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Redox-responsive, reversibly-crosslinked thiolated cationic helical polypeptides for efficient siRNA encapsulation and delivery

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ABSTRACT

Cationic helical polypeptides, although highly efficient for inducing membrane penetration, cannot stably condense siRNA molecules via electrostatic interactions, which greatly limit the gene knockdown efficiency. By developing and crosslinking the thiolated polypeptide via formation of disulfide bonds post formation of the polypeptide/siRNA complexes, we were able to obtain stable complexes without compromising the helical secondary structure as well as the membrane activity of the polypeptide. As such, the stable polypeptide/siRNA complex was able to notably protect the siRNA cargo from nuclease digestion in the extracellular environment, while the functions of the polypeptide/siRNA complex for effective cellular internalization and endosomal escape are still largely preserved. Because the disulfide is susceptible to cleavage in response to intracellular redox triggers, siRNA release from the complex is expected upon redox triggering by glutathione (GSH) intracellularly and was actually observed upon redox triggers mediated by glutathione (GSH). With the collective contribution of the potent membrane activity and redox-responsive cargo release profiles, the crosslinked complexes enable efficient gene silencing without appreciable cytotoxicity, thus providing a potential strategy for polypeptide-based intracellular siRNA delivery.

1. Introduction

RNA interference (RNAi) has recently emerged as a promising therapeutic strategy for the treatment of various genetic disorder-related diseases by silencing the expression of therapeutically relevant target genes in a precise and specific manner [1]. Small interfering RNA (siRNA) duplexes, usually 20–25 base pairs in length, are oligonucleotides that can regulate silencing of the target genes by triggering sequence-specific mRNA degradation [2–5]. Despite their efficiency, their high molecular weight, hydrophilicity, and negative charge prevent siRNA from penetrating cell membranes. siRNA also suffer from degradation by ubiquitous RNase [6–8]. Therefore, effective carriers which can protect siRNA from degradation and facilitate their delivery to the cytosol are crucial to effective RNAi.

In the past decade, a variety of materials, such as cationic polymers, lipids, proteins, and peptides [9–15], were explored as siRNA delivery vectors. Although the cationic polymers facilitate cellular internalization, they enter the cells mainly via the endocytosis mechanism, which thereafter experience endosomal entrapment and lysosomal degradation. Cell penetrating peptides (CPPs), such as HIV-TAT, Arg9, penetratin, and melittin, are sequence-specific, membrane-active short oligopeptides that can efficiently enter the cytosol via direct membrane penetration. However, CPPs are usually too short (with 25 amino acid residues or less) and lack sufficient cationic charge density to efficiently condense and deliver siRNA. As such, they often function as membrane-active ligands conjugated to existing delivery vehicles to improve the delivery efficiency.

We recently developed a cationic polypeptide poly(γ-(4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl)-L-glutamate) (PPABLG) for gene delivery. PPABLG with its stabilized helical structure exhibits excellent membrane activity, which has been shown to facilitate intracellular internalization and endosomal escape of DNA cargos [16–20]. In comparison to plasmid DNA, siRNA molecules have fewer charged groups, lower molecular weight and higher stiffness, which makes it difficult for them to form stable complexes with cationic polymers, especially with helical polypeptides with rigid, rod-like structures [19,21]. siRNA loosely bound to helical polypeptide can still be attacked by nucleases and is therefore vulnerable to enzymatic degradation before reaching the target site. Therefore, it is of particular importance to improve the siRNA condensation efficiency of cationic, helical polypeptides.

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Herein, we designed and synthesized a thiolated cationic helical copolypeptide, poly(γ-(4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl)-L-glutamate)-*r*-poly(γ-(4-((2-mercaptoethyl)aminomethyl)benzyl)-L-glutamate) (PPABLG-SH). The cationic PPABLG segment affords positive charges to loosely condense the oppositely charged siRNA, and the pendent thiol groups on the side chain terminals can be crosslinked to form compact complexes that allow stable siRNA encapsulation as well as protection against nuclease-assisted siRNA degradation. The potent membrane activity of the helical PPABLG-SH promotes intracellular delivery of siRNA cargos which can be promptly released in the cytosol upon redox-triggered disulfide cleavage to allow efficient mRNA knockdown. The physicochemical properties of the polypeptide/siRNA complexes, intracellular kinetics, gene silencing efficiency, and cytotoxicity were evaluated. This study thus provides insights into the design of cationic helical polypeptides for siRNA delivery.

