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Amphiphilic polyallylamine based polymeric micelles for siRNA delivery to the gastrointestinal tract: *In vitro* investigations

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ABSTRACT

Targeting the gastrointestinal (GI) tract represents a promising strategy for local or systemic delivery of nucleic acid-based therapeutics. The development of a nano-carrier for siRNA delivery *via* the GI tract would enable localised therapy for a range of gastrointestinal diseases. Previously, nano-sized polymeric micelles (PM) formed by a variety of amphiphilic poly(allylamine) (PAA) derivatives have shown potential for oral delivery of hydrophobic drugs and bioactive peptides. The aim of this study was to evaluate the ability of these amphiphilic PAA-based PM for siRNA delivery *via* the GI tract. The physicochemical characteristics of PAA·siRNA transfection complexes and their biological efficacy *in vitro* were investigated. Physicochemical profiles demonstrated that PAAs and siRNA self-assembled into complexes with nano-sized diameters (150–300 nm) and cationic surface charge (+ 20 to 30 mV). The PAA·siRNA complexes were stable in the presence of salt solutions and simulated gastric/intestinal fluids. In undifferentiated Caco-2 cells, PAA·siRNA complexes achieved up to 35% cellular uptake, with successful siRNA release from endosomes/lysosomes and significant luciferase gene knockdown. These results highlight the potential of these nano-sized PM for siRNA oral delivery *via* the GI tract for treatment of gastrointestinal diseases.
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1. Introduction

In the last decade, the progression of RNA interference (RNAi) therapy from an experimental technology into a feasible clinical approach to treat a wide range of human diseases has been remarkable (Bumcrot et al., 2006; Davidson and McCray, 2011; Kim and Rossi, 2007). The future of RNAi therapeutics is highly dependent on achieving successful delivery of the effector molecule, small interfering RNA (siRNA) (Bumcrot et al., 2006; Guo et al., 2010; Whitehead et al., 2009). Recently, delivery *via* the gastrointestinal (GI) tract has attracted an increased level of interest (O'Neill et al., 2011a). Indeed, oral administration of small interfering RNA (siRNA) has many merits, including non-invasive access, patient acceptability and therapeutic versatility in the treatment of gastrointestinal cancer and inflammatory bowel disease (IBD) (Aouadi et al., 2009; Kriegel and Amiji, 2011; Wilson et al., 2010). The GI tract, however, poses various morphological and physiological obstacles, including the presence of mucus, salts and enzymes and pH fluctuations, which can restrict the stability and therapeutic efficacy of siRNA (Martien et al., 2006; O'Neill et al., 2011a). Therefore, the design of robust non-viral delivery systems is paramount to future success (Akhtar, 2009; O'Neill et al., 2011a).

Previous attempts at non-viral delivery of nucleic acids to the GI tract have largely involved polymeric nano- or micro-particles, while liposomes have typically demonstrated poor stability in the gut (O'Neill et al., 2011a). Polymeric nanoparticles made of chitosan (Bowman et al., 2008) or poly(lactide-co-glycolide) PLGA (He et al., 2005), polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO-PPO-PEO) non-ionic micelles (Chou et al., 2009) and thioketal nanoparticles (TKNs) (Wilson et al., 2010) have been employed as oral vectors for siRNA delivery. One recent strategy involved a multi-compartmental oral delivery system combining both nano- and micro-particle technologies. Aouadi and colleagues designed a delivery system consisting of a polyethylenimine (PEI)-coated tRNA core, with siRNA held in between the PEI layers – this was encapsulated by glucan-based microparticles (GeRPs) (Aouadi et al., 2009). This delivery system was administered to the GI tract of mice by oral gavage, whereupon the microparticles were phagocytosed by macrophages and transported to distal organs including the liver, lungs and spleen, with significant reduction in the target mRNA expression

Abbreviations: Ch2.5, cholesteryl modified poly(allylamine); GI, gastrointestinal; MTT, 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP, nanoparticles; PAA, poly(allylamine); Pa2.5, palmitoyl modified poly(allylamine); PM, polymeric micelles; QPa2.5, quaternised cholesteryl modified poly(allylamine); QCh2.5, quaternised palmitoyl modified poly(allylamine); RNAi, RNA interference; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; siRNA, small interfering RNA.

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(Aouadi et al., 2009). Another noteworthy approach was the NiMOS (nanoparticles-in-microsphere oral system) delivery system. Here, plasmid DNA was encapsulated in type B gelatin nanoparticles, which were further encapsulated in poly-(epsilon-caprolactone) (PCL) microparticles, for oral administration (Bhavsar and Amiji, 2007, 2008). This strategy enabled successful delivery and expression of the interleukin-10 gene in an acute colitis mouse model and a subsequent therapeutic response (Bhavsar and Amiji, 2008).

Recently, a promising strategy has been developed for oral hydrophobic drug and protein delivery, which utilises polymeric micelles (PM). PM are nano-sized self-assemblies formed by amphiphilic block copolymers or hydrophobically modified polymers. Upon aggregation of the hydrophobic moieties of the polymers in an aqueous environment, a hydrophobic core is formed and the hydrophilic moieties of the polymers maintain the aqueous solubility of these micellar nano-structures. Although PMs have been extensively explored as hydrophobic drug solubilisers for intravenous administration, their promising potential in oral drug delivery has recently been discovered (Gaucher et al., 2010). Research has shown that despite the complex and harsh physiological environment in the GI tract, these PM are able to retain the hydrophobic payload upon dilution in the GI tract and promote oral absorption of hydrophobic drugs (Gaucher et al., 2010).

