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Quantitative measurement of delivery and gene silencing activities of siRNA polyplexes containing pyridylthiourea-grafted polyethylenimines

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ABSTRACT

The activity of synthetic interfering nucleic acids (siRNAs) relies on the capacity of delivery systems to efficiently transport nucleic acids into the cytosol of target cells. The pyridylthiourea-grafted 25 KDa polyethylenimine (πPEI) is an excellent carrier for siRNA delivery into cells and it was extensively investigated in this report. Quantification of the siRNA-mediated gene silencing efficiency indicated that the πPEI specific delivery activity at the cell level may be measured and appears relatively constant in various cell lines. Delivery experiments assaying inhibitors of various entry pathways or concanamycin A, an inhibitor of the H^+/ATPase vacuolar pump showed that the πPEI/siRNA polyplexes did not require any specific entry mode but strongly relied on vacuolar acidification for functional siRNA delivery. Next, πPEI polyplexes containing a siRNA targeting the transcription factor HIF-1α, known to be involved in tumor progression, were locally injected into mice xenografted with a human glioblastoma. A 55% reduction of the level of the target mRNA was observed at doses comparable to those used *in vitro* when the πPEI delivery activity was calculated per cell. Altogether, our study underscores the usefulness of "simple"/rough cationic polymers for siRNA delivery despite their intrinsic limitations. The study underscores as well as that bottom–up strategies make sense. The *in vitro* experiments can precede *in vivo* administration and be of high value for selection of the carrier with enhanced specific delivery activity and parallel other research aiming at improving synthetic delivery systems for resilience in the blood and for enhanced tissue-targeting capacity.

1. Introduction

Small interfering RNAs (siRNAs) are 21–22 nt long RNA duplexes that trigger sequence specific mRNA degradation, leading to the silencing of a selected gene [1]. This oligonucleotide-mediated process offers not only novel opportunities for research purposes and validation of therapeutic targets but also fights any disease with an identifiable molecular target. The molecular site of action of siRNAs is in the cytosol of the cells, and this represents an unreachable site since nucleic acids do not have the intrinsic ability to diffuse across the cellular plasma membrane. High-pressure tail vein injection of siRNAs (hydrodynamic delivery method) promotes accumulation of siRNA in the liver and their therapeutic potential [2,3]. Further confirmation and progress toward clinical applications have been achieved (for review, see [4–6]) and are mostly based on the formulation of siRNA with either cationic polymers/oligomers or within lipidic vesicles for delivery to the liver [7,8], to tumors [9–22] or to the mononuclear phagocyte system for immunomodulation therapy [23–25].

We have previously modified polyethylenimine (PEI) with hydrophobic elements to decrease the aqueous solubility of PEI at pHs above 7. This property enhances the polyplex stability by complementing the electrostatic siRNA–polymer association with hydrophobic caging association [26–28]. A careful tuning of the hydrophobic/hydrophilic balance yielded hydrophobic PEI derivatives that nonetheless became soluble at endosomal acidic pHs, enabling hydrophobic caging association to vanish for a favorable siRNA release within the cell. The N-3-pyridyl, N′-PEI thiourea (πPEI) appears as the leading delivery reagent for its excellent siRNA delivery activity and its low cellular toxicity. It also assembles with siRNAs at high concentrations to form about 100 nm diameter polyplexes with spherical morphologies and promising *in vivo* siRNA-mediated gene silencing. Before engaging in further *in vivo* evaluation, we thought it necessary to quantify the siRNA-mediated gene silencing process using πPEI as delivery reagent. The process, represented in Fig. 1, engages at least three partners, the siRNA, the delivery reagent and the cell, each having its own activity or life. The process may be modulated as well by the extracellular medium or by the size/stability/stoechiometry of the

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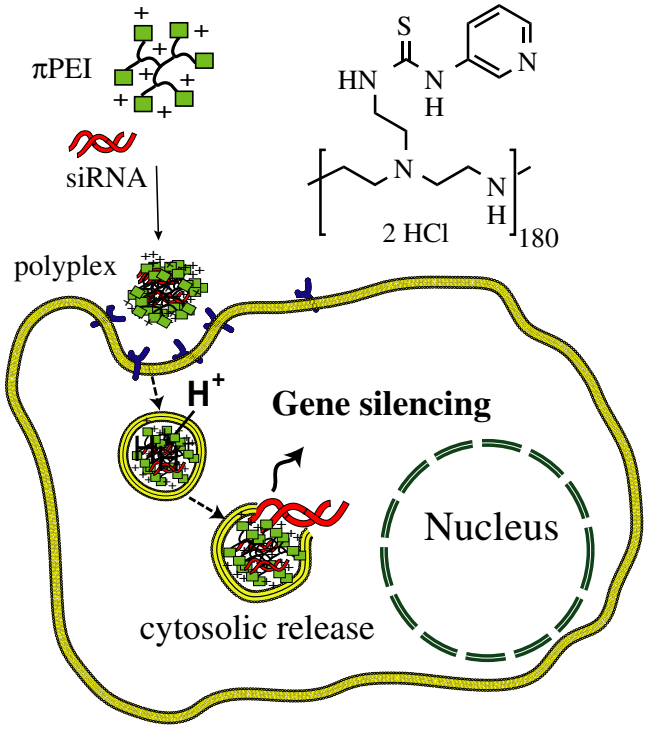

Fig. 1. Schematic representation of siRNA delivery pathway using πPEI.

polyplexes. This investigation was conducted to precisely determine the specific activity of the delivery agent and provide further evidence for the validity of the "proton sponge"-mechanism of PEI-based nucleic carriers [29].

