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Synthesis and characterization of rabies virus glycoprotein-tagged amphiphilic cyclodextrins for siRNA delivery in human glioblastoma cells: *In vitro* analysis

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ABSTRACT

In man brain cancer is an aggressive, malignant form of tumour, it is highly infiltrative in nature, is associated with cellular heterogeneity and affects cerebral hemispheres of the brain. Current drug therapies are inadequate and an unmet clinical need exists to develop new improved therapeutics. The ability to silence genes associated with disease progression by using short interfering RNA (siRNA) presents the potential to develop safe and effective therapies. In this work, in order to protect the siRNA from degradation, promote cell specific uptake and enhance gene silencing efficiency, a PEGylated cyclodextrin (CD)-based nanoparticle, tagged with a CNS-targeting peptide derived from the rabies virus glycoprotein (RVG) was formulated and characterized. The modified cyclodextrin derivatives were synthesized and co-formulated to form nanoparticles containing siRNA which were analysed for size, surface charge, stability, cellular uptake and gene-knockdown in brain cancer cells. The results identified an optimised co-formulation prototype at a molar ratio of 1:1.5:0.5 (cationic cyclodextrin:PEGylated cyclodextrin:RVG-tagged PEGylated cyclodextrin) with a size of 281 ± 39.72 nm, a surface charge of 26.73 ± 3 mV, with efficient cellular uptake and a 27% gene-knockdown ability. This CD-based formulation represents a potential nanocomplex for systemic delivery of siRNA targeting brain cancer.

1. Introduction

The specific silencing of target genes using short interfering RNA (siRNA) via the catalytic RNA interference (RNAi) pathway is of significant interest for the treatment of a wide range of diseases. The delivery of siRNA to the site of action in the body remains a serious challenge. siRNAs can be readily degraded by endogenous nucleases, and permeability across biological membranes is hindered by the negative charge of the molecule. A number of different approaches to siRNA delivery have been proposed, including the use of cationic lipids, cell penetrating peptides and polymers. Several treatments based on these methods are now in clinical trials (Gooding et al., 2012; Kanasty et al.). Nanoparticle-based drug/gene delivery devices are gaining interest for targeting central nervous system (CNS) diseases as current treatment modalities are invasive and lack efficacy (Malhotra and Prakash, 2011; O'Mahony et al., 2013a). Site-specific delivery of nanoparticles can be achieved by active-targeting that follows receptor-mediated endocytosis of the nano-formulations. Such targeted nano-formulations have advantages over general chemotherapeutic drugs as the healthy tissue is not affected by the adverse effect therapy.

Brain cancer is the most aggressive and malignant primary tumour affecting glial cells (astrocytes, oligodendrocytes, and ependymal cells) in humans (Maher et al., 2001). Recent advances in nanoparticulate formulations for brain cancer include the use of various organic and inorganic materials for therapeutic delivery. Organics include polymers such as PLGA (Chang et al.; Zhou et al.), lipids (Gaillard et al.; Krauze et al.), dendrimers (He et al.; Sarin et al.) while among inorganics gold (Hainfeld et al.; Joh et al.), silica (Wan et al.) and iron oxide (Ren et al.) have been used.

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Organic materials are preferred over inorganic materials, due to the biodegradability of the nanoparticle device after delivery. Nanoparticles offer the versatility of coating with a variety of targeting moieties for cell-specific delivery. Some of the most commonly used targeting ligands for CNS delivery include transferrin (Chang et al.; Ren et al.), lactoferrin (Xie et al.), trans-activating transcriptional activator (Malhotra et al., 2013b), aptamers (Gao et al., 2012a; Gao et al.), and angiopep (Xin et al.). In addition, the rabies virus glycoprotein (RVG), known to specifically bind to the nicotinic acetylcholine receptors (nAchR) on neuronal cells, has been used as a targeting ligand to potentiate receptor-mediated endocytosis of siRNA nanoparticles as a means of crossing the blood–brain barrier (BBB) (Son et al.; Tao et al., 2012). Nanoparticles are also often modified with poly(ethylene) glycol (PEG) in order to reduce aggregation, immunogenicity, and ionic interaction with serum proteins (Alexis et al.).

In the present study, we have utilized modified β-cyclodextrin derivatives to formulate nanoparticles capable of delivering siRNA to human glioblastoma cells. Cyclodextrins (CDs) are cyclic non-reducible oligosaccharides, consisting of glucopyranose units linked together via α-(1-4) glycoside bonds. Cationic amphiphilic CDs have shown successful delivery of siRNA to neuronal cells (O'Mahony et al., 2012a,b) and achieved knockdown of the Huntington's gene following direct injection into the brain of the R6/2 mouse model with acceptable levels of toxicity (Godinho et al., 2014a; Godinho et al.).

We have previously shown that a neutral PEGylated CD (**CD2** in Fig. 1) was able to reduce nanoparticle aggregation when co-formulated with a cationic amphiphilic CD derivative (**CD1** in Fig. 1) (O'Mahony et al., 2012c), but also inhibited cellular uptake, probably due to masking of the cationic charges which are necessary for cell adhesion. In this study, in order to reinstate cellular uptake while simultaneously maintaining the reduced cationic nature of the co-formulation, the attachment of a targeting ligand, RVG, was investigated. As previously described by us, copper(I)-catalysed 'click' chemistry was used to PEGylated the secondary face of β-CD (**CD2** in Fig. 1). In this case, however, the PEG_{500} chains (**CD3** in Fig. 1) were terminated in reactive amino groups. PEG_{500} chains were chosen in this study due to the ease of synthesis, as longer chains can result in steric crowding at the secondary face of the cyclodextrin that prohibits full substitution. We have previously shown that although longer PEG chains lead to lower aggregation *in vitro*, this difference does not translate to a significant improvement in pharmacokinetic properties (Godinho et al., 2014b). The additional amine functionality at the end of the PEG chains was used to attach a maleimide linker in order to conjugate the RVG peptide as a targeting ligand (**CD4** in Fig. 1). Co-formulations of the three modified CDs (**CD1**, **CD2 and CD4**) complexed with siRNA were prepared, characterized and assessed for the ability to transfect brain cancer cells.

2. Material and methods

2.1. Materials

Negative control fluorescein-labelled scrambled siRNA (sense sequence 5′-UUC UCC GAA CGU GUC ACG U) and GAPDH siRNA (sense sequence 5′-GGU CGG AGU CAA CGG AUU U) were obtained from Qiagen (United Kingdom).

Anhydrous DMF was purchased from Aldrich in Sureseal™ bottles over molecular sieves and stored under nitrogen. Anhydrous DCM was obtained from a PureSolv™ solvent purification system. Cyclodextrin (Aldrich) was dried at 120 °C under high vacuum for 12 h before use. Triphenylphosphine (Aldrich) was recrystallised from ethanol. For solid phase peptide synthesis, protected amino acids and HBTU were purchased from Merck, HOBt from Iris, and solvents and Rink Amide resin from Applied Biosystems. All other chemicals were obtained from Aldrich and used without further purification unless otherwise stated.

2.2. Synthesis of modified cyclodextrins

In all cases reactions were monitored by TLC on a precoated aluminium plates of silica gel (Merck $60F_{254}$ 0.25 mm). Carbohydrates were visualised by dipping in 5% sulphuric 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-T of Premier mass spectrometer (Waters Corporation, USA) using a matrix of either trans-2-[3-(4-te rt-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) or α-cyano-4-hydroxycinnamic acid (CHCA).

The cationic amphiphilic cyclodextrin, SC12-CD-click-propylamine (**CD1 in** Fig. 1) and the neutral PEGylated derivative, SC12-CD-click-PEG500, (**CD2 in** Fig. 1), were synthesized as previously described (O'Mahony et al., 2012c).

2.2.1. Synthesis of SC12-CD-click-PEG500-amine

The synthesis, outlined in Scheme 1, is as follows;

1-Boc-amino-ω-azido-PEG_{500} (1)

1-amino-ω-azido-PEG_{500} (Iris Biotech GmbH, Germany) (502 mg, 0.95 mmol), di-tert-butyl dicarbonate (229 mg, 1.05 mmol) and diisopropylethylamine (183 μl, 1.05 mmol) were dissolved in dichloromethane (20 mL) and stirred for 17 h at room temperature (RT). The reaction mixture was concentrated *in vacuo*, and the residue was purified by column chromatography over silica gel (dichloromethane–methanol: 9–1). The product was obtained as a yellow oil (579 mg, 97%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.72–3.57 (m, 38H, PEG-CH_2), 3.54 (t, J = 5.2 Hz, 2H, O—CH_2), 3.42–3.35 (m, 2H, NHCH_2), 3.30 (d, J = 5.0 Hz, 2H, N_3—CH_2), 1.44 (s, 9H, $3CH_3$)

^{13}C NMR (126 MHz, $CDCl_3$) δ 85.1 (s, CCH_3), 70.7–70.4 (m, PEG-CH_2), 70.18 (s, O—CH_2), 70.16 (s, O—CH_2), 50.6 (s, N_3—CH_2), 40.3 (s, NH—CH_2), 28.4 (s, CH_3), 27.4 (s, CH_3).

ESI-MS (m/z) (% relative intensity, ion): 625.76 (100, M— H), 626.75 (35, M^-), 661 (20, M + Cl).

HRMS (ESI$^+$-TOF) (m/z): $[M + Na]^+$ calcd for $C_{27}H_{54}N_4O_{12}Na$, 649.3636; found, 649.3625.

2.2.2. Heptakis[6-bromo-2-O-(N-(ω-Boc-amino-PEG_{500}-yl)-1′H-triazole-4′-yl-methyl)]-β-cyclodextrin (2)

Heptakis(6-bromo-6-deoxy-2-*O*-propargyl)-β-cyclodextrin (169 mg, 0.092 mmol) (O'Mahony et al., 2012c), 1-Boc-amino-ω-azido-PEG_{500} (579 mg, 0.92 mmol), copper(II) sulphate pentahydrate (23 mg, 0.092 mmol) and sodium ascorbate (55 mg, 0.28 mmol) were dissolved in dimethylformamide (10 mL) and water (10 mL) and stirred for 17 h at 80 °C. The mixture was concentrated *in vacuo* and the residue taken up in methanol, filtered and purified over Sephadex LH20 (methanol 100%) to give the product as a yellow oil (510 mg, 89%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (s, 1H, triazole CH), 5.18–4.75 (m, 4H, NH, H-1, OH-3), 4.61 (d, J = 19.1 Hz, 2H, OCH_b), 4.10–3.14 (m, 38H, PEG-CH_2, OCHa, H-3, H-5, H-6a, H-6b, NHCH_2), 1.44 (s, 7H, $3CH_3$).

^{13}C NMR (126 MHz, $CDCl_3$) δ 156.0 (s, C═O), 124.6 (s, triazole CH), 101.4 (s, C-1), 85.6 (s, C-4), 79.1 (s, CCH_3), 72.8 (s, C-3),