 2H, CH, Phε), 6.80 (d, *J* = 9 Hz, 2H, CH, Phα), 6.57 (t, NH), 4.50-4.45 (m, 1H, CHα Lys), 4.38 (d, *J* = 6.9 Hz, 2H, CH_2 Fmoc,), 4.21-4.18 (m, 1H, CH Fmoc,), 3.82 (s, 3H, OMe), 3.80-3.36 (m, 196H, PEG; OMe; CH_2ε, Lys), 2.36-1.41 (m, 6H, CH_2β, Lys; CH_2δ, Lys; CH_2γ Lys).

^{13}C NMR (100 MHz, $CDCl_3$) δ 175.28, 174.07, 162.02, 156.19, 149.70, 149.42, 143.97, 128.81, 127.60, 126.98, 121.59, 119.90, 113.97, 113.59, 97.40, 70.54, 55.41, 54.41, 53.60, 47.25, 38.77, 32.96, 28.83, 22.51.

2.3.2.4 Ad-PEG5000-Lys (Anisamide)$_2$ (L)

To a solution of **K** (210mg) in DMF (5ml) was added piperidine (1ml). The reaction mixture was stirred at room temperature for 2 h. DMF was evaporated under pressure. The residue was taken up in a min of DCM and precipitated by addition of Et_2O. The precipitate was filtered and washed with Et_2O then loaded on a LH-20 gel filtration chromatography (MeOH 100 %) to afford the free amino intermediate which was used without further purification (137 mg, 68 %). A solution of this crude amine (137 mg) in dry DCM (10 ml) was cooled to 0 ºC. Adamantane carbonyl chloride (5 eq, 27 mg) and DIPEA (10 eq, 47 µL) were added. The reaction mixture was stirred at room temperature overnight then the solvent was evaporated under reduced pressure. The residue was loaded on a LH-20 gel filtration chromatography (MeOH 100 %) to afford compound **L** (113 mg, 80 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (s, 1H, NH), 7.76 (d, *J* = 8.8 Hz, 2H, CH, Phε), 7.43 (d, *J* = 9 Hz, 2H, CH, Phα), 6.87 (d, *J* = 9 Hz, 2H, CH, Phε), 6.80 (d, *J* = 9 Hz, 2H, CH, Phα), 4.47 (m, 1H, CHα Lys), 3.81-3.40 (m, 176H, PEG; OMe; CH_2ε, Lys), 2.35-1.41 (m, 11H, CH_2β, Lys; CH_2δ, Lys; CH_2γ Lys; CH and CH_2 ADAM).

^{13}C NMR (100 MHz, $CDCl_3$) δ 174.05, 169.31, 162.01, 156.18, 128.81, 121.58, 113.96, 113.58, 70.53, 55.34, 39.16, 38.78, 36.51, 32.94, 30.19, 28.82, 28.11, 25.47, 22.59.

2.4 Cell Culture

DU145 cells (human prostate cancer cell line, kindly donated by the Conway Research Institute, University College Dublin, Dublin, Ireland) and PC3 cells (human prostate cancer cell line, European Collection of Cell Cultures (ECACC), UK) were maintained in RPMI-1640 medium, supplemented with 10 % fetal bovine serum (FBS), 2 mM L-Glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. VCaP cells (human prostate cancer cell line,

Sigma, Wicklow, Ireland) were maintained in DMEM and supplemented with 10 % FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. VCaP cells were cultured and seeded in Poly-D-Lysine (1x) coated tissue culture flasks and wells. All cells were cultured in a humidified, 5 % CO_2 atmosphere tissue culture incubator at 37 °C.

2.5 Isothermal Calorimetry

A MicroCal™ PEAQ-ITC microcalorimeter (S/N MAL1119955, Malvern, United Kingdom) was used to investigate the ability of Adamantane-PEG5000 and Adamantane-PEG5000-2xAnisamide to form inclusion complexes with Dilysine-CD. The analysis was performed using the MicroCal™ PEAQ-ITC analysis software. The corresponding samples for each experiment were loaded into the sample cell and the titrant was loaded into the syringe, using 280 µL and 40 µL respectively. The reference cell was loaded with Milli-Q water. After a 60-second delay, a typical experiment involved a series of 19 injections of 2 µL of the titrant solution, plus an initial injection of 0.4 µL and a spacing of typically 120 seconds between injections. The data point from the initial injection was discarded before the data analysis. The sample cell was stirred at 750 rpm throughout the experiment and the experimental temperature was maintained at 25 °C. The feedback/gain mode was set to high. Consecutive titrations were combined using the freeware software CONCAT. The area under each peak of the resulting heat profile was integrated, normalized by the concentrations, and plotted against the molar ratio of ligand to protein using MicroCal PEAQ-ITC Analysis Software. The resulting binding isotherms were fitted by nonlinear regression using a one set of sites model. The stoichiometry of the interaction (n), the equilibrium dissociation constant (KD), and the change in enthalpy (ΔH) were obtained during the fitting of all titration data. Gibbs free energy change (ΔG) was calculated from the following equation $\Delta G=-RT \ln Ka$ and the entropy (ΔS) from $\Delta G = \Delta H - T\Delta S$.

2.6 Preparation of Complexes with siRNA

Dilysine-CD and adamantane derivatives were soluble in aqueous solutions and were dissolved at a concentration of 1 µg/µl in ddH_2O. These aqueous solutions were stored at -20 °C until use and were thawed and sonicated at room temperature for 30 min, before forming nanocomplexes with siRNA. The Dilysine-CD.siRNA nanocomplexes were formed at a fixed mass ratio (mr) of 10. Specifically, 30 µg of Dilysine-CD and 3 µg of siRNA were separately dissolved in ddH_2O to give a final volume of 50 µl each. The siRNA (50 µl) was then added to the Dilysine-CD solution (50 µl) and vortexed at a mild speed for 10 seconds, to give a final volume of 100 µl and was left to stabilize at room temperature 30 min. Required amounts of adamantane-PEG derivatives were prepared (to form untargeted and targeted nanoparticles) in separate Eppendorf vials to which Dilysine-CD.siRNA complexes were added. Specifically, for untargeted nanoparticles, Ad-PEG-500 and Ad-PEG-5000 were used in the molar ratio of 0.5:0.5 to 1 mole Dilysine-CD. For targeted nanoparticles Ad-PEG500, Ad-PEG5000 and Ad-PEG5000-2xAA were used in the molar ratio of 0.5:0.25:0.25 to 1 mole Dilysine-CD. The Dilysine-CD.siRNA complexes containing adamantane derivatives were then kept at 900 rpm at 25 °C for 30 mins in a thermomixer after which the nanoparticles were brought to 1 ml of total volume with ddH_2O. The nanoparticles were kept at room temperature for 30 min to stabilize, before performing any further analysis.

2.7 Size, Charge and Aggregation

Particle size and zeta potential were measured in RNase-free water using a Malvern Nano-ZS (Malvern Instrument, UK) with negative control non-silencing siRNA. Complexes were prepared as detailed above. For size and charge measurement, each sample contained 3 µg siRNA (at Dilysine-CD.siRNA mass ratio 10) in 1 ml RNase-free water. Nanoparticle stability in high salt solution was assessed by preparing complexes to a final volume of 1 ml in OptiMEM Reduced Serum Media (Gibco) (50 %) as previously employed (14).

Complexes were incubated at 37 ºC and particle size was measured at 1 h, 4 h and 24 h. Each sample was measured in triplicate.

2.8 Transmission Electron Microscopy

The morphology of the CD.siRNA complexes was visualised using transmission electron microscopy (TEM). Complexes were prepared as detailed above using negative control non-silencing siRNA. 15 µl of the CD.siRNA complex was mixed with 15 µl 2 % (w/w) uranyl acetate and applied to 400 mesh carbon-film copper grids (Agar Scientific) for a couple of minutes. Images were taken using a JEOL 2000 FXII transmission electron microscope.

2.9 Binding and Release Gel

The ability of the cyclodextrins to bind and release siRNA was assessed using gel electrophoresis. For the binding study, complexes were prepared as detailed above and mixed with 1x loading buffer. Samples were loaded onto a 1 % agarose gel containing SafeView^{TM} (NBS Biologicals Ltd, England) (6 µl/100 ml) (14). For the release study, complexes were incubated with 5 µl Heparin (1,000 U/ml) for 60 minutes and loaded onto the gel. Each well contained 0.3 µg siRNA. Electrophoresis was carried out at 90 V for 45 minutes using a Tris-borate-EDTA buffer. Naked siRNA was used as a control.