To evaluate πPEI delivery activity *in vivo*, U87 tumor-xenografted mice were injected with πPEI/siRNA polyplexes targeting the transcription factor HIF-1α known to be involved in tumor progression [30,31]. Results indicated that the *in vivo* dose for intratumoral administration could be calculated from *in vitro* delivery experiments by a simple rule of three.

2. Materials and methods

2.1. Materials

Colorimetric and fluorescence analyses were performed on 96-well plates using a Safas Xenius spectrofluorometer (Safas, Monaco, France). The HIF-1α siRNA was a StealthTM RNAi from Invitrogen Life technologies, Saint Aubin, France). The sense sequence was 5′-UAC UCA GAC GUU UGG AUC AAG UUA A. A scrambled sequence (Stealth™ RNAi Negative Control duplexes, Invitrogen™), also named siNeg hereafter, was used to take into account induction of non specific cellular events caused by introduction of oligonucleotides into the cells. This duplex was not homologous to any known human or mouse gene. The other siRNAs were purchased from Eurogentec (Seraing, Belgique), annealed at 100 μM (or 1.5 μg μL^{-1}) final siRNA concentration and stored in aliquots at −80 °C. Sense sequences of siRNA duplexes were as follows and terminated with 2′deoxythymine at the 3′end. EGFPLuc siRNA (siEL): 5′-CUU ACG CUG AGU ACU UCG A, Untargeted siRNA duplex (*siC*): 5′-CGU ACG CGG AAU ACU UCG A, Jab1 siRNA: 5′-GGA UCA CCA UUA CUU UAA G. Cy5 or Atto488 were on the 5′end of the sense strand of siEL and *siC*, respectively.

Plastic tubes were guaranteed RNAse-free by the manufacturers. The lactate dehydrogenase (LDH) activity in the cell culture medium was measured using a commercial kit (Cytotoxicity Detection Kit Plus, Roche Applied Science). LDH activity was reported relative to the maximal LDH release. This value was obtained by complete cell lysis using the lysis solution included in the kit. Buffered solutions were sterilized by filtration through 0.22 μm Millex-GS filter units and all solutions were prepared and kept sterile by working under a class II microbiological safety cabinet. Branched PEI 25 kDa (40,872-7, batch 09529KD-466), heparin sodium salt (Grade 1-A, >180 USP/mg, reference H3149) and the other chemicals were purchased from Sigma-Aldrich (St Quentin, France) and were used as supplied. Chemical synthesis and work-ups were performed under a chemical fume hood. The pyridyl-grafted PEI (πPEI, 30% grafting) was prepared as hydrochloride salts according to a described procedure [28]. The polymer concentration was calculated relative to ethylenimine units using a molecular weight of 120 g mol^{-1}, which was estimated by adding the molecular weight of hydrochloride (36 g mol^{-1}) to that of the polymer unit (84 g mol^{-1}). The polymer was dissolved in RNAse free-water (Millipore, reference H20MB0501) and the pH was adjusted to 6.0 with NaOH 1 M. Stock solutions (0.2 M; 24 mg mL^{-1}) stored at 4 °C remained active for at least 6 months.

2.2. Synthesis of fluorescently labeled πPEI (green-πPEI)

6-Carboxyfluorescein N-hydroxysuccinimide ester (2.4 μmol), freshly dissolved in methanol (1 mL), was added at once to a methanol solution of PEI (0.2 M in ethylenimine, 1 mL). The solution was stirred in the dark overnight at room temperature. The methanol was removed under reduced pressure. The residue was then dissolved in DMF (1 mL)/CH_2Cl_2 (1 mL) and was reacted at room temperature for 4 h with 3-pyridyl isothiocyanate (60 μmol) in CH_2Cl_2 (1 mL). The CH_2Cl_2 was removed under reduced pressure and the residual material was taken-up in 0.1 M aqueous HCl (2 mL). Extensive dialysis using a SpectraPor 12–14 kDa membrane against water (0.1 L, 2 changes over a 48 h period), aqueous 100 mM NaCl (0.1 L, 24 h) followed by water (0.2 L, 2 changes over a 48 h period) and lyophilization afforded the fluorescently labeled polymer (14 mg, 58% yield).