One example is the use of PM formed by novel poly(allylamines) (PAAs) modified with different hydrophobic pendant groups and subsequently modified with hydrophilic moieties (Cheng et al., 2010; Clare et al., 2012; Hoskins et al., 2010; Thompson et al., 2011). In one of these studies, PAA modified with 5% hydrophobic cholesteryl pendants (ChPAAs) demonstrated significant enhancement of oral absorption of a model hydrophobic drug, griseofulvin, when administered intragastrically in rats (Clare et al., 2012). Another study looked at PAA modified with palmitoyl pendant groups, which were subsequently modified by quaternising with methyl iodine (QPa2.5) (Thompson et al., 2008). This polymer, QPa2.5, has been investigated for intestinal delivery of proteins and peptides. The cationic nature of the polymeric micelles enables interaction with negatively charged proteins to form nano-sized complexes. Unlike hydrophobic drugs, proteins and peptides encounter significant degradation in GI tract fluids. Interestingly, these complexes demonstrated protection of insulin from proteolytic degradation by pepsin and trypsin (Thompson et al., 2010) and facilitated transport of the protein across Caco-2 monolayers *in vitro* (Thompson et al., 2011). Intrajejunal administration of QPa2.5 complexed with a peptide, salmon calcitonin, retained the bioactivity of this peptide and resulted in a reduction of serum calcium in rats (Cheng et al., 2010).

Based on these results, we hypothesised that modified PAAs could be further applied to the delivery of siRNAs to the intestine. Therefore, in the current study, the potential for both unquaternised PAAs (Ch2.5 and Pa2.5) and quaternised PAAs (QPAAs) (QCh2.5 and QPa2.5) as novel non-viral vectors for siRNA delivery was investigated. Their chemical structures are illustrated in Supplementary Figure 1. These modified PAAs were systematically assessed for their ability to complex siRNA, protect it from degradation in GI fluids and achieve effective delivery into an intestinal cell model.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2013.02.050.

2. Materials and methods

2.1. Materials

Caco-2 cell line was purchased from the European Collection of Cell Cultures (ECACC), UK. Luciferase GL3 siRNA (sense sequence 5′–CUU ACG CUG AGU ACU UCG A–3′), fluorescein-siRNA (sense sequence 5′–UUC UCC GAA CGU GUC ACG U–3′, modified by 3′-fluorescein on sense sequence) and non-silencing siRNA (sense sequence 5′–UUC UCC GAA CGU GUC ACG U–3′, no modification) were obtained from QIAGEN, USA. Reporter Lysis Buffer, luciferin and RNase ONE Ribonuclease were obtained from Promega, WI, USA. BCA® Protein Assay Reagent was purchased from Pierce, Thermo Scientific, LysoTracker Red was purchased from Molecular Probes, Invitrogen, USA. All other materials were purchased from Sigma–Aldrich. All reagents were used as recommended by suppliers.

Samples of cholesteryl modified PAA (Ch2.5; molar ratio of monomer and hydrophobic pendant group = 1:0.025), quaternised cholesteryl modified PAA (QCh2.5), palmitoyl modified PAA (Pa2.5; molar ratio of monomer and hydrophobic pendant group = 1:0.025) and quaternised palmitoyl modified PAA (QPa2.5) were synthesised as previously described (Thompson et al., 2008).

2.2. Polymeric micelles (PM) and formation of complexes in aqueous solution

PM were formed by dissolving PAA derivatives in aqueous solutions (*i.e.*, phosphate buffer saline (PBS) for particle size and deionised (DI) water for all other experiments), followed by sonication at room temperature (RT) for a set time period. PAA concentration was 1 mg/ml. PAA·siRNA complexes were prepared by aliquoting the PAA solution into a solution of siRNA (in RNase-free water) at different mass ratios (MRs) of PAAs and siRNA (MR 5–50). The mixture was incubated for 30 min at RT to achieve complexation.

2.3. Determination of siRNA binding by gel retardation assay

The ability of PAAs to complex siRNA was analysed by gel retardation (Guo et al., 2012a; O'Mahony et al., 2012c). Briefly, complexes of amphiphilic PAA and siRNA (containing 0.5 μg siRNA) at different MRs were prepared as described above and loaded onto 1% (w/v) agarose gels in Tris/Borate/EDTA (TBE) buffer (Sigma) containing ethidium bromide. Electrophoresis was performed at 120 V for 30 min and the resulting gels were visualised under UV.

2.4. Particle size and zeta potential of PAA·siRNA complexes

Particle size and zeta potential measurements were carried out using a Malvern Zetasizer Nano (Malvern Instruments, UK) (Guo et al., 2012b; O'Mahony et al., 2012b). Briefly, PBS (filtered through a 0.2 μm membrane) was added to PAA·siRNA complexes to final volume of 1 ml and incubated for 0, 2 and 24 h prior to particle size measurements. For zeta potential measurements, DI water (filtered through a 0.2 μm membrane) was added to PAA·siRNA complexes to final volume of 1 ml. The concentration of PAAs was fixed at 1 mg/ml.

2.5. siRNA stability studies in SGF/SIF

Simulated gastric fluid (SGF)/simulated intestinal fluids (SIF) were prepared based on formulas from the United States Pharmacopoeia (2006, *USP 29*, United States Pharmacopoeial Convention Inc.). Briefly, SGF was prepared by dissolving 2.0 g of sodium chloride and 3.2 g pepsin in 7.9 ml of hydrochloric acid and water was added to 1 L. For SIF, 6.8 g of monobasic potassium phosphate was dissolved in 250 ml of water and was added to 190 ml of 0.2 N sodium hydroxide and 400 ml of water. 10.0 g of pancreatin was added to this solution, mixed, and the final solution was adjusted with 0.2 N sodium hydroxide to a pH of 7.5 ± 0.1 and diluted with water to 1 L. Samples consisting of siRNA (1 μg) formulated with

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