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NANOMEDICINE

Design of an inhalable dry powder formulation of DOTAP-modified PLGA nanoparticles loaded with siRNA

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ABSTRACT

Matrix systems based on biocompatible and biodegradable polymers like the United States Food and Drug Administration (FDA)-approved polymer poly(DL-lactide-co-glycolide acid) (PLGA) are promising for the delivery of small interfering RNA (siRNA) due to favorable safety profiles, sustained release properties and improved colloidal stability, as compared to polyplexes. The purpose of this study was to design a dry powder formulation based on cationic lipid-modified PLGA nanoparticles intended for treatment of severe lung diseases by pulmonary delivery of siRNA. The cationic lipid dioleoyltrimethylammoniumpropane (DOTAP) was incorporated into the PLGA matrix to potentiate the gene silencing efficiency. The gene knock-down level *in vitro* was positively correlated to the weight ratio of DOTAP in the particles, and 73% silencing was achieved in the presence of 10% (v/v) serum at 25% (w/w) DOTAP. Optimal properties were found for nanoparticles modified with 15% (w/w) DOTAP, which reduced the gene expression with 54%. This formulation was spray-dried with mannitol into nanocomposite microparticles of an aerodynamic size appropriate for lung deposition. The spray-drying process did not affect the physicochemical properties of the readily re-dispersible nanoparticles, and most importantly, the *in vitro* gene silencing activity was preserved during spray-drying. The siRNA content in the powder was similar to the theoretical loading and the siRNA was intact, suggesting that the siRNA is preserved during the spray-drying process. Finally, X-ray powder diffraction analysis demonstrated that mannitol remained in a crystalline state upon spray-drying with PLGA nanoparticles suggesting that the sugar excipient might exert its stabilizing effect by sterical inhibition of the interactions between adjacent nanoparticles. This study demonstrates that spray-drying is an excellent technique for engineering dry powder formulations of siRNA nanoparticles, which might enable the local delivery of biologically active siRNA directly to the lung tissue.

1. Introduction

Diseases residing in the airways constitute a major global health problem. Lung cancer is already the leading cause of cancer-related deaths in the USA [1], and is together with lower respiratory infections and tuberculosis among the top 10 causes of death worldwide according to the World Health Organization [2]. Thus, efficient therapies leading to high and sustained local concentration of potent drugs in the lung are urgently needed.

The concept of RNA interference, mediated by small interfering RNA (siRNA), provides an opportunity for a whole new class of highly specific and efficacious drugs directed towards disease-associated genes [3]. Delivering siRNA to the desired target tissue in the organism (e.g. the lungs) is not a trivial task since the physicochemical characteristics of siRNA prevent passive diffusion across the plasma membrane of most cell types. It is becoming increasingly clear that realization of the therapeutic potential of siRNA depends on developing efficient and safe delivery vehicles that can overcome the pertinent bottleneck for siRNA-based drugs: Delivery [4].

Polymeric matrix systems based on biocompatible and biodegradable polymers are promising siRNA carriers due to characteristics like a favorable safety profile, controlled release properties and improved colloidal stability, as compared to polyplexes [5]. The United States Food and Drug Administration (FDA)-approved polymer poly (DL-lactide-co-glycolide acid) (PLGA) has for these reasons been widely used for the engineering of both nano- and micro-sized particles loaded with a variety of drugs [6–9]. Nanoparticles are of particular interest for siRNA delivery since a size within the nano-range allows for efficient tissue penetration and cellular uptake [10].

We previously optimized the formulation and processing parameters for obtaining a high siRNA encapsulation into PLGA nanoparticles [11–13]. The PLGA matrix was shown to protect the siRNA against

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nuclease degradation and to provide a sustained, triphasic release of biologically active siRNA [12]. However, unmodified PLGA nanoparticles are poor transfection reagents for siRNA compared to other delivery vehicles based on cationic lipids or polymers. This could be due to sub-optimal particle characteristics for cellular uptake and membrane permeation and/or a too slow release of the siRNA. Therefore, cationic excipents such as chitosan [14–16], polyethyleneimine (PEI) [17–19], cationic lipids [20–22] and polyamines [23] have been added to PLGA-based carrier systems to improve the transfection properties [24].

In addition to the development of an appropriate carrier system, dosage form design and product stability are important pharmaceutical issues to address in the early development phase of siRNA therapeutics. Spray-drying is an excellent technique for processing suspensions of nanoparticles into more stable dry powder formulations with precisely engineered functional properties such as a well-defined particle size and an optimal density suitable for inhalation therapy [25], which would allow for local delivery of siRNA directly to diseased tissue in the lung.

We recently reported a quality-by-design based optimization of formulation and processing parameters for the spray-drying of siRNA-loaded, unmodified PLGA nanoparticle suspensions with sugar excipients into nanocomposite microparticles [13]. Importantly, spray-drying did not compromise the gene silencing activity and there was a minor loss of siRNA during the solidification process. This formulation approach elegantly combines the properties of the nanoparticles with respect to cellular uptake, sustained release and RNase protection with the characteristics of micro-sized particles displaying a size distribution suitable for deposition in lungs, where the nanocomposite microparticles disintegrate rapidly resulting in nanoparticle release [26].

The purpose of this study was to design an optimized formulation by modifying the PLGA matrix with the cationic lipid excipient dioleoyltrimethylammoniumpropane (DOTAP) to improve the gene silencing activity of the nanoparticles followed by processing of the resulting nanoparticles in the presence of a sugar excipient into a spray-dried powder. The requirements for the final product were i) a dry powder of nanocomposite microparticles with characteristics suitable for deposition in the lower airways, ii) easy re-dispersion of the powder upon addition of water to release the nanoparticles, and most importantly, iii) preservation of the nanoparticle size as well as biological activity of the siRNA-loaded carrier during the spray-drying process.

2. Materials and methods

2.1. Materials

Dicer substrate asymmetric duplex siRNAs directed against enhanced green fluorescent protein (EGFP) and negative control firefly luciferace (FLuc) were provided by Integrated DNA Technologies (IDT, Coralville, IA, USA) as dried, annealed, purified and desalted duplexes [27]. The siRNAs had the following sequences: EGFP, sense 5'-pACCCUGAAGUUCAUCUGCACCACcg-3', antisense 5'-CGGUGGUGCAGAUGAACUUCAGGGUCA-3', FLuc sense 5'-pGGUUCCUGGAACAAUUGCUUUUAca-3', antisense 5'-UGUAAAAGCAAUUGUUCCAGGAACCAG-3', where lower case letters are 2'-deoxyribonucleotides and underlined capital letters are 2'-O-methylribonucleotides. RNase-free diethyl pyrocarbonate (DEPC)-treated Milli-Q water was used for all buffers and dilutions. PLGA (lactide:glycolide molar ratio 75:25, Mw: 20 kDa) was from Wako Pure Chemical Industries (Osaka, Japan) and polyvinylalcohol (PVA) 403 (80% degree of hydrolysis) from Kuraray (Osaka, Japan). DOTAP was from Avanti Polar Lipids (Alabaster, AL, USA), Quant-iT™ RiboGreen® RNA Reagent and SYBR® Green II RNA gel stain were acquired from Molecular Probes, Invitrogen (Paisley, UK), and additional chemicals were obtained commercially at analytical grade.

2.2. Preparation of PLGA nanoparticles

The nanoparticles were produced by the double emulsion solvent evaporation method as reported previously [13], but with a few minor changes. In brief, 125 μl of an 8 μM siRNA solution in TE-buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was added to 250 μl of a DOTAP/PLGA solution in chloroform at a DOTAP content ranging from 0 to 25% (w/w). The total concentration of DOTAP and PLGA was kept constant at 60 mg/ml. The mixture was sonicated using a UP100H ultrasonic processor (Hielscher Ultrasonics, Teltow, Germany) for 90 s to obtain a water-in-oil (w_1/o) emulsion. A volume of 1 ml 2% (w/v) PVA in water was added to the emulsion, and a second sonication of 60 s was performed, resulting in a water-in-oil-in-water ($w_1/o/w_2$) double emulsion. The double emulsion was subsequently diluted with 5 ml of 2% (w/v) PVA in water and left under agitation overnight to evaporate the chloroform. The dispersion was centrifuged for 12 min at 4 °C and 18,000 ×*g*, the supernatant was discarded, and the pellet containing the nanoparticles was re-dispersed in water. Centrifugation and re-dispersion of the nanoparticle pellet was repeated three times to ensure removal of PVA before further characterization.

2.3. Size, zeta-potential and morphology

The mean particle diameter (Z-average) and polydispersity index (PDI) of the nanoparticles were determined by dynamic light scattering, and the surface charge of the particles was estimated by analysis of the zeta-potential as described previously [13]. Both types of measurements were performed in triplicate and on three different batches of nanoparticles diluted in water to approximately 0.5 mg/ml at 25 °C using a Malvern NanoZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. The surface morphology was examined by scanning electron microscopy (SEM) using a JSM-6320 F scanning electron microscope (JEOL, Tokyo, Japan). The nanoparticle suspension was placed on a Nucleopore Track-Etch Membrane (Whatman, Maidstone, UK) with 100 nm pore sizes, and the samples were dried at ambient conditions for 24 h. The dried membranes were attached to silicon wafer using double-sided carbon tape and subsequently sputter-coated with gold prior to examination.

2.4. siRNA extraction

The siRNA was extracted from the nanoparticle matrix by dissolving a known fraction of the nanoparticle suspension, corresponding to approximately 1 mg of nanoparticles in 200 μl chloroform followed by the addition of 500 μl TE buffer. The mixture was rotated end-over-end for 90 min at room temperature to facilitate the extraction of siRNA from the organic phase and into the aqueous phase. After incubation, the two phases were separated by centrifugation at 4 °C and 18,000 ×*g* for 20 min, and the aqueous supernatant was collected and incubated at 37 °C for 5 min to allow evaporation of the residual chloroform. The siRNA concentration in the supernatant was measured by the RiboGreen® RNA reagent according to the manufacturer's instructions using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with fluorescence detection at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Each sample was assayed in triplicate.

2.5. Localization of siRNA during the nanoparticle formation process

The localization of siRNA during the preparation process was visualized by confocal microscopy of the primary emulsion prepared as described above. The organic phase was stained with the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD, Molecular Probes, Eugene, OR, USA) at a final concentration of 10 μg/ml in the organic phase. EGFP siRNA end-labeled with Alexa Fluor 488 (IDT) was added to the water phase at a final concentration