2. Materials and methods

2.1. Materials and cell lines

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous tetrahydrofuran (THF), hexane, and dimethylformamide (DMF) were dried by a column packed with 4 Å molecular sieves. Nitrobenzene was treated by CaH_2 followed by distillation under reduced pressure. γ-(4-vinylbenzyl)-L-glutamate *N*-carboxyanhydride (VB-L-Glu-NCA) was prepared as previously reported [22]. PPABLG was synthesized following previously published procedures and its molecular weights (MWs) were determined by gel permeation chromatography (GPC) [17].

Pierce BCA assay kit and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from ThermoFisher Scientific (Rockford, IL, USA). Lipofectamine™ 2000 (LPF) and 3-(4,5-dimethylthiahiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Invitrogen (Carlsbad, CA, USA). siRNA against the GL2 luciferase gene (*siGL2*) with target sequence 5′-CGT ACG CGG AAT ACT TCG A-3′ was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The negative control siRNA containing scrambled sequences were purchased from Qiagen (Valencia, CA, USA). siRNA labeling kit with the FAM dye was purchased from Life Technologies (Grand Island, NY, USA). Bright-Glo luciferase assay kit was purchased from Promega (Madison, WI, USA).

HeLa-Luc cells stably expressing GL2 luciferase were purchased from the Signosis, Inc. (Santa Clara, CA, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% hygromycin B (Roche, Indianapolis, IN, USA).

2.2. Instrumentation

^{1}H NMR spectra were recorded on a Varian U500 MHz spectrometer. Infrared spectra were recorded on a Perkin-Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film (Perkin-Elmer, Santa Clara, CA, USA). Lyophilization was performed on a FreeZone lyophilizer (Labconco, Kansas City, MO, USA).

2.3. Synthesis and characterization of PPABLG-SH

VB-L-Glu-NCA (29 mg, 0.1 mmol) was dissolved in a mixture of DMF (1 mL) and nitrobenzene (30 μL) in a glove box, followed by addition of hexamethyldisilazane (HMDS) (20 μL, 0.1 M, M/I = 50) and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) solution (20 μL, 0.01 M) in DMF. FTIR was used to monitor the polymerization until the conversion reached 99% (24 h) to obtain poly(γ-(4-vinylbenzyl)-L-glutamate) (PVBLG). Tetrabutylammonium fluoride solution (1 M in THF, 50 μL), benzyl chloroformate (25 μL), and *N*,*N*-diisopropylethylamine (DIEA, 25 μL) were added and the solution was stirred for 3 h to cleave the N – Si bond and protect the terminal amino groups. The solution was then concentrated under vacuum and the resulting polymer was purified by precipitation from cold ethyl ether. PVBLG was then dissolved in chloroform (15 mL) and oxidized by O_3 at −78 °C. Dimethyl sulfide (1 mL) was added and the solution was stirred at room temperature (RT) overnight before most solvent was removed under vacuum. The product poly(γ-(4-aldehydobenzyl)-L-glutamate) (PABLG) was then purified by precipitation from methanol and dried under vacuum. The obtained PABLG (15 mg) was dissolved in DMF (1 mL), 2-aminoethanethiol hydrochloride (6 mg dissolved in DMSO, 0.5 molar equivalents relative to the aldehyde) was added. After stirring at 50 °C for 24 h, 1-(2-aminoethyl)piperidine (50 μL, 6.7 molar equivalents relative to the aldehyde) was added, and the solution was stirred at 50 °C for another 24 h. After reduction at 50 °C for another 24 h using borane–pyridine complex, the final polypeptide was purified through ultrafiltration (molecular weight cut-off = 3 kDa) against water filled with nitrogen at 4 °C and lyophilized. ^{1}H NMR (500 MHz, TFA-*d*): δ 7.49 (m, 4H, ArH), 5.30 (br s, 2H, ArCH_2O–), 4.88 (br s, 2H, ArCH_2NH–), 4.49 (s, 1H, α–H), 3.99 (s, 4H, –OCH_2CH_2–), 3.92–3.83 (br m, 6H, –HNCH_2CH_2N– and –NCH_2CH_2CH_2CH_2CH_2–), 3.15 (m, 2H, –HNCH_2CH_2N–), 2.75 (s, 2H, –COCH_2CH_2–), 2.37 (br m, 2H, –COCH_2CH_2–), 2.14–1.52 (br m, 6H, –NCH_2CH_2CH_2CH_2CH_2– and –CH_2-S*H*).

2.4. Quantitation of the thiol groups by Ellman's reagent

Ellman's reagent was prepared by dissolving 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer (0.5 M, pH = 8) at the concentration of 0.3 mg/mL. Ellman's reagent (0.3 mg/mL, 50 μL) was added into the PPABLG-SH solution (0.5 mg/mL, 50 μL) which was incubated for 20 min in the dark at RT before measurement of absorbance at 405 nm [23].

2.5. Preparation and characterization of crosslinked polypeptide/siRNA complexes

siRNA and polypeptide were dissolved in DEPC-treated water at 0.1 mg/mL and 1 mg/mL, respectively, and then mixed at the polypeptide/siRNA weight ratios of 5, 10, 15, 20, 30 and 40 followed by pipetting for 30 s and incubation in the glass vial filled with O_2 at 37 °C for 2 h. As the control group, un-crosslinked complexes were formed by mixing polypeptide and siRNA and incubation in the glass vial filled with N_2 at 37 °C for 15 min. The obtained crosslinked and un-crosslinked complexes were characterized for size and zeta potential by dynamic laser scanning (DLS) on a Malvern Zetasizer (Herrenberg, Germany). The gel retardation assay was used to evaluate the siRNA condensation by polypeptides. Complexes were loaded on a 4% low-melting agarose gel at 200 ng siRNA/well followed by electrophoresis at 70 V for 60 min. Naked siRNA was used as a control, and siRNA migration in the agarose gel was visualized by a Gel Doc imaging system (Biorad, Hercules, CA, USA) following staining with ethidium bromide (EB). To quantitatively measure the percentage of siRNA condensed, the EB exclusion assay was performed as follows [24]. siRNA solution was prepared in 140 mM NaCl and 20 mM HEPES at pH 7.2 and polypeptide was dissolved in DEPC-treated water at 1 mg/mL. The crosslinked complexes prepared as described above were stained with EB at the siRNA/EB weight ratio of 10 and RT for 1 h before quantification of the fluorescence intensity (λ_{ex} = 510 nm, λ_{em} = 590 nm). The siRNA condensation efficiency (%) was calculated according to the following equation:

$$\text{siRNA condensation efficiency}\,(\%) = \left(1 - \frac{F - F_{EB}}{F_0 - F_{EB}}\right) \times 100$$

where F_{EB}, F, and F_0 represent the fluorescence intensity of pure EB solution, siRNA/EB solution, and siRNA/EB/polypeptide solution, respectively.

In order to evaluate the siRNA release from the crosslinked complexes, complexes were mixed with GSH at various concentrations (4.5 μM represents the extracellular GSH concentration and 10 mM represents the intracellular GSH concentration) and incubated up to 120 min. The percentage of siRNA released from the complexes and the particle sizes were quantified as described above.

2.6. Stability of siRNA

In order to evaluate the stability of siRNA following RNase treatment, free siRNA and polypeptide/siRNA complexes were treated with fetal bovine serum at a final concentration of 50% for 4 h at 37 °C. Heparin was added to dissociate the siRNA from the complexes, and samples were immediately loaded on a 4% agarose gel followed by electrophoresis at 70 V for 1 h. The siRNA stability was visualized by a Gel Doc imaging system following staining with EB.

2.7. In vitro cell uptake

To allow visualization and quantification of cellular internalization, siRNA was labeled with the FAM dye using the *silencer*® siRNA labeling kit and used to form complexes with polypeptide as described above. HeLa-Luc cells were cultured in serum-containing DMEM, seeded in 96-well plates at 1×10^4 cells/well, and cultured for 24 h before cell uptake. The medium was replaced by serum-free DMEM (100 μL/well) and crosslinked PPABLG-SH/FAM–siRNA complexes were added at 20, 40, 60, 80 and 100 nM siRNA/well. After incubation at 37 °C for 4 h, cells were washed with cold PBS containing heparin (20 U/mL) for 3 times and lysed with the RIPA lysis buffer containing 0.5% SDS which could dissociate FAM–siRNA from the lipo- and polyplexes (100 μL/well) at RT for 20 min [25,26]. The FAM–siRNA content in the lysate was determined by spectrofluorimetry (λex = 492 nm, λem = 518 nm) and the total protein level was determined by the BCA kit. Uptake level was expressed as the amount of FAM–siRNA associated with 1 mg cellular protein. To further evaluate the cell uptake mechanism, the cell uptake study was performed at 4 °C during the 2-h period to block the energy-dependent endocytosis. The cell uptake level was determined as described above, and results were expressed as percentage uptake of the control cells which were incubated with crosslinked complexes at 37 °C for 2 h.

We then probed the pore formation ability of the complexes by monitoring the uptake level of fluorescein-tris(hydroxymethyl) methanethiourea (FITC–Tris), which is the non-reactive form of a hydrophilic and membrane-impermeable dye. HeLa-Luc cells were cultured in serum-containing DMEM, seeded in 96-well plates at 1×10^4 cells/well, and cultured for 24 h. The medium was replaced by serum-free DMEM (100 μL/well) into which crosslinked PPABLG-SH and FITC–Tris were added at 2 μg/well and 0.2 μg/well, respectively. Free FITC–Tris without polypeptides was added as a control. After incubation at 37 °C for 2 h, cells were washed with cold PBS containing heparin (20 U/mL) for 3 times and lysed with the RIPA lysis buffer (100 μL/well). The FITC–Tris content in the lysate was determined by spectrofluorimetry ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm) and the total protein level was determined by the BCA kit. Uptake level was expressed as ng FITC–Tris associated with 1 mg cellular protein.

Internalization and intracellular distribution of FAM–siRNA in HeLa-Luc cells were further visualized by confocal laser scanning microscopy (CLSM). HeLa-Luc cells cultured on coverslips in 6-well plate were treated by complexes in serum-free DMEM (2 mL) at 60 nM FAM–siRNA/well. Following incubation at 37 °C for 4 h, cells were washed three times with PBS containing heparin (20 U/mL), fixed with 4% paraformaldehyde, and stained with DAPI (10 μg/mL) and Lysotracker® Red (200 nM) before observation by CLSM (LSM700, Zeiss, Germany).

To explore the serum-resistant ability of the crosslinked complexes, cells were incubated with complexes in DMEM containing 10% FBS throughout the 4-h uptake experiment at 37 °C, and the uptake level was quantified as described above.

2.8. In vitro gene knockdown

HeLa-Luc cells were cultured in serum-containing DMEM, seeded in 96-well plates at 1×10^4 cells/well, and cultured for 24 h before the transfection study. The medium was replaced by serum-free DMEM (100 μL/well) and polypeptide/*siGL2* complexes were added at predetermined concentrations (20, 40, 60, 80 and 100 nM siRNA/well). After incubation at 37 °C for 4 h, the medium was replaced by DMEM containing 10% FBS (100 μL/well) and cells were further incubated for 24 h before quantification of luciferase expression using a Bright-Glo luciferase assay kit and measurement of the cellular protein level by a BCA kit. Luciferase expression level was evaluated as relative luminescence unit (RLU) associated with 1 mg of cellular protein (RLU/mg protein). The silencing efficiency was indