Journal of Controlled Release 157 (2012) 418–426

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

# Pyridylthiourea-grafted polyethylenimine offers an effective assistance to siRNA-mediated gene silencing *in vitro* and *in vivo*

Gaelle Creusat [a], Jean-Sébastien Thomann [a], Anne Maglott [b], Bénédicte Pons [c], Monique Dontenwill [b], Eric Guérin [d], Benoit Frisch [a], Guy Zuber [a,*]

[a] Laboratoire de Conception et Application de Molécules Bioactives, CNRS — Université de Strasbourg UMR 7199, Faculté de Pharmacie, 74, route du rhin, 67400 Illkirch, France
[b] Laboratoire de Biophotonique et Pharmacologie, CNRS — Université de Strasbourg UMR7213, Faculté de Pharmacie, 74, route du rhin, 67400 Illkirch, France
[c] Laboratoire d'Innovation Thérapeutique CNRS — Université de Strasbourg UMR 7200, Faculté de Pharmacie, 74, route du rhin, 67400 Illkirch, France
[d] Physiopathologie et Recherche Translationnelle, EA 4438, Université de Strasbourg, 3 avenue Molière, 67200 Strasbourg, France

ARTICLE INFO

*Article history:*
Received 16 July 2011
Accepted 6 October 2011
Available online 12 October 2011

*Keywords:*
siRNA delivery
Nanovector
Transfection
Polymer

ABSTRACT

Success of synthetic interfering nucleic acids (siRNAs)-based therapy relies almost exclusively on effective, safe and preferably nanometric delivery systems which can be easily prepared, even at high concentrations. We prepared by chemical synthesis various self-assembling polymers to entrap siRNAs into stable polyplexes outside cells but with a disassembly potential upon sensing endosomal acidity. Our results revealed that pyridylthiourea-grafted polyethylenimine (πPEI) followed the above-mentioned principles. It led to above 90% siRNA-mediated gene silencing *in vitro* on U87 cells at 10 nM siRNA concentration and did not have a hemolytic activity. Assembly of siRNA/πPEI at high concentration was then studied and 4.5% glucose solution, pH 6.0, yielded stable colloidal solutions with sizes slightly below 100 nm for several hours. A single injection of these concentrated siRNA polyplexes into luciferase-expressing human glioblastoma tumors, which were subcutaneously xenografted into nude mice, led to a significant 30% siRNA-mediated luciferase gene silencing 4 days post-injection. Our results altogether substantiate the potential of self-assembling cationic polymers with a pH-sensitive disassembly switch for siRNA delivery *in vitro* and also *in vivo* experiments.



## 1. Introduction

Small interfering RNA duplex (siRNA) generates sequence-selective mRNA degradation, resulting in an effective silencing of the targeted gene [1]. This gene-silencing mechanism is of great value for analyzing the function of a single gene in cultured cells and holds great potential as gene-specific therapeutics [2]. High pressure tail vein injection of siRNAs (hydrodynamic delivery method) enables translocation of nucleic acids into the liver cells and provided the first demonstrations of the therapeutic potential of siRNAs [3,4]. Unfortunately, this method is too risky to be used on humans, especially on unhealthy individuals. Further progress has been achieved by formulating siRNAs within lipidic vehicles either for delivery to the liver [5] or to lung cancer cells [6]. Cationic polymers have also been used for siRNA complexation and *in vivo* delivery. Promising results were obtained for tumor therapy either using simple cationic systems [7–9], PEG-protected [10] or targeted multifunctional ones [11,12].

The polyethylenimine (PEI) is one of the most studied cationic DNA transfection reagents [13] and has been evaluated as well for siRNA delivery [14]. Encouraging siRNA-mediated gene silencing results were obtained *in vivo* with low molecular weight branched PEI [8,15] despite the weak cohesion of siRNA/PEI polyplexes in presence of cell surfaces [16]. At an *in vitro* cellular level, successful siRNA delivery with PEI showed to rely on high siRNA dose, on a branched polymer and on specific polyplex formation conditions [17]. Strategies to increase the stability of the siRNA polyplexes were developed [18]. One is to modify the polycation backbone with hydrophobic elements [9,19]. In this vein, we recently reported that modification of the "proton-sponge" polyethylenimine (PEI) 25 kDa with tyrosine led to a self-assembling polymer (PEIY) with effective siRNA delivery abilities at a cellular level [20]. Besides providing increased extracellular stability, non-covalent interactions have the advantage of potential reversibility. A further detailed investigation indicated that mildly acidic conditions (pH 6.0), such as ones encountered in PEI-buffered endosomes [21], weaken the non-covalent tyrosine–tyrosine interactions and may facilitate release of siRNA at the right time for optimal delivery [22]. However, effective siRNA-mediated gene silencing was observed with large polymolecular aggregates (>500–800 nm) [20]. This feature asks question about suitability of such polyplexes for *in vivo* administration that relies on preparation of concentrated and stable colloids of sizes around 100 nm. We also found tyrosine-modified PEI to have a toxicological profile unsuited for *in vivo* administration.

* Corresponding author. Fax: +33 3 68 85 43 06.
*E-mail address:* zuber@unistra.fr (G. Zuber).

0168-3659/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2011.10.007

In this study, we tuned the chemical structure of self-assembling polymers for *in vivo* administration and focused our efforts to improve the biocompatibility of the siRNA carrier. We synthesized various novel hydrophobic polyethylenimines starting from the commercial branched polymer of 25 kDa. We also evaluated their siRNA delivery efficiency and their toxicological profile and selected N-3-pyridyl, N′-PEI thiourea (πPEI) as the most suitable siRNA vehicle for *in vivo* administration. Single injection of siRNA polyplexes in subcutaneous implanted U87 tumors resulted in a significant siRNA-mediated luciferase silencing at about 30% relative to controls.

## 2. Materials and methods

### 2.1. Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Invitrogen (Cergy Pontoise, France). Branched PEI 25 kDa (40,872-7, batch 09529KD-466) and the other chemicals were purchased from Sigma-Aldrich (St Quentin, France) and were used as supplied. Water was deionized on a Millipore Milli-Q apparatus. Before use, regenerated cellulose dialysis membranes (SpectraPor 4, 12–14 kD, SpectrumLabs) were soaked in MilliQ water (200 mL, 3 times, 8 h each) to remove preservatives. Chemical synthesis and work-ups were performed under a chemical fume hood and plastic tubes were guaranteed RNAse-free by the manufacturers. Experiments involving cell lines were performed according to the biosafety level 2 guidance. NMRI nude mice (strain Crl:NMRI-Foxn1$^{nu}$) were obtained from Elevage Janvier, Le Genest-St-Isle, France. Animal experimentation was conducted according to French regulations at the Inserm U682 animal facility. HEPES buffered saline ×2 (HBS) contained 20 mM HEPES, 240 mM NaCl and 1.5 mM $Na_2HPO_4$. Buffered solutions and water were sterilized by filtration through 0.22 μm pore membrane. The polymers and siRNA solutions were prepared using sterile media. All solutions were kept sterile by working under a class II microbiological safety cabinet. UV/Vis analyses were performed on a Shimadzu UV2401PC spectrometer. NMR spectra were performed on a Bruker DPX 400 MHz spectrometer. The modification degree of the polymer was determined relative to ethylenimine (EI) residues by integration of $^1$H NMR signals and was of 30 +/− 5% for all polymer otherwise indicated.

### 2.2. Succinimidyl ester of vanillic acid

A solution of N, N′-DiCyclohexylCarbodiimide (DCC) (20 g, 97 mmol) in $CH_2Cl_2$ (25 mL) was added dropwise at 0–4 °C to a solution of N-hydroxysuccinimide (8.9 g, 77 mmol) and vanillic acid (11.8 g, 70 mmol) in ethylacetate (80 mL)/DMF (20 mL). The reaction mixture was then stirred overnight at room temperature. The DiCyclohexylUrea (DCU) was removed by filtration, washed with ethyl acetate (100 mL) and the combined organic phases were washed with saturated NaCl (100 mL), saturated $NaHCO_3$ (twice 100 mL), saturated NaCl (100 mL) and then dried over $MgSO_4$. The solvent was then removed under reduced pressure to give the product as a yellow solid (16.5 g; 70% yield). $^1$H NMR ($CDCl_3$) δppm: 2.9 (s, 4H), 3.9 (s, 3H), 6.9 (d, J = 8.4 Hz, 1H), 7.55 (d, J = 2.0 Hz, 1H), 7.75 (dd, J = 2.0 Hz, J = 8.4 Hz, 1H), 8.0 (s, 1H). $^{13}$C NMR ($CDCl_3$) δppm: 25.7, 56.2, 112.4, 114.8, 116.5, 125.9, 146.7, 152.2, 161.5, 169.5. ES-MS: (M calculated for $C_{12}H_{11}NO_6$: 265.057) found: 288.049 ($[MNa]^+$).

### 2.3. Succinimidyl ester of salicylic acid

A solution of DCC (10.9 g, 53 mmol) in $CH_2Cl_2$ (5 mL) was added dropwise at 0–4 °C to a solution of N-hydroxysuccinimide (6.19 g, 53.8 mmol) and salicylic acid (6.16 g, 44.7 mmol) in DMF (30 mL). The reaction mixture was then stirred overnight at room temperature and the DCU was removed by filtration and washed with ethyl acetate (200 mL). The combined organic phases were washed with saturated $NaHCO_3$ (twice 200 mL), citric acid 5% (200 mL), saturated NaCl (200 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure to give the product as a yellow solid (10.1 g, 95% yield). $^1$H NMR ($CDCl_3$) δppm: 2.9 (s, 4H), 6.95 (t, J = 7.2 Hz, 1H), 7.05 (d, J = 8.0 Hz, 1H), 7.58 (td, J = 7.2 Hz, J = 1.6 Hz, 1H), 8.0 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H). $^{13}$C NMR ($CDCl_3$) δppm: 25.6, 108.1, 118.0, 120.0, 130.1, 137.9, 161.9, 165.0, 169.1. ES-MS: (M calculated for $C_{11}H_9NO_5$: 235.048) found: 258.038 ($[MNa]^+$).

### 2.4. Succinimidyl ester of nicotinic acid

A solution of DCC (7.0 g, 33.98 mmol) in $CH_2Cl_2$ (10 mL) was added dropwise at 0–4 °C to a solution of N-hydroxysuccinimide (4.1 g, 35.6 mmol) and nicotinic acid (3.89 g, 31.6 mmol) in DMF (30 mL). The reaction mixture was then stirred overnight at room temperature and the DCU was removed by filtration and washed with ethyl acetate (200 mL). The combined organic phases were washed with saturated $NaHCO_3$ (twice 200 mL), citric acid 5% (200 mL), saturated NaCl (200 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure to give the product as a white solid (5.7 g, 80% yield). $^1$H NMR ($CDCl_3$) δppm: 2.8 (s, 4H), 7.2–7.43 (m, 1H), 8.31–8.35 (m, 1H), 8.82–8.84 (m, 1H), 9.26 (d, J = 1.6 Hz). $^{13}$C NMR ($CDCl_3$) δppm: 25.7, 121.7, 123.6, 137.8, 151.4, 155.2, 160.8, 168.9. ES-MS: (M calculated for $C_{10}H_8N_2O_4$: 220.049) found: 221.056 ($[MH]^+$).

### 2.5. N-3-pyridyl-, N′-PEI–thiourea (πPEI) 1

A solution of 3-pyridyl isothiocyanate (460.4 mg; 3.46 mmol) in $CH_2Cl_2$ (40 mL) was added dropwise at room temperature to a solution of PEI (500 mg; 11.62 mmol) in $CH_2Cl_2$ (40 mL). After 30 min, the solvent was evaporated under reduced pressure. The residue was dissolved in water (40 mL) and the solution was adjusted to pH 4.0 by addition of hydrochloric acid 3 M. Dialysis against water (1 L volume; 2 changes over 48 h) and freeze-drying gave 650 mg of the pyridyl PEI–thiourea. The modification degree was estimated at 28% relative to ethylenimine. $^1$H NMR ($D_2O$) ∂ppm: 3.95–2.5 (m, 4H, $NHCH_2CH_2NH$), 4.2 (t, 0.5H, $CH_2NCS$), 7.5 (m, 0.25H, CHaro), 7.86 (m, 0.25H, CHaro), 8.45 (m, 0.5H, CHaro). $\lambda_{max}$ (ε calculated for ethylenimine unit): 245 nm (2660 $M^{-1}.cm^{-1}$). Average molecular weight (MW): 122.0 g/mol.

### 2.6. N-3-pyridyl-, N′-PEI–thiourea 15% 2

A solution of 3-pyridyl isothiocyanate (230 mg; 1.73 mmol) in $CH_2Cl_2$ (50 mL) was added dropwise at room temperature to a solution of branched polyethylenimine (500 mg; 11.62 mmol) in $CH_2Cl_2$ (40 mL). After 30 min, TLC indicated full consumption of the isothiocyanate. The solvent was then evaporated under reduced pressure. The residue was dissolved in water (40 mL) and the solution was adjusted to pH 4.0 by addition of hydrochloric acid 3 M. Dialysis against water (1 L volume; 2 changes over 48 h) and freeze-drying gave 515 mg of pyridyl PEI-thiourea 15%. $^1$H NMR ($D_2O$) ∂ppm: 4.02–2.65 (m, 4H, $NHCH_2CH_2NH$) 4.24 (t, 0.3H, $CH_2NCS$), 7.82 (m, 0.15 H, CHaro), 9.1–8.1 (m, 0.3H, CHaro). MW: 100 g/mol.

### 2.7. N-4-aminophenyl, N′-PEI–thiourea 3

A solution of *tert*-butyl 4-isothiocyanatophenylcarbamate (500 mg; 2.02 mmol) in $CH_2Cl_2$ (50 mL) was added dropwise to a solution of PEI (265 mg; 6.16 mmol) in $CH_2Cl_2$ (40 mL). The reaction was then stirred for 30 min at room temperature and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in aqueous HCl 3 M (30 mL) and the solution was carefully adjusted to pH 4.0 with aqueous NaOH 6 M. Dialysis against water (2 changes over 24 h) and freeze drying provided N-(4-aminophenyl), N′-PEI–thiourea (650 mg) as a yellow powder. $^1$H NMR ($D_2O$) ∂ppm: 4.0–2.67 (m, 3.3H, $NHCH_2CH_2NH$), 4.21 (m, 0.7H, $CH_2NCS$), 7.35 (bm, 1.4H, CHaro). Average MW: 140 g/mol.