2.10 Serum Stability

Serum stability was evaluated by incubating the formulations with 50 % FBS for various time periods (30 min, 1 h, 2 h, 4 h, 8 h and 24 h) and stored at -20 °C until gel electrophoresis was performed. Each time point contained 0.35 µg of siRNA for all nanoparticle formulations. To inactivate the serum, samples were incubated in a bath incubator at 80 °C for 5 min, followed by addition of Heparin (10 µl at 1000 U/ml) and kept at room temperature for an hour. All the samples were mixed with a 1x Blue Juice Gel loading buffer (Invitrogen, U.S.A) and were added to the wells of a 1.5 % (w/v) agarose (Sigma, MO, USA) solution, prepared with 1x

Tris borate EDTA (TBE) buffer. 6 µl of SafeView™ (NBS Biologicals Ltd., England) was added to the 100 ml of 1 % (w/v) agarose-TBE solution. The electrophoresis was carried out at 90 V for 30 min in TBE buffer. The bands were visualised by UV using DNR Bioimaging Systems MiniBis Pro and Gel capture US B2 software.

2.11 Cellular Uptake

DU145 cells ($1x10^4$ cells/well), VCaP cells ($5x10^4$ cells/well) and PC3 cells ($1x10^4$ cells/well) were seeded in complete growth media 24 hours prior to transfection in 96-well plates. CD.siRNA complexes were prepared as detailed previously using FAM-labelled siRNA (Sigma). After 24 hours, 50 µL of the CD.siRNA complex (prepared in ddH_2O) was added to the cells with 150 µl serum-free and antibiotic-free transfection media. The cells were then incubated at 37 °C in 5 % CO_2 for 24 h, after which the quantitative measurement of transfection was performed by measuring fluorescence intensity using a Perkin Elmer Victor2 1420 fluorescent plate reader (excitation 485 nm, emission 535 nm). Samples were normalised to untreated control by subtracting the background fluorescence of untransfected cells.

2.12 MTT

The MTT assay was used to assess the cell viability after transfection with the complexes as previously employed (14, 16). DU145 cells ($2x10^4$ cells/well), VCaP cells ($5x10^4$ cells/well) and PC3 cells ($2x10^4$ cells/well) were seeded 24 h prior to transfection in 96-well plates. CD.siRNA complexes were prepared as previously described using non-silencing siRNA (Sigma). Transfection was carried out using 50 nM siRNA in antibiotic-free media for 24 h. After this time period the media was removed and replaced with 100 µl serum-free media and 20 µl MTT reagent (5 mg/ml solution in PBS). Cells were incubated for 4 h at 37 ºC after which 100 µl DMSO was added to each well. Absorbance was measured at 570 nm using a

UV plate reader. The results are express as the percentage cell viability relative to untreated controls.

2.13 Competitive Uptake

DU145 cells ($2x10^4$ cells/well), VCaP cells ($5x10^4$ cells/well) and PC3 cells ($2x10^4$ cells/well) were seeded in 96-well plates in antibiotic-free medium 24 h prior to transfection. After 24 h and prior to transfection, cells were pre-incubated with 100 μM Ad-PEG5000-2xAA in serum-free and antibiotic-free medium for 1 h. CD.siRNA complexes (prepared with FAM-labelled non-silencing siRNA) were then added to the media and co-incubated with the free Ad-PEG5000-2xAA for a further 5 hours in serum-free and antibiotic-free medium (37 °C with 5 % CO_2) at a final siRNA concentration of 50 nM. Cell uptake was done by measuring fluorescence intensity using a Perkin Elmer Victor2 1420 fluorescent plate reader (excitation 485 nm, emission 535 nm). Samples were normalised to untreated control by subtracting the background fluorescence of untransfected cells.

2.14 Gene Knockdown

Complexes were prepared as detailed above. DU145 cells ($5\ x10^4$ cells/well), VCaP cells ($1.5x10^5$ cells/well) and PC3 cells ($5x10^4$ cells/well) were seeded 24 h prior to transfection in 24-well plates. Complexes were added to the cells in serum-free and antibiotic-free media to give a final concentration of 100 nM siRNA. After 24 h the media was changed to serum-containing medium. 48 h after the initial transfection experiment, cells were lysed open and RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) as per manufacturer's instructions. A NanoDrop ND-1000 UV-vis Spectrophotometer was used to quantify the concentration of RNA in the samples. First-strand complementary DNA (cDNA) was generated from the total RNA 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 s 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 (23).

2.15 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 except in **Figure 12** where a two-tailed unpaired student *t*-test was used to compare PLK1 siRNA knockdown with its non-silencing counterpart.

3 Results/Discussion

3.1 Synthesis

A cationic cyclodextrin modified with a dilysine group on the primary face (**Figure 1 (a)**). was successfully synthesized (**Figure 3 (a)**). The β-cyclodextrin was modified to include two lysine amino acids on the primary face as it was anticipated that the presence of two cationic residues would lead to enhanced siRNA complexation efficiency. In addition, β-cyclodextrin is known to form inclusion complexes with lipophilic guest molecules (2). One such molecule is adamantane, a highly hydrophobic entity with a diameter that fits well into the β-cyclodextrin inner cavity resulting in a high binding constant (24). Such inclusion complex formation has previously been exploited for the targeted delivery of gene therapy agents to cancer cells (18). In a similar fashion, the CD.siRNA nanoparticle was modified utilising the inclusion complex formation strategy with three different PEGylated adamantane derivatives; Ad-PEG500 and Ad-PEG5000 (**Figure 1 (b) and (c)**) and Ad-PEG5000-2xAA (**Figure 1 (d)**). The Ad-PEG derivatives were chosen to mask the cationic charge. The Ad-PEG5000-2xAA molecule (synthesis described in **Figure 4 (b)**) was prepared with two anisamide-targeting moieties on the terminal end of the PEG chain. Anisamide was selected as the targeting ligand as it is known to increase cellular uptake via binding to the sigma receptor on prostate cancer cells (17, 25). Two anisamide ligands were used to further enhance clustering on the cell surface and subsequent receptor-mediated uptake.

3.2 Physicochemical Characterisation

3.2.1 ITC

To confirm the ability of the Ad-PEG derivatives to form inclusion complexes with the Dilysine-CD, isothermal calorimetry (ITC) was used (26, 27). This technique is based on the change in heat when two molecules interact and can be employed to determine the

stoichiometry of the interaction (N) as well as the dissociation constant (K_D) (27). In addition, it gives details on the heat released or taken in during the reaction (the enthalpy change, ΔH) and allows the entropy (ΔS) and Gibbs free energy (ΔG) to be calculated (27).

Isothermal calorimetry was used in this study to investigate the thermodynamics of the interaction between the Dilysine-CD and the Ad-PEG5000 derivatives (PEG5000 or PEG5000-2xAA). Representative ITC data plots are shown in **Figure 5** and the thermodynamic properties of the interaction are listed in **Table 1**.

It is reported that each β-cyclodextrin molecule is capable of interacting with a single adamantane molecule yielding a maximum N value of 1 (28). As shown in **Table 1** the N values for Ad-PEG5000 and Ad-PEG5000-2xAA are 0.587 and 0.783 respectively indicating that the cyclodextrin cavities are not fully saturated. This is most likely due to steric hindrance caused by the long PEG5000 chains on the adamantane molecule as previously reported (28). In addition, the ΔH for both PEG chains is negative, indicating that energy is released upon binding due to the favourable interaction between the β-cyclodextrin cavity and the hydrophobic adamantane derivative (28). This is further supported by the unfavourable entropic contribution (i.e. positive -TΔS). The Ad-PEG5000 and Ad-PEG5000-2xAA derivatives form complexes with K_D values of 12.8 and 89.8 μM respectively. These are consistent with those previously reported for the interaction between alkylated cyclodextrin and mannose-adamantane conjugates (K_D 13.9-76.9 μM) (26).

The lower K_D value for the Ad-PEG5000 derivative indicates that it forms a tighter inclusion complex with the Dilysine-CD relative to the Ad-PEG5000-2xAA derivative. However, the results presented confirm that the PEGylated adamantane derivatives are capable of forming inclusion complexes with the Dilysine-CD.

