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PEGylated cyclodextrins as novel siRNA nanosystems: Correlations between polyethylene glycol length and nanoparticle stability

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ABSTRACT

Silencing disease-related genes in the central nervous system (CNS) using short interfering RNA (siRNA) holds great promise for treating neurological disorders. Yet, delivery of RNAi therapeutics to the brain poses major challenges to non-viral systems, especially when considering systemic administration. Cationic nanoparticles have been widely investigated for siRNA delivery, but the tendency of these to aggregate in physiological environments limits their intravenous application. Thus, strategies to increase the stability of nanoparticles have been developed. Here, we investigated the ability of modified cationic amphiphilic or PEGylated amphiphilic cyclodextrins (CD) to formulate stable CD.siRNA nanoparticles. To this end, we describe a simple method for post-modification of pre-formed cationic CD.siRNA nanoparticles at their surface using PEGylated CDs of different PEG lengths. PEGylated CD.siRNA nanoparticles presented reduced surface charges and increased stability in physiological salt conditions. Stability of PEGylated CD.siRNA nanoparticles *in vitro* increased with both PEG length and PEG density at the surface. Furthermore, in a comparative pharmacokinetic study, increased systemic exposure and reduced clearance were achieved with CD-formulations when compared to naked siRNAs. However, no significant differences were observed among non-PEGylated and PEGylated CD.siRNAs suggesting that longer PEG lengths might be required for improving stability *in vivo*.

1. Introduction

Therapeutic gene silencing by harnessing the endogenous RNA interference (RNAi) pathway using synthetic short interfering RNAs (siRNA) holds great promise for the treatment of neurological disorders, such as Huntington's disease (Sah, 2006; Thakker et al., 2006). However, silencing disease-related genes in the central nervous system (CNS) presents a significant challenge for traditional non-viral delivery systems. In addition to the difficulty of transfecting neuronal cells, siRNA nanoparticles must overcome multiple biological barriers, not least of all, the blood brain barrier (BBB), which limits the diffusion of these nanoparticles to the brain (O'Mahony et al., 2013b). Indeed, the most successful preclinical studies so far have bypassed the challenge of systemic delivery and used stereotaxic injections into specific structures within the brain (Bonoiu et al., 2011; Cardoso et al., 2010, 2008; Godinho et al., 2013) and/or infusion into the intracerebroventricular (i.c.v.) system (Thakker et al., 2004, 2005). Although gene expression knockdown has been efficiently achieved using the strategies mentioned above, direct and continuous administration into the brain may be less practical when transferring to the clinical setting. Therefore, the development of efficient and non-toxic non-viral formulations for systemic administration and subsequent transport across the BBB has received great attention (O'Mahony et al., 2013b).

Cyclodextrins (CD) are starch-derived molecules which have been successfully modified to form cationic amphiphilic siRNA delivery systems (O'Mahony et al., 2012). We have previously

Abbreviations: ANOVA, one-way analysis of variance; AUC, area under the curve; AUMC, area under the moment curve; BBB, blood-brain barrier; CL, clearance; CNS, central nervous system; CD, cyclodextrin; DIW, deionised water; DLS, dynamic light scattering; ELS, electrophoretic light scattering; FBS, foetal bovine serum; i.c.v., intracerebroventricular; i.v., intravenous; MRT, mean residence time; Mw, molecular weight; PDI, polydispersity index; PEG, polyethylene glycol; PEI, polyethylenimine; PK, pharmacokinetic; RNAi, RNA interference; RT, room temperature; SD, standard deviation; SEM, standard error of mean; SI, supplementary information; siRNA, short interfering RNA; Vss, steady-state volume distribution.

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used this non-viral vector to deliver specific siRNAs to silence the expression of the mutant Huntingtin gene in the R6/2 mouse brain through stereotaxic injections into the striatum (Godinho et al., 2013). However, further modifications are required to improve the suitability of such cationic CD formulations for systemic administration, including in the first instance, enhancing their stability in physiological salt and serum conditions. Polyethylene glycol (PEG), a polymer of ethylene oxide commonly used in medical applications, has been widely used to confer "steric" stability to nanoparticles, reducing interactions with plasma and blood components (Gref et al., 2000; Monfardini and Veronese, 1998). In addition, PEGylation has also been shown to minimise recognition of nanoparticles by the mononuclear phagocyte system (Gref et al., 2000; Owens and Peppas, 2006). By these means, PEGylation has prolonged the circulating times of several non-viral vectors including CD-containing polymers (Davis, 2009; Pun and Davis, 2002), lipid-based nanoparticles (Li et al., 2007; Sonoke et al., 2008) and cationic polymers (e.g. polyethylenimine (PEI)) (Malek et al., 2009). In order to optimise the stability of nanoparticles, different PEG lengths and polymer densities have been evaluated, however, no general consensus has yet been reached on the ideal PEG length or polymer density (Gref et al., 2000; Kunath et al., 2002; Mao et al., 2006). Indeed, this is likely to be dependent on the vector type and on the PEGylation strategy used for modification of the nanoparticles.

Different strategies for PEGylating nanoparticles have been employed including: chemical methods to covalently couple PEG chains to the non-viral vector (Guo et al., 2012; Mao et al., 2006); and physical methods, such as post-insertion (Mendonça et al., 2009; Morille et al., 2011; O'Mahony et al., 2013a) and co-formulation (O'Mahony et al., 2013c), used to incorporate PEG into the final formulation. Regarding CD-based formulation approaches, our group has previously described a co-formulation strategy whereby a cationic amphiphilic CD and a PEGylated amphiphilic CD were blended together prior to siRNA complexation. This PEGylation method yielded nanoparticles with increased stability in physiological salt conditions (O'Mahony et al., 2013c). Alternatively, pre-formed CD.siRNA nanoparticles have also been surface-modified by post-insertion of PEGylated lipids yielding a formulation with an enhanced pharmacokinetic (PK) profile when compared to naked siRNAs (O'Mahony et al., 2013a). However, although different methods for PEGylating CD-based nanoparticles have been reported, the influence of PEG length and polymer density on the properties of these delivery systems warrants further investigation.

The present study aims to determine the influence of PEG density and length on the properties of CD nanosystems. To this end, a post-PEGylation approach was used to modify pre-formed cationic CD.siRNA nanoparticles at the surface using different PEGylated amphiphilic CDs containing a range of molecular weight (Mw) PEGs. Physicochemical characteristics and *in vitro* stability in physiological salt conditions were investigated. Finally, an optimal polymer density was selected and the PK behaviour of PEGylated CD.siRNA nanoparticles was assessed *in vivo* and compared to non-PEGylated CD.siRNA nanoparticles and naked siRNA.

2. Materials and methods

2.1. Synthetic siRNAs

Synthetic duplexed siRNAs were obtained from Sigma–Aldrich (France) or QIAGEN (United Kingdom). Huntingtin target siRNAs (HTTsiRNA) as per Wang et al. (Wang et al., 2005) sense strand, 5′-GCCUUCGAGUCCCUCAAGUCC-3′; antisense strand, 5′-ACUUGAGG-GACUCGAAGGCCU-3′. FAM-labelled siRNA (FAMsiRNA): sense strand, 5′-[6FAM] UUCUCCGAACGUGUCACGUdTdT-3′; antisense strand, 5′-ACGUGACACGUUCGGAGAAdTdT-3′.

2.2. Preparation of PEGylated CD.siRNA nanoparticles

The synthesis of the modified cationic amphiphilic β-cyclodextrin (SC12CD Click Propylamine) and PEGylated amphiphilic β-cyclodextrin (SC12CD Click PEG500) has been previously described (O'Mahony et al., 2012). PEGylated amphiphilic β-cyclodextrins with larger Mw PEG chains (SC12CD Click PEG1000, SC12CD Click PEG2000) were synthesised using the same method. SC12CD Click Propylamine and SC12CD Click PEGx will hereafter be referred to as cationic CD and PEG CDs, respectively. Modified amphiphilic CDs were dissolved in chloroform and chloroform evaporated under a stream of gaseous nitrogen. Prior to formulation with siRNAs, modified amphiphilic CDs were reconstituted in sterile deionised water (DIW) and sonicated for 60 min. Complexes with anionic siRNA were formed using the cationic CD at mass ratio 10 (μg of CD : μg of siRNA). Equal volumes of cationic CD and siRNA solution were mixed together and incubated at room temperature (RT) for 20 min. Thereafter, PEGylated CDs were added to pre-formed cationic CD.siRNA nanoparticles in defined molar ratios between cationic CD and PEGylated CD. A specific volume of each of the PEGylated CDs was gently mixed with corresponding pre-formed CD.siRNA nanoparticles and further incubated at RT for 20 min.

For *in vivo* studies, CD.siRNA nanoparticles were prepared as outlined above in 5% glucose and concentrated by ultrafiltration using Vivaspin 500 centrifugal units (Sartorius, Germany) to a final concentration of 0.267 μg/μL of siRNA, as previously described (Godinho et al., 2013).

2.3. Physicochemical characterisation

Binding and complexation of siRNA was confirmed by gel retardation assay. CD.siRNA nanoparticles containing ~0.3 μg of siRNA were mixed with 2 μL of Blue Juice Loading Buffer (Invitrogen, Carlsbad, CA) and sufficient DIW to a final volume of ~30 μL. Samples were loaded in a 2% agarose gel and electrophoresis performed at 90 mV for 20 min in Tris-acetate-Ethylenediaminetetraacetic acid buffer (Fisher Scientific, Fair Lawn, NJ). The gel was post-stained using GelRed™ nucleic acid stain (Biotium, Hayward, CA) and visualised using the DNR Bioimaging Systems and Gel Capture version 7.0.9 software.

Size and surface charge measurements were carried out using dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively. CD.siRNA nanoparticles, containing ~3 μg of siRNA, were diluted up to 1 mL with filtered sterilised DIW and assessed by DLS and ELS using a Malvern Zetasizer Nano ZS. A total of five readings for size and charge were taken per sample and the refractive index (1.33) and viscosity (0.8872 mPa s) of water were used for data analysis.

2.4. In vitro *stability studies in physiological buffer conditions*

The stability of PEGylated CD.siRNA nanoparticles was investigated in salt-containing medium (optiMEM®, Invitrogen, UK) and in foetal bovine serum (FBS, Sigma–Aldrich, Germany). Complexes were prepared as above, diluted up to 1 mL in optiMEM® or FBS and incubated at 37 °C. Size measurements were carried out at different time points (4, 24, and 48 h) using a Malvern Zetasizer Nano ZS.

2.5. Comparative pharmacokinetic study

Male 8-week old Balb/c mice (~24 g) were obtained from Harlan Laboratories (United Kingdom). Mice were given