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Biophysical characterization of hyper-branched polyethylenimine-graft-polycaprolactone-block-mono-methoxyl-poly(ethylene glycol) copolymers (hy-PEI-PCL-mPEG) for siRNA delivery

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ABSTRACT

A library of mono-methoxyl-poly(ethylene glycol)-block-poly(ε-caprolactone) (mPEG-PCL) modified hyperbranched PEI copolymers (hy-PEI-PCL-mPEG) was synthesized to establish structure function relationships for siRNA delivery. These amphiphilic block-copolymers were thought to provide improved colloidal stability and endosomal escape of polyplexes containing siRNA. The influence of the mPEG chain length, PCL segment length, hy-PEI molecular weight and the graft density on their biophysical properties was investigated. In particular, buffer capacity, complex formation constants, gene condensation, polyplex stability, polyplex size and zeta-potential were measured. It was found that longer mPEG chains, longer PCL segments and higher graft density beneficially affected the stability and formation of polyplexes and reduced the zeta-potential of siRNA polyplexes. Significant siRNA mediated knockdown was observed for hy-PEI25k-$(PCL900\text{-}mPEG2k)_1$ at N/P 20 and 30, implying that the PCL hydrophobic segment played a very important role in siRNA transfection. These gene delivery systems merit further investigation under *in vivo* conditions.

1. Introduction

Gene silencing by short interfering RNA (siRNA) offers tremendous promise for the treatment of many genetic and acquired diseases. The discovery of siRNA in mammalian cells [1] provides a new and much more effective strategy to induce the degradation of specific mRNA sequences that may regulate diseased cells [2]. Similar to plasmid DNA (p-DNA), siRNA also consists of double-stranded nucleic acids. They possess a phosphodiester backbone with the same negative charge to nucleotide ratio, and can interact electrostatically with cationic agents [3]. The duration of siRNA therapeutic effects was reported to be longer than that of p-DNA [4]. Additionally, siRNA needs to be delivered to the cytosol only [5]. The development of safe and efficient non-viral carriers for siRNA remains a challenging task.

Among the vast family of non-viral gene delivery systems, poly (ethylenimine) (PEI) and its derivatives have taken a prominent position due to their high positive charge density. They are able to effectively condense nucleic acids into homogenous polyplexes with sizes of ≤100 nm, which are capable of transfecting cells efficiently *in vitro* as well as *in vivo*[6]. Recent reports showed that PEI can facilitate efficient delivery of siRNA both *in vitro* and *in vivo*[7]. The molecular weight of PEI was a critical factor influencing the toxicity and transfection efficiency [8]. PEI with higher molecular weight, for example, 25 kDa, exhibited both higher transfection efficiency and higher toxicity than other smaller PEIs [9,10]. The dilemma of the correlation of toxicity with transfection efficiency has been the key obstacle for the application of PEI *in vivo*. Many strategies to overcome these problems have been proposed. For instance, the introduction of hydrophilic and hydrophobic segments in PEI molecules [11–13], the cross-linking of small PEI molecules via disulfide bonds [14] or ester bonds and/ or amide-based PEI derivatives [15,16], the modification of polyplexes' surface to shield the positive charge [17], and the conjugation of PEI with ligands [18].

Amphiphilic polymer structures containing mPEG as the hydrophilic component and PCL as flexible hydrophobic segments grafted onto branched PEI (hy-PEI) molecules could hypothetically form micelles exhibiting a core-corona structure. These carriers could improve the solubility and colloidal stability of polyplexes in aqueous solution and biological fluids. Also transfection efficiency could be improved due to facilitated transmembrane transport [19,20]. Moreover, the core-corona arrangement could offer the possibility of multi-functionality [21] whereby the co-delivery of siRNA (corona) and hydrophobic markers or drugs (core) could be envisaged.

Previous results demonstrated that this strategy could be promising. The cytotoxicity decreased with increasing molecular weights of the PCL and mPEG segments. Hy-PEI-PCL-mPEG with very short PCL segments

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displayed higher transfection efficiency compared to hy-PEI25k. The hy-PEI-PCL-mPEG copolymers also exhibited the cleavage of ester bonds in aqueous solution [20,22]. Despite these initial results knowledge about relationships between the copolymer structure and function as a gene delivery vector is still limited. To demonstrate that these copolymers can be used for efficient siRNA delivery, further investigations were required. We hypothesized that hy-PEI-PCL-mPEG might show controllable stability of polyplexes, and transfection properties for siRNA as a function of different polymer compositions.

Hence a library of hy-PEI-PCL-mPEG copolymers with varying mPEG length, short PCL segments, graft density and two molecular weights of hy-PEI (10 kDa and 25 kDa) was synthesized to explore the influence of polymer compositions on the physicochemical properties of the polymers as well as the relationship to efficiency of siRNA transfection to enable successful application of this type of polymer for siRNA delivery in the future.

2. Experimental

2.1. Materials

Poly(ethylene glycol) mono-methyl ether (mPEG) (MW, 550 Da, 2 kDa, 5 kDa) and caprolactone were purchased from Fluka (Taufkirchen, Germany). Acryloyl chloride and tin (II) 2-ethylhexanoate ($SnOct_2$) were from Sigma-Aldrich (Taufkirchen, Germany). Hy-PEIs with molecular weights of 25 kDa (hy-PEI25k) and 10 kDa (hy-PEI10k) were obtained from BASF (Ludwigshafen, Germany) and Polysciences Inc. (Eppelheim, Germany) respectively. 2′-*O*-Methylated 25/27mer DsiRNA targeting Firefly Luciferase and a Non-Coding control DsiRNA were obtained from Integrated DNA Technologies (Leuven, Belgium). Other reagents of analytical quality were used without further purification. As abbreviations hy-PEIα, PCLβ, mPEGγ, mPEGγ-PCLβ and hy-PEIα-(PCLβ-mPEGγ)n were used. Herein n represents the graft density of mPEG-PCL on PEI while α, β and γ describe the molecular weight of PEI, PCL and mPEG segments, respectively.

2.2. Synthesis and characterization

Hy-PEI-PCL-mPEG was synthesized as described previously [20]. Briefly, mPEG, ε-caprolactone and $SnOct_2$ were reacted in a round-bottom flask at 120 °C under stirring for 24 h. One mmol of dried products was mixed with 2 mmol of triethylamine and 2 mmol of acryloyl chloride in 40 ml of toluene and stirred for 8 h at 80 °C, followed by the removal of triethylamine hydrochloride and precipitation of the polymer by addition of cold n-hexane. The dried precipitate (based on the molar ratio of hy-PEI) was finally reacted with PEI in chloroform at 45 °C for 8–24 h (higher graft density required longer reaction time). The copolymer was characterized by ^{1}H NMR and ^{13}C NMR spectroscopy, which verified its structure and enabled calculation of its composition. Gel permeation chromatography (GPC) demonstrated the absence of unreacted mPEG-PCL diblock copolymer and PEI homopolymers. No further purification steps were necessary. Details of the characterization are shown in Table 1 of the supplementary materials.

2.3. Buffer capacity of hyPEI-PCL-mPEG

1 ml of polymer aqueous solution at the concentration of 0.01 and 0.05 M (based on the repeat unit of PEI) was titrated by aliquots of standard 0.1 M HCl at each time point and the pH response was monitored at room temperature [23] by a Hanna bench top pH meter 210 (Hanna Instrument, Germany) fitted with a microelectrode Model 421 (Inlab, Mettler Toledo, Schwerzenbach, Switzerland). The titration was stopped at pH 2. All samples were titrated in triplicate. The buffer capacity (β) was calculated from the titration curves as reported earlier, $\beta = dC_{HCl}/d_{pH}$[24,25]. The individual amino group of the copolymers was considered as a mono-protic base B. So pKa = pH + log ($[BH^+]/[B]$). The concentration fractions of [B] and [BH^+] in the copolymer solution were defined as [23,24]

$$\alpha_{BH^+} = \alpha_1 = \frac{[BH^+]}{[BH^+] + [B]} = \frac{[H^+]}{[H^+] + Ka} \quad (1)$$

$$\alpha_B = \alpha_0 = \frac{[B]}{[BH^+] + [B]} = \frac{Ka}{[H^+] + Ka} \quad (2)$$

Here, α_1 and α_0 represent the percentage of protonated and unprotonated nitrogen atoms respectively.

2.4. Complex stability of copolymers

The complex stability of copolymers was described by the complex formation constant (K), which was calculated as follows: K = [Cu − PEI]/[Cu] [PEI] [24], where, [Cu-PEI] stands for the concentration of copper and PEI bound in the complex. Titration was performed with 0.1 M copper sulfate as titrant [26]. All solutions used in this assay were prepared in 5% potassium acetate at pH 5.5. Copper sulfate solution was added to the polymer solution (1 mg/ml), and the optical density at 285 nm was recorded by an Ultrospec 3000 UV/visible spectrophotometer (GE Healthcare, Germany). To quantify the amount of PEI in copolymers, a standard titration curve was used. All samples were titrated in triplicate.

2.5. Preparation of polyplexes

Distilled water, PBS buffer (pH 7.4, 0.15 M), 5% glucose, HBG buffer (pH 7.4, 10 mM), and sodium acetate solution (pH 5.0, 0.10 M) were selected as media for polyplex preparation. All buffer solutions were filtered through 0.20 μm pore-size filters (Nalgene syringe filter, Sigma-Aldrich, Taufkirchen, Germany) before use. Ten μl of stock copolymer solution (1 mg/ml based on hy-PEI) were diluted with buffers to a final volume of 50 μl in microcentrifuge tubes. siRNA stock solution was also diluted in the same buffers used for copolymers to a final volume of 50 μl. Equal volumes of siRNA aliquots and the diluted copolymer solutions were mixed by pipetting and incubated for 20 minutes for complex formation.

2.6. Characterization of polyplexes

The particle size and zeta-potential of the polyplexes were monitored using a Malvern Zetasizer Nano ZS (Malvern Instrument, Herrenberg, Germany) as described previously [22]. The size of the polyplexes was measured in a disposable low volume cuvette (100 μl, Uvette, Eppendorf, Wesseling–Berzdorf, Germany). Zeta-potential measurements were then carried out in the standard clear capillary electrophoresis cell (Malvern, Herrenberg, Germany) at 25 °C by diluting 100 μl of polyplexes solution with an additional 600 μl of buffer to give a final siRNA concentration of 11 ng/μl. The salt stability was determined by the size changes of the polyplexes after incremental amounts of 3 M NaCl solution were added stepwise to the polyplex solution with vortexing. The total salt concentration required for aggregation of the polyplexes was recorded. All experiments were performed in triplicate.

2.7. Ethidium bromide complexation assay

The ethidium bromide complexation assay was performed using a PERKIN ELMER fluorescence spectrometer LS 50B (PerkinElmer Instruments, Rodgau, Germany) with an excitation wavelength of 510 nm (10 nm slit) and an emission wavelength of 590 nm (10 nm slit). 4 μg of anti-luc siRNA was mixed with various amounts of polymer in HBG (containing 5% glucose buffered with 10 mM HEPES)