Biomaterials 33 (2012) 6551–6558

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Enhancing in vivo circulation and siRNA delivery with biodegradable polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol) copolymers

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ARTICLE INFO

Article history:
Received 15 May 2012
Accepted 23 May 2012
Available online 16 June 2012

Keywords:
siRNA delivery
Biodegradable polycations
Non-viral polymeric nanocarriers
In vivo biodistribution and pharmacokinetics
SPECT imaging

ABSTRACT

The purpose of this study was to enhance the in vivo blood circulation time and siRNA delivery efficiency of biodegradable copolymers polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol) (hy-PEI-g-PCL-b-PEG) by introducing high graft densities of PCL-PEG chains. SYBR® Gold and heparin assays indicated improved stability of siRNA/copolymer-complexes with a graft density of 5. At N/P 1, only 40% siRNA condensation was achieved with non-grafted polymer, but 95% siRNA was condensed with copolymer PEI25k-(PCL570-PEG5k)$_5$. Intracellular uptake studies with confocal laser scanning microscopy and flow cytometry showed that the cellular uptake was increased with graft density, and copolymer PEI25k-(PCL570-PEG5k)$_5$ was able to deliver siRNA much more efficiently into the cytosol than into the nucleus. The in vitro knockdown effect of siRNA/hyPEI-g-PCL-b-PEG was also significantly improved with increasing graft density, and the most potent copolymer PEI25k-(PCL570-PEG5k)$_5$ knocked down 84.43% of the GAPDH expression. Complexes of both the copolymers with graft density 3 and 5 circulated much longer than unmodified PEI25 kDa and free siRNA, leading to a longer elimination half-life, a slower clearance and a three- or fourfold increase of the AUC compared to free siRNA, respectively. We demonstrated that the graft density of the amphiphilic chains can enhance the siRNA delivery efficiency and blood circulation, which highlights the development of safe and efficient non-viral polymeric siRNA nanocarriers that are especially stable and provide longer circulation in vivo.

1. Introduction

RNA interference (siRNA) promises great advantages for emerging therapeutic applications to silence disease genes [1,2]. In contrast to the efficient and reliable siRNA-mediated gene silencing in vitro, only limited silencing of target gene expression in vivo has been achieved. One of the reasons is that naked siRNA is very unstable in blood due to rapid enzymatic degradation, rapid excretion, and non-specific uptake by the reticuloendothelial system [3]. Therefore, it is a formidable challenge to design and synthesize effective siRNA delivery systems, which are stable but biodegradable and long circulating in vivo [4].

Copolymers hy-PEI-g-(PCL-b-PEG)$_n$ are amphiphilic biodegradable non-viral polymeric gene delivery agents, which have shown a prominent gene delivery efficiency [5–7]. Positively charged polyethylenimine (PEI) is the functional component which condenses the genetic material due to electrostatic interactions and offers the buffer capacity with protonable amino groups to achieve successful endosomal escape of polyplexes [8,9]. Hydrophobic poly(caprolactone) (PCL) increases the biodegradability of the copolymers and affects the hydrophilic–hydrophobic balance of the polymer to enhance uptake of the complexes through cell membranes [5,6]. PEG has been widely used as a classical polymer to modify the surface of delivery systems like hydrophobic colloids [10] or other polymeric delivery agents such as polycation hy-PEI, resulting in decreased cytotoxicity, non-specific interaction of complexes with serum components and a prolonged blood circulation [11–14]. We hypothesize that the effect of PEG on prolonged circulating and

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doi:10.1016/j.biomaterials.2012.05.055

the protection of siRNA with PCL-PEG chains depends not only on its content in a copolymer (length or percentage), but also on the structure or the shape of the amphiphilic copolymer. Therefore, we designed and synthesized a panel of biodegradable amphiphilic copolymers hy-PEI-g-PCL-b-PEG with different graft densities of PCL-PEG chains as polymer-based siRNA delivery systems. We expected that the PEG-PCL grafting degree would improve not only the gene silencing, but also the circulating time in vivo. In our study, the physicochemical properties of these siRNA/polymer-complexes were characterized with respect to particle size, zeta potential, and stability against competing heparin anions. Real-Time-PCR and confocal laser scanning microscopy were used to measure the in vitro knockdown efficiency and cell uptake. The siRNA delivery efficiency and biodistribution of these triblock amphiphilic copolymers under in vivo conditions was determined using single photon emission computed tomography (SPECT) imaging with ^{111}In radiolabeled siRNA [15] and fluorescently labeled copolymer. Our study provides us with an insight into advantageous structures and shapes of copolymers to promote the rapid development of safe and efficient non-viral polymeric siRNA delivery nanocarriers that are especially stable in vivo.

2. Methods and materials

2.1. Materials

hy-PEI with a molecular weight of 25 kDa was obtained from BASF. Poly(-ethylene glycol) mono-methyl ether (mPEG) (5 kDa) and ε-caprolactone were purchased from Fluka (Taufkirchen, Germany). Heparin sodium salt was from Sigma–Aldrich Laborchemikalien GmbH (Seelze, Germany) and all other chemicals were obtained from Sigma–Aldrich (Steinheim, Germany). hy-PEI-g-PCL-b-PEG was synthesized as reported previously [5,6]. Lipofectamine™2000 (LF) was bought from Invitrogen (Karlsruhe, Germany). Amine-modified firefly luciferase DsiRNA with a C6-NH2 linker at the 3′of the sense strand, negative control sequence, hGAPDH-DsiRNA, and TYE546-DsiRNA were obtained from Integrated DNA Technologies (IDT, Leuven, Belgium). Balb/c mice were bought from Harlan Laboratories (Horst, The Netherlands). The chelator 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (p-SCN-Bn-DTPA) was purchased from Macrocyclics (Dallas, TX, USA). The radioactive $^{111}InCl_3$ was purchased from Covidien Deutschland GmbH (Neustadt a.d. Donau, Germany).

2.2. Polyplex preparation

Polyplexes were formed by diluting a calculated volume of 1 mg/mL polymer (based on PEI25 kDa) with 5% glucose solution in the first step. Equal volumes of diluted polymer and siRNA solution were mixed followed by vigorous pipetting before letting the polyplexes form at room temperature for 20 min. The N/P ratio (=nitrogen/phosphorus-ratio) was calculated as described earlier [6].

2.3. Dynamic light scattering and zeta potential analysis

Polyplexes were prepared with hGAPDH-DsiRNA as described above at different N/P ratios and measured in a disposable low volume UVette (Eppendorf, Wesseling–Berzdorf, Germany) using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). The measurements in 5% glucose solution were conducted in triplicates according to a previous work [16].

2.4. SYBR® gold assay and heparin assay: binding and protection efficiency and stability against competing polyanions

The ability of hy-PEI-g-PCL-b-PEG copolymers to condense siRNA into small polyplexes was studied in SYBR® Gold assays. Briefly, polyplexes were formed at different N/P ratios from 0 to 20. Polyplex solutions contained 1 μg hGAPDH-DsiRNA and the according amount of polymer in each well of FluoroNunc 96 well plates (Nunc, Thermo Fisher Scientific, Langenselbold, Germany). For heparin assays, polyplexes were prepared in solutions at different N/P-ratio like in the SYBR® Gold assay described above. Additionally, 20 μL heparin solution with a concentration of 1.5 IU/μmol siRNA was added to the polyplex solutions in each well of the 96-well plate (Perkin Elmer, Rodgau-Jügesheim) and the polyplex solutions were incubated for 20 min at 25 °C, before 20 μL diluted SYBR® Gold solution were added. The measurement of fluorescence was conducted according to a previous study in quadruplets [17].

2.5. RT-PCR

SKOV3 cells were used for knockdown experiments and the hGAPDH scilencing was measured with RT-PCR according to our previous work [18]. Briefly, 500,000 cells per well were seeded in 6-well-plates 24 h prior to transfection and transfected with 100 pmol of siRNA in triplicates. Real-Time PCR was performed using QuantiFast™ SYBR® Green PCR Kit and Hs_GAPDH and Hs_ACTB_2_SG Primers (Qiagen, Germany) and the RotorGene3000 real-time PCR thermal cycler (Qiagen, Hilden, Germany).

2.6. Flow cytometry

SKOV3 cells were seeded in 24-well plates at a density of 60,000 cells per well 24 h before transfection with polyplexes consisting of 50 pmol of Alexa488-labeled siRNA at N/P 5. Cells were incubated with the polyplexes in triplicates for 4 h at 37 °C and were then washed with PBS buffer (with Ca^{2+} and Mg^{2+}). The fluorescence bound on the cell-surface was quenched by 5 min incubating with 0.4% trypan blue before the cells were trypsinized for 5 min and spun down in serum containing medium and fixed with CellFIX (BD Biosciences, San Jose, CA). Treated and untreated cells (as negative control) cells were measured using a FACSCantoII (BD Biosciences, San Jose, CA) with excitation at 488 nm and the emission filter set to a 530/30 bandpass. In each measurement, cells were gated to evaluate 10,000 viable cells, and the geometric mean fluorescence intensity (MFI) was calculated as the mean value of 3 independent measurements. Data acquisition and analysis was performed using FACSDiva software (BD Biosciences, San Jose, CA) [17].

2.7. Confocal laser scanning microscopy (CLSM)

For CLSM, polymers were first labeled with FITC as described before complexation with TYE546-DsiRNA [19] to detect polymer and siRNA in parallel. SKOV3 cells were seeded in 8 well-chamber slides (Lab-Tek; Rochester, NY, USA) 24 h before transfection. For sample preparation and confocal microscopy on a Zeiss Axiovert 100 Microscope and a Zeiss LSM 510 scanning device (Zeiss, Oberkochen, Germany), steps and settings were chosen as previously described [20].

2.8. siRNA radiolabeling and purification

Amine-modified siRNA (IDT, Leuven, Belgium) was coupled with p-SCN-Bn-DTPA, radiolabeled with $^{111}InCl_3$ and purified using Absolutely RNA miRNA columns (Agilent, Waldbronn, Germany) as described before [19].

2.9. In vivo imaging, pharmacokinetics and biodistribution

Balb/c mice at the age of 6 weeks (~20 g) were used for in vivo experiments. All animal experiments were carried out according to the German law of protection of animal life and were approved by an external review committee for laboratory animal care. Balb/c mice were anesthetized with xylazine and ketamine, and 5 animals per group were injected with polyplexes containing 35 μg of radiolabelled siRNA and the corresponding amount of FITC labeled polymer at N/P 5. Complexes were injected i.v. into the tail vein. For the pharmacokinetics study, 25 μL blood samples were drawn at different time points within 2 h and measured using a Gamma Counter Packard 5005 (Packard Instruments, Meriden, CT). After 2 h, biodistribution was recorded using three-dimensional SPECT and planar gamma camera imaging (Siemens AG, Erlangen, Germany). Finally, animals were sacrificed and the biodistribution in dissected organs was quantified using a Gamma Counter Packard 5005.

2.10. Flow cytometric quantification of cellular tissue uptake

As described above, mice were injected with polyplexes containing 35 μg siRNA and FITC labeled polymers at the N/P 5. Animals were sacrificed 2 h after injection, and the organs were dissected. The intracellular uptake within the organs was determined by flow cytometry. To obtain organ homogenates, lungs and livers were incubated in 2 mg/mL collagenase D solution (Roche, Mannheim, Germany) at 37 °C for 20 min before sieving the tissue through 100 μm nylon cell strainers (BD Falcon, Heidelberg, Germany). Cells were then suspended in 10 mL of PBS and centrifuged at 350 g for 10 min. The cells were resuspended, once again centrifuged and resuspended in 500 μL 4% paraformaldehyde for flow cytometry. The internalization of fluorescently labeled polyplexes into lung and liver cells was measured on a FACSCantoTM II (BD Biosciences, San Jose, CA) with excitation at 488 nm and emission filter set to 530/30 bandpass. 10,000 viable cells were evaluated in each experiment and results are the mean values of 3 independent measurements.

2.11. Statistics

Results are given as mean values and standard deviation (SD). Statistical evaluation, calculation of the AUC and two way ANOVA were performed using Graph Pad Prism 4.03 (Graph Pad Software, La Jolla, CA).