3.2.2 The Influence of PEGylation on Size and Charge-Preliminary Screening of Formulations

The change in particle size and zeta potential of the cyclodextrin nanoparticle was investigated by varying the amounts of PEGylated adamantane derivatives in the formulation. A mixture of adamantane derivatives was added to give a total cyclodextrin:adamantane molar ratio of 1:1. After optimisation experiments, the Dilysine-CD was complexed with siRNA at mass ratio of 10. A mixture of two Ad-PEG lengths was chosen following an optimisation experiment investigating stability in high salt solution **(Supplementary Figure 1)**. These results indicated that the PEG500 chain length did not prevent salt-induced aggregation to the same extent as a mixture of both PEG lengths, i.e Ad-PEG500 and Ad-PEG5000-2xAA **(Supplementary Figure 1).** Hence, a combination of the two PEG lengths was selected for use in the formulation. The final optimised targeted nanoparticle, in terms of size and charge, contained a mixture of the three adamantane derivatives (1:0.5:0.25:0.25 Dilysine-CD:Ad-PEG500:Ad-PEG5000:Ad-PEG5000-2xAA). Correspondingly the untargeted nanoparticle did not contain any anisamide-targeted PEG (1:0.5:0.5 Dilysine-CD:Ad-PEG500:Ad-PEG5000). The composition of these nanoparticles as well as their size and charge properties are summarised in **Table 2**.

As shown in **Table 2** the unPEGylated Dilysine-CD formulation had the lowest particle size (115.3 nm) and the highest surface charge (+ 27.8 mV). Both the targeted and untargeted formulations (which contained a mixture of PEG500 and PEG5000 adamantane derivatives) had a larger nanoparticle diameter (288.9 and 283.2 nm respectively) relative to the Dilysine-CD. This is in agreement with previous reports whereby the addition of a PEG group to a cyclodextrin gene delivery vector resulted in increased nanoparticle diameter (10, 29). Furthermore, the addition of PEG to the nanoparticle successfully reduced the surface charge from + 27.8 mV for the Dilysine-CD to ~10-14 mV for both the targeted and untargeted

nanoparticles. In addition, all formulations demonstrated polydispersity values (PDI) less than 0.4 (**Table 2**). Thus the targeted formulation demonstrates favourable pharmaceutical properties for use in gene delivery.

3.2.3 Aggregation in Physiological Media

To facilitate *in vivo* application, stability in biological fluid is critical. Depending on the surface properties, nanoparticles may interact with electrolytes, proteins and cellular surfaces in the blood leading to reduced efficacy (30). In addition, aggregation in physiological fluids *in vivo* can result in life-threatening conditions (12). As previously outlined, PEG is frequently included in drug delivery formulations to shield the surface of nanoparticles against undesirable interactions with the bioenvironment (30).

The stability of the three cyclodextrin-based formulations in a physiologically-relevant environment was assessed by measuring the increase in nanoparticle size following incubation in a high salt OptiMEM solution at 37 ºC over a 24 h period (**Figure 6**) (6, 10, 14, 16). As previously reported for other cationic cyclodextrins (9, 10, 14-16), the highly cationic unPEGylated Dilysine-CD aggregated in high salt solution. In contrast, the combination of PEGylated adamantane derivatives used in both the targeted and untargeted formulations prevented aggregation up to 24 h (**Figure 6**), thus supporting the incorporation of PEG500 and PEG5000 into the formulation.

3.2.4 Morphology of the Complexes

The morphology of the cyclodextrin complexes (unPEGylated Dilysine-CD, targeted and untargeted) was analysed by Transmission Electron Microscopy (TEM) (14, 17, 28) (**Figure 7 (a)**). The unPEGylated Dilysine-CD formed spherical nanoparticles when complexed with siRNA. On addition of Ad-PEG (as in the case of the both targeted and untargeted formulations) a noticeable increase in nanoparticle size was detected, further corroborating

the results obtained by dynamic light scattering (**Table 2**). In addition a distinctive 'shielding' PEG layer is visible in both targeted and untargeted formulations as previously reported (14).

3.2.5 Ability of Complexes to Bind and Release siRNA

The degree of binding will influence protection and release of siRNA after cellular uptake. Thus the binding and release properties of the cyclodextrin-based delivery vectors were assessed. As observed in **Figure 7 (b)**, the unPEGylated Dilysine-CD formulation at mass ratio 10 was able to both bind and release siRNA. The addition of Ad-PEG to the formulation (both targeted and untargeted) did not interfere with either siRNA binding or release.

3.2.6 Serum Stability

To ensure efficacy after systemic administration *in vivo* siRNA must be protected from degradation by nuclease enzymes (31). Hence the ability of nanoparticles to protect siRNA from nuclease digestion was investigated after incubation in 50 % serum for up to 24 hours and analysed by gel electrophoresis as previously employed (7, 17, 31, 32). Naked siRNA was partially degraded after 8 h incubation in 50 % serum and was fully degraded after 24 h. In contrast, an siRNA band was still visible in cyclodextrin samples for up to 24 h indicating enhanced protection against serum nucleases (**Figure 8**).

3.3 Uptake of Dilysine-CD.siRNA Formulations in Prostate Cancer Cells

The physicochemical characterisation data of the Dilysine-CD.siRNA nanoparticles identified the optimum prototype nanoparticle with potential for targeted delivery to prostate cancer cells. The uptake of fluorescent siRNA over 24 hours in three different sigma-receptor positive prostate cancer cells lines (DU145, VCaP and PC3 cells) was assessed using targeted and untargeted nanoparticles. The cationic unPEGylated Dilysine-CD was included for comparative purposes.

As demonstrated in **Figure 9**, the targeted formulation had significantly higher uptake relative to the untargeted formulation in all three prostate cell lines tested ($P<0.001$), giving a strong indication that this nanoparticle was taken into the cells via receptor-mediated endocytosis. Thus, the targeted formulation was further investigated for cell toxicity.

3.4 MTT

A major hurdle that must be overcome in the area of gene therapeutics is biocompatibility of delivery vectors to avoid cellular toxicity (33). The MTT assay is a widely used method of assessing eukaryotic cell viability by measuring the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide by cellular mitochondrial reductase enzymes (34). It is frequently used to assess the toxicity of gene therapy delivery vectors and is a good measure of cell metabolism, proliferation and viability (33). Hence the MTT assay was used to assess prostate cancer cell viability 24 hours post-transfection in serum-containing media as previously reported (8, 10, 14, 16). The results for the three different prostate cancer cell lines (DU145, VCaP and PC3 cells) are shown in **Figure 10**. Cell viability was maintained at >80 % following transfection with each of the cyclodextrin-based formulations (Dilysine-CD, targeted and untargeted).

3.5 Competitive Uptake Inhibition

To investigate if uptake into prostate cancer cells occurs specifically via the sigma receptor, cells were pre-incubated with excess free Ad-PEG5000-2xAA. The specificity of the anisamide ligand for the sigma receptor has been extensively studied (17, 25, 35, 36). Such procedures have been previously employed for competitive uptake experiments (28). Results from the competitive uptake experiment in the three different prostate cancer cells lines (DU145 cells, VCaP cells and PC3 cells) are shown in **Figure 11**.

As shown in **Figure 11 (a)**, in the absence of excess free anisamide the targeted formulation had significantly higher uptake in DU145 cells relative to the untargeted formulation 5 hours after transfection ($P<0.05$). However when excess free anisamide was co-incubated with the formulation, the uptake of the targeted nanoparticle was reduced to the same level as the untargeted nanoparticle. In contrast the uptake of the untargeted nanoparticle was unaffected by the absence or presence of free anisamide. This indicates that, in DU145 cells, the targeted nanoparticle is taken up via sigma receptor-mediated endocytosis whereas untargeted nanoparticles are not (**Figure 11 (a)**).

A similar result was found in VCaP cells (**Figure 11 (b)**) where the targeted nanoparticle had significantly higher uptake than the untargeted in the absence of excess free Ad-PEG5000-2xAA ($P<0.001$). However, following the competitive study, uptake of both nanoparticles were equal, demonstrating that uptake of the targeted nanoparticle in VCaP cells is via the sigma receptor.

The results obtained in PC3 cells showed no difference in uptake between the targeted and untargeted nanoparticles in the absence of excess free Ad-PEG5000-2xAA under these experimental conditions. In contrast, the targeted nanoparticle had significantly higher uptake relative to the untargeted nanoparticle at 24 h as seen in **Figure 9**. This effect was not observed at 5 h (**Figure 11**) and suggests that a longer period of transfection (24 h versus 5 h) is required to observe a significantly higher level of uptake of the targeted nanoparticle in PC3 cells. Following co-incubation with excess free Ad-PEG5000-2xAA the uptake of both targeted and untargeted nanoparticles was equally reduced (**Figure 11 (c)**). The reduced cellular uptake of the untargeted nanoparticle was surprising and suggests that the excess free Ad-PEG5000-2xAA ligand may have caused steric hindrance and inhibited non-specific uptake pathways in PC3 cells. The inconclusive results to try to demonstrate receptor-mediated uptake of the targeted nanoparticle may be rationalised by either of two

explanations. Firstly, the conditions for the competitive uptake experiment were optimised in DU145 cells. It is possible that further optimisation may be required to prove receptor-mediated uptake into PC3 cells. Secondly, PC3 cells have lower sigma receptor expression than both DU145 and VCaP cells (manuscript in preparation). Hence it is possible that receptor-mediated uptake of the targeted nanoparticle via binding to the sigma receptor is not a dominant mechanism for cellular uptake in PC3 cells. However, as previously shown, the uptake of the targeted nanoparticle 24 h post-transfection was significantly higher than the untargeted (**Figure 9 (c)**) and hence it was included for comparative purposes in the gene knockdown experiment to see if the higher level of uptake would correspond to higher knockdown of the PLK1 gene (**Figure 12 (c)**).

It is recognised that, despite the absence of the anisamide-targeting moiety, the untargeted nanoparticle underwent high levels of non-specific cellular uptake. This non-specific uptake pathway is also likely to be present for the targeted nanoparticle whereby uptake is further enhanced by the anisamide targeting ligand. However, as the sigma receptor is overexpressed in prostate cancer cells, the presence of the targeting ligand should enhance accumulation of the nanoparticle at its intended site of action relative to the untargeted nanoparticle *in vivo.*

3.6 PLK1 Gene Knockdown

Polo-like kinase 1 (PLK1) is a serine-threonine-protein kinase enzyme whose overexpression has been linked with a variety of cancers, including prostate cancer where it is associated with a higher tumor grade (37). It plays a key role in cellular mitosis and high levels are associated with increased cell proliferation (38). Recently, targeting the PLK1 gene has been identified as a potential therapy for prostate cancer (38-40). With this in mind, the PLK1 gene was selected for mRNA knockdown with our targeted cyclodextrin-based nanoparticle.

As demonstrated in **Figure 12**, the targeted formulation delivering PLK1 siRNA mediated significant knockdown relative to the corresponding non-silencing siRNA in all cell lines tested (minimum $P<0.01$) (**Figure 12 (a), (b), (c)**). In contrast, the untargeted formulation did not reduce expression of the PLK1 gene. This was surprising as the untargeted formulation mediated high levels of siRNA uptake at 24 h (**Figure 9**). The lack of transfection by this vector could indicate that the vector may have been entrapped in the endosome. As demonstrated in **Table 1**, the affinity of the targeting Ad-PEG5000-2xAA for the cyclodextrin cavity was lower than that of Ad-PEG5000. It is possible that this instability allowed the formulation to break apart inside the cell to enhance siRNA release from the targeted nanoparticle which did not occur to the same extent with the untargeted formulation. The successful gene knockdown mediated by the targeted formulation further re-enforces the utility of the anisamide ligand in the formulation to target the sigma receptor and facilitate gene knockdown.

It is recognised that 30 % gene knockdown is unlikely to be powerful enough to observe a clinical effect. However, the scope of this paper was to validate the design and chemical synthesis of an anisamide-targeted adamantane derivative to form an inclusion complex with a cationic cyclodextrin for the treatment of prostate cancer. The formulation will be further optimised in the future to improve upon the results obtained in this study.

4 Conclusion and Future perspective

In conclusion, the ability of a cationic dilysine β-cyclodextrin to form inclusion complexes with adamantane derivatives was successfully utilised to prepare anisamide-targeted siRNA nanocomplexes. The nanoparticles were in the nanoscale size range, close to neutral, resisted aggregation in high salt solution and successfully protected siRNA against serum-induced nuclease degradation. The mechanism of uptake of the targeted formulation into DU145 and VCaP prostate cancer cells was shown to be via receptor-mediated endocytosis. The targeted formulation mediated high siRNA uptake into three prostate cancer cell lines (DU145, VCaP and PC3 cells). Cell viability was maintained at greater than 80 % after treatment and the targeted formulation achieved efficient silencing of the PLK1 gene which is implicated in a wide variety of cancers (41). The nanoformulation holds potential for the treatment of other types of cancer by incorporating a PEGylated adamantane derivative with a different targeting ligand on the terminal end (18). This is the first time that the ability of cyclodextrins to form inclusion complexes with adamantane derivatives has been exploited for the targeted delivery of siRNA to prostate cancer cells via the sigma receptor.

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Figure 1: Chemical structures of Dilysine-CD and adamantane derivatives used. (a) Dilysine-CD. (b) Adamantane-PEG500 (n=10). (c) Adamantane-PEG5000 (n=113). (d) Adamantane-PEG5000-divalent-Anisamide.

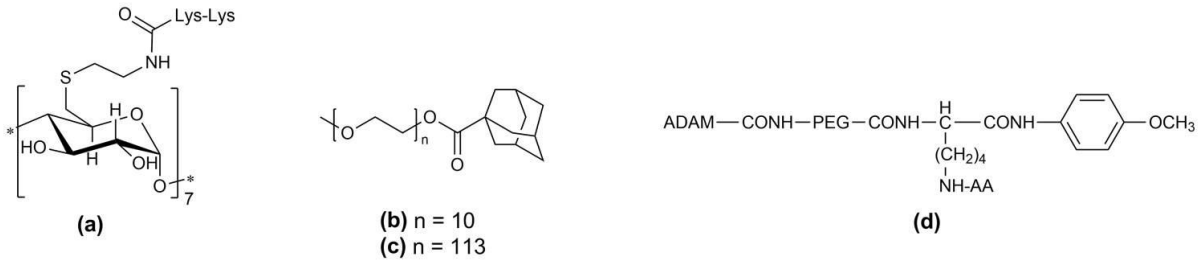

Figure 2: Schematic showing the formulation strategy of the anisamide-targeted and untargeted Dilysine-CD derivatives. (a) Complexation of the Dilysine-CD with siRNA and co-formulation with different PEGylated adamantane derivatives. (b) The targeted nanoparticle. (c) The untargeted nanoparticle.

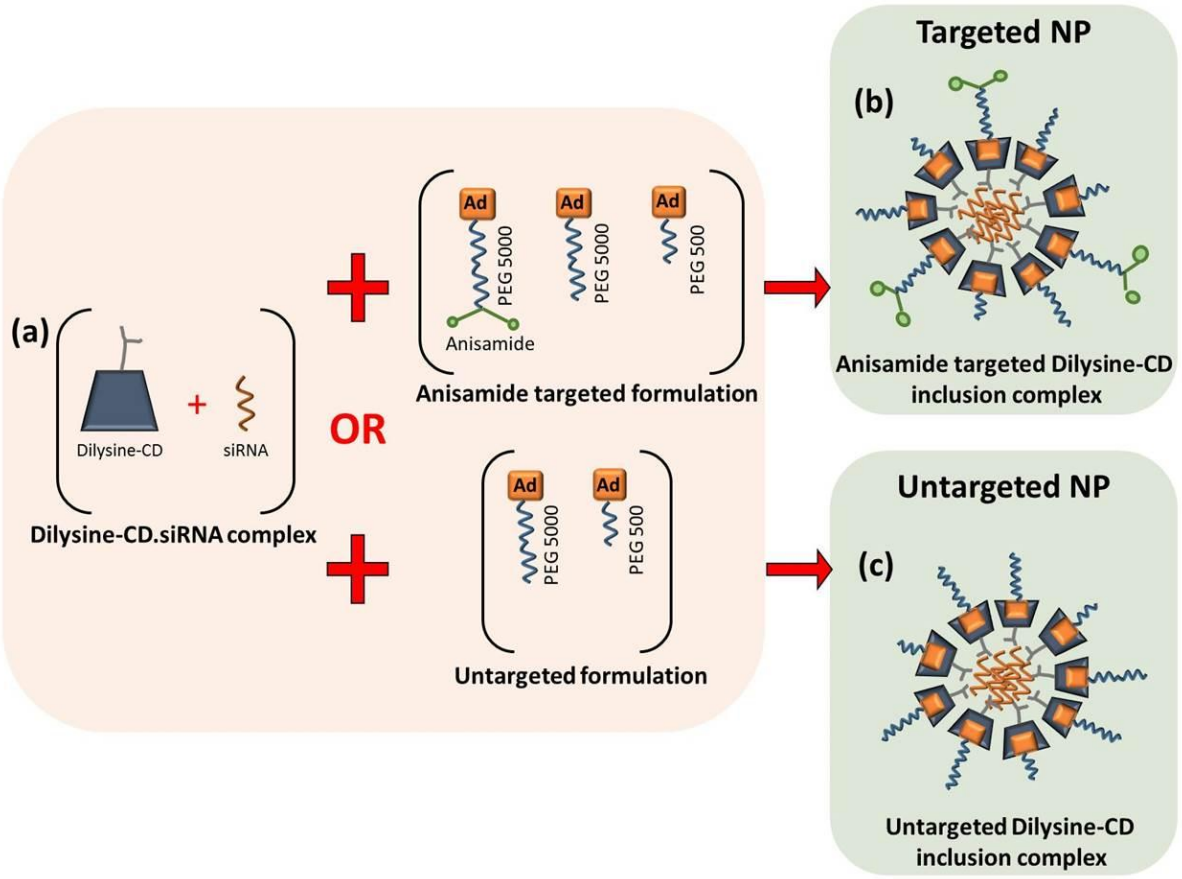

Figure 3: Synthesis of Dilysine-CD (A). (i) Boc anhydride, DIPEA, DMF/water (2/1), rt, 17 h (72%). (ii) N-hydroxysuccinimide, EDC, anhydrous DCM, rt, 17 h under N2 (43%). (iii) 2-(Boc-amino)-ethanethiol, NaH, DMF/water, 80°C, 17 h under N2. (iv) DCM/TFA (1/1), rt, 6h (42% over two steps).(v) DIPEA, DMF, rt, 48 h under N2 (85%); (vi) DCM/TFA (1/1), rt, 6h (100%).

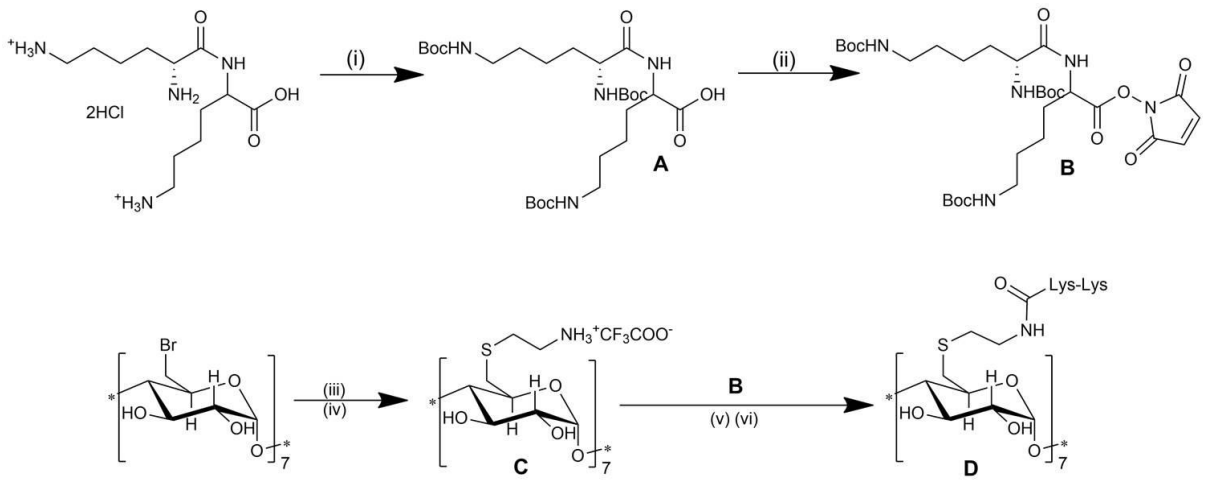

Figure 4: Synthesis of PEGylated adamantane derivatives. (a) Synthesis of Adamantane-PEG500 (E) and Adamantane-PEG5000 (F). DIPEA, DMAP, anhydrous DCM, rt, 17 h, N2. (b) Synthesis of Adamantane-PEG5000-divalent Anisamide (L) (Ad-PEG5000-2xAA).(i) EDC, TEA, DCM, rt, 17h (61%). (ii) HCl, dioxane, 0°C, 2 h,rt, 2h (80%). (iii) EDC, TEA, anisic acid DCM, rt,17 h (47%). (iv) Piperidine, DMF, rt, 2 h. (Q.Y.). (v) Fmoc-PEG5000-OSu, DCM, DIPEA, rt, 3 days (71%). (vi) Piperidine, DMF, rt, 2 h. then Adamantane carbonyl chloride, DIPEA, rt, 17 h (80%)

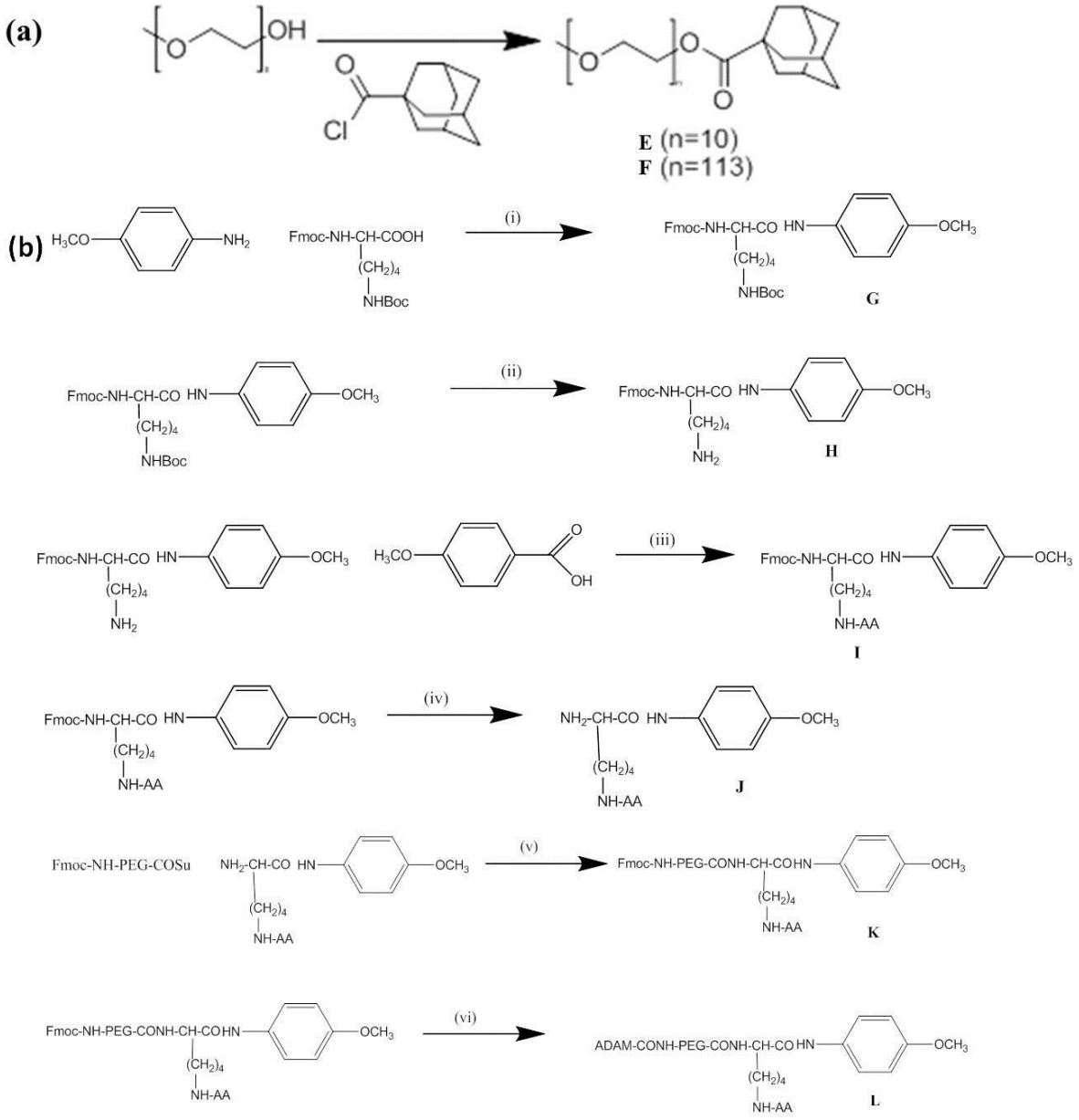

Figure 5: Binding isotherms and signature plot of the titration of (a) Ad-PEG5000 and (b) Ad-PEG5000-2xAA into Dilysine-CD. Raw data (top) and integrated heats (bottom) as a function of the molar ratio (Ad=Adamantane, AA=Anisamide).

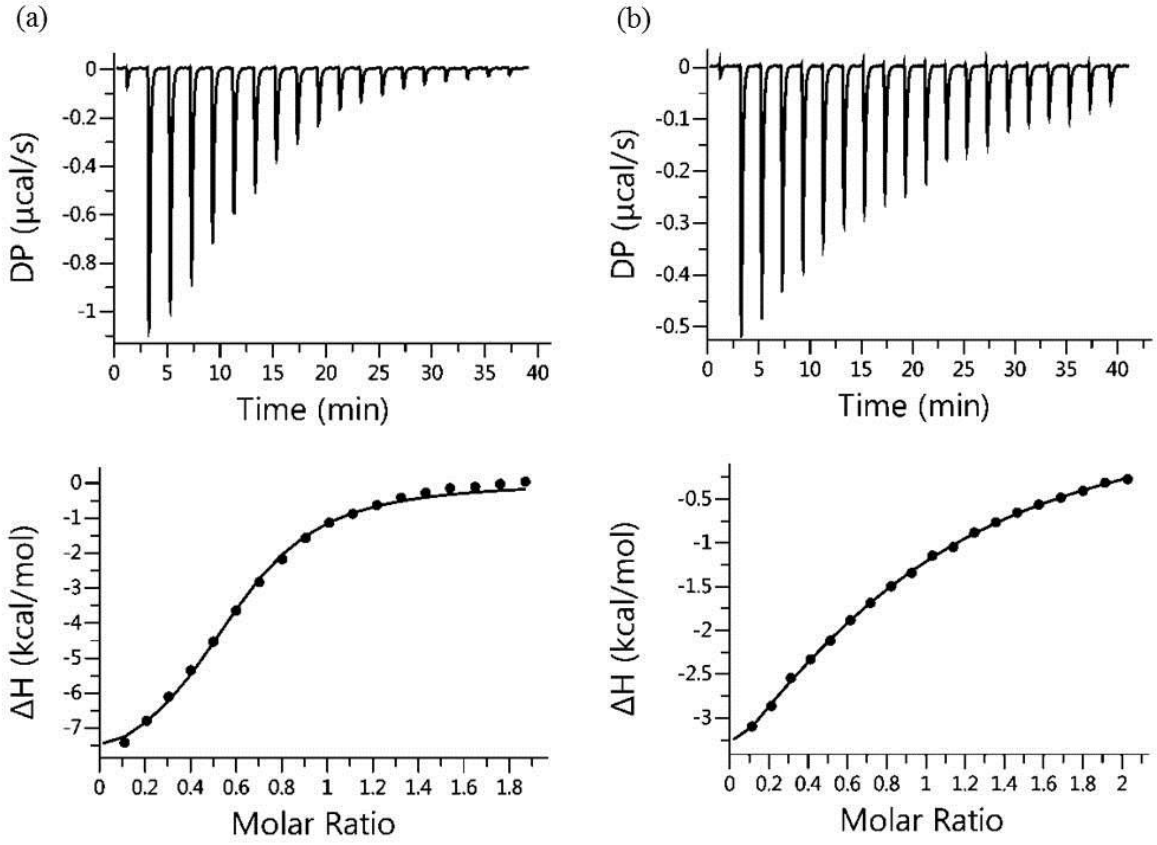

Figure 6: Stability of nanoparticles in high salt solution. Particle size was measured in OptiMEM at 1, 4 and 24 h. (n=3±S.D.)

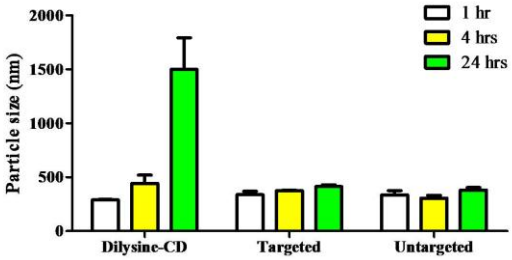

Figure 7: Transmission Electron Microscopy (TEM) and siRNA of optimised nanocomplexes. (a) TEM (scale bar = 500 nm) (b) Binding and release study.

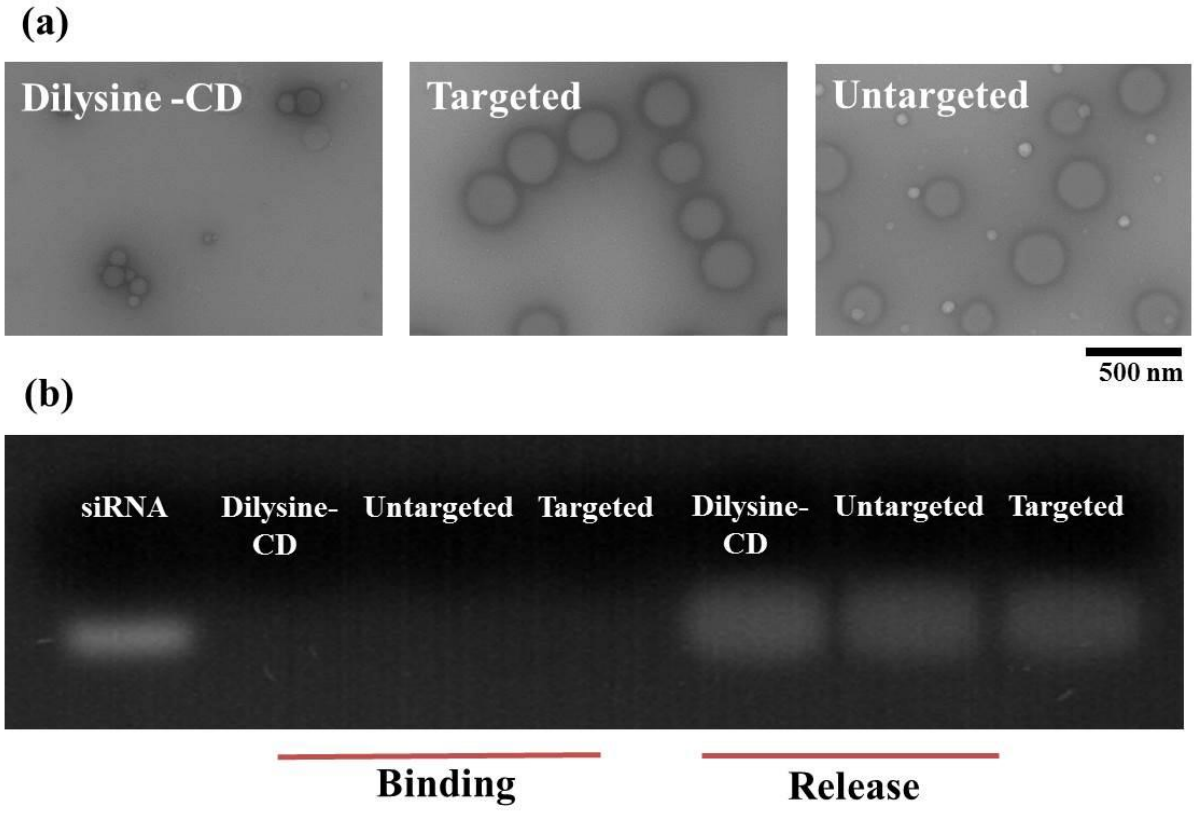

Figure 8: Serum stability of optimised nanocomplexes after incubation in 50 % Fetal Bovine Serum for up to 24 h at 37 °C. (a) Naked siRNA. (b) Dilysine-CD. (c) Targeted. (d) Untargeted.

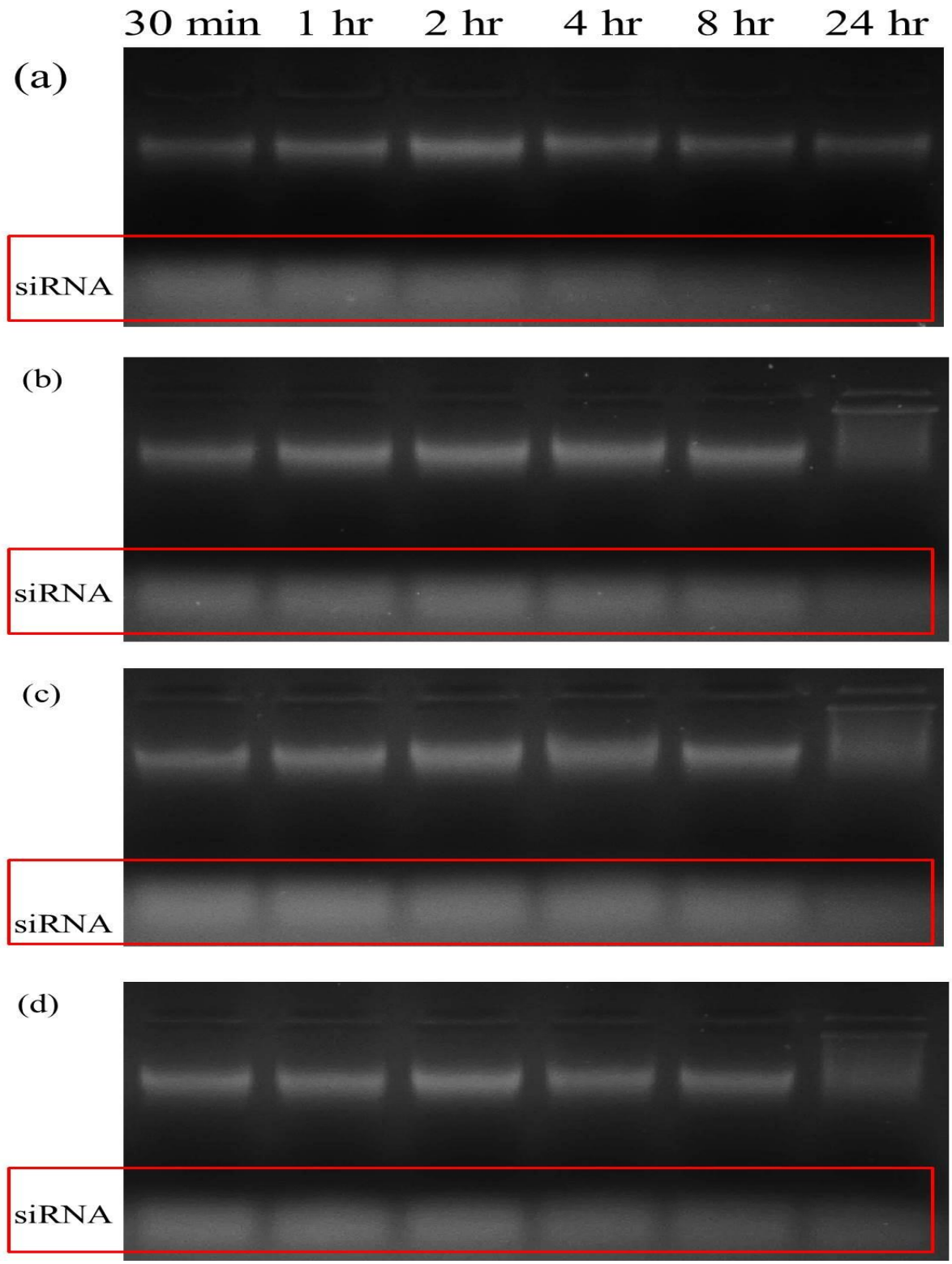

Figure 9: Fluorescent siRNA uptake into prostate cancer cells at 24 h. Fluorescence of cells was measured 24 h after transfection using a fluorescent plate reader. (a) DU145 cells (b) VCaP cells. (c) PC3 Cells. (Lipo=Lipofectamine 2000. siRNA=Naked siRNA. n=3/4±S.D.)

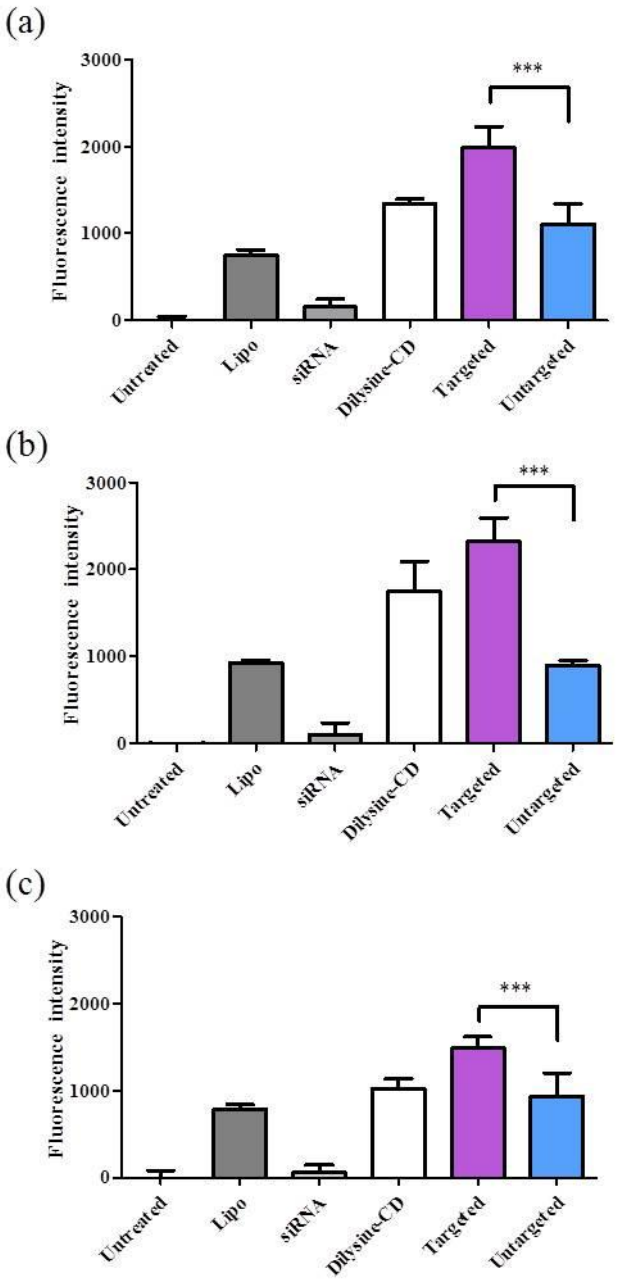

Figure 10: MTT assay measuring viability of prostate cancer cells 24 h post-transfection using 50 nM siRNA. (a) DU145 cells (b) VCaP cells (c) PC3 cells. (n=3-4±S.D.)

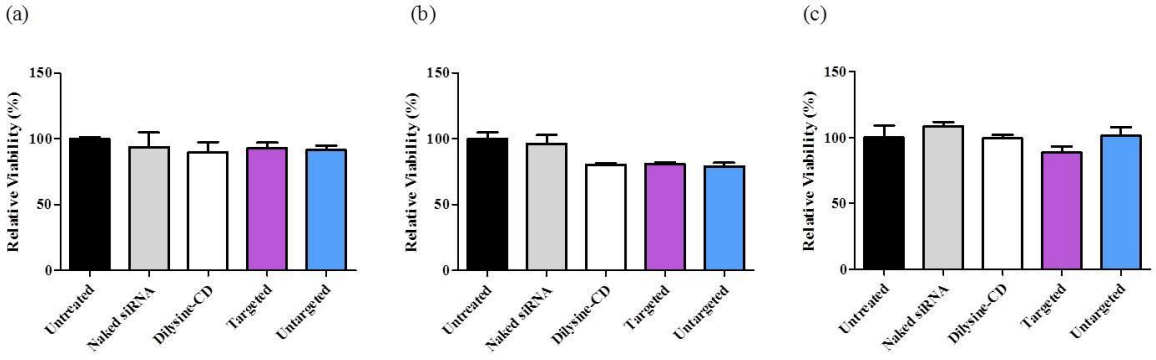

Figure 11: 5 h competitive uptake in the absence/presence of 100 µM free Ad-PEG5000-2xAA ligand. (a) DU145 cells. (b) VCaP cells. (c) PC3 cells. (n=3-4±S.D)

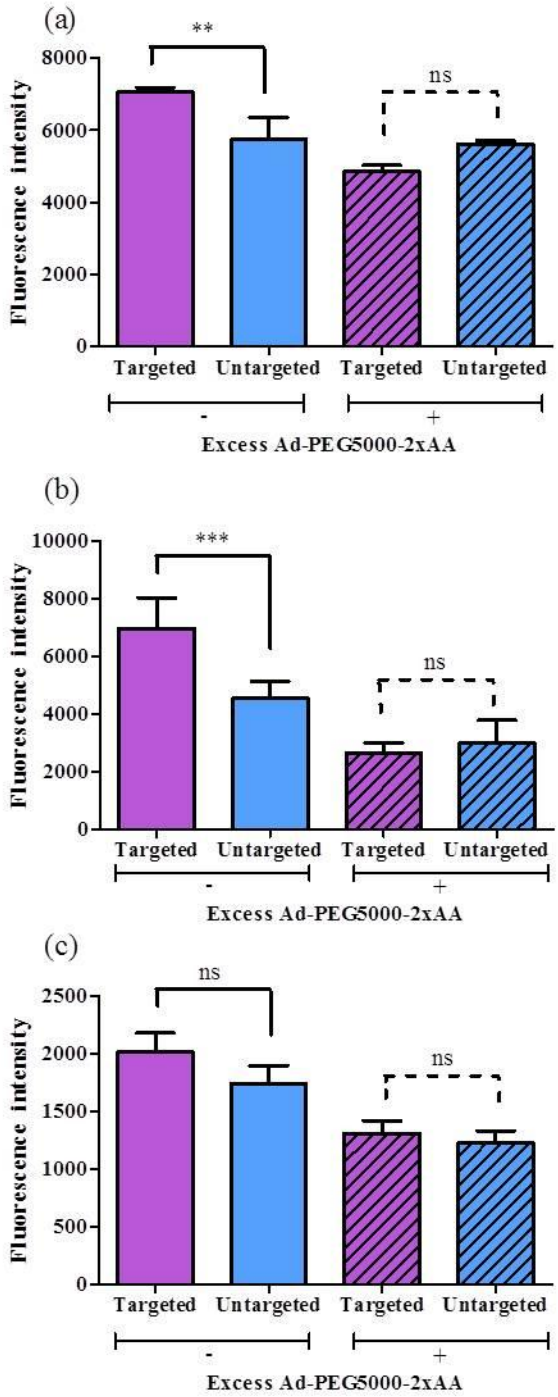

Figure 12: 48 h PLK1 mRNA knockdown using 100 nM siRNA. PLK1 gene expression was normalised to the non-silencing counterpart (either targeted or untargeted) whose expression was set as 100 %. (a) DU145 cells. (b) VCaP cells. (c) PC3 cells. (n=3-5±S.D.).

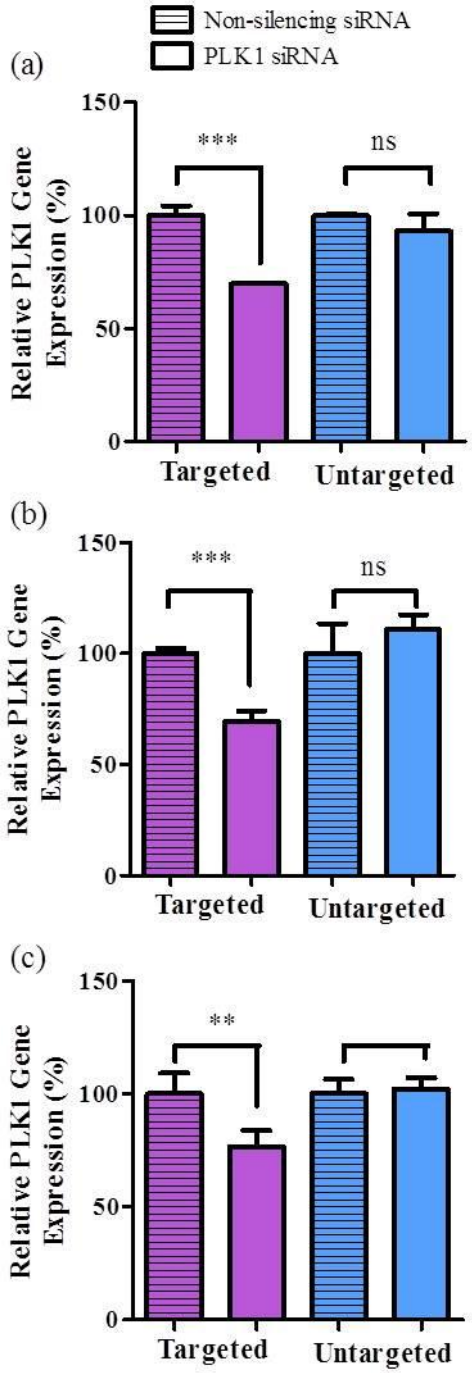

Table 1: Isothermal Calorimetry showing the thermodynamic properties of Dilysine-CD in complexation with Ad-PEG5000 and Ad-PEG5000-2xAA. N refers to the stoichiometry of the interaction between the Dilysine-CD and adamantane derivative, K_D is the dissociation constant, ΔH is the enthalpy change, ΔG is the Gibbs free energy and ΔS is the entropy of the reaction. The lower value of ΔH for the Ad-PEG5000 derivative relative to Ad-PEG5000-2xAA indicates higher binding between this adamantane derivatives and Dilysine-CD. (*Ad=Adamantane, AA=Anisamide).*

Sample	[Ad] (μM)	[CD] (μM)	N (sites)	K_D (μM)	ΔH (kcal/mol)	ΔG (kcal/mol)	-TΔS (kcal/mol)
Ad-PEG5000 into Dilysine-CD	958	100	0.587 (±3.5e-3)	12.8 (±493e-3)	-9.79 (± 0.128)	-6.68	3.11
Ad-PEG5000-2xAA into Dilysine-CD	980	100	0.783 (±2.4e-2)	89.8 (±14)	-8.11 (± 0.968)	-5.52	2.59

Table 2

Composition of formulations tested as well as their size and charge properties. The number of moles of cationic Dilysine-CD was set as 1 and the total ratio of Ad-PEG to CD was kept constant at 1. The Ad-PEG was composed of different PEGylated derivatives. (Dilysine-CD = Dilysine-modified cyclodextrin. Ad-PEG500 = Adamantane modified with a PEG500 chain. Ad-PEG5000 = Adamantane modified with a PEG5000 chain. Ad-PEG5000-2xAA = Adamantane modified with a PEG5000 chain with two anisamide targeting moieties on the terminal end of the PEG). Size, charge and polydispersity index (PDI) measurements were taken in RNase free water using 0.3 μg siRNA (n=3 ± S.D.)

Formulation name	**Dilysine-CD: siRNA mass ratio**	**Number of moles relative to Dilysine-CD**			**Size (nm) (± S.D.)**	**Charge (+mV) (± S.D.)**	**PDI (± S.D.)**
		Ad-PEG500	Ad-PEG5000	Ad-PEG5000-2xAA			
Dilysine-CD	10	-	-	-	115.3 (± 5.8)	27.8 (± 2.1)	0.047 (± 0.046)
Targeted	10	0.5	0.25	0.25	288.9 (± 17.0)	10.28 (± 2.1)	0.395 (± 0.005)
Untargeted	10	0.5	0.5	-	283.2 (± 11.8)	14.1 (± 1.2)	0.372 (± 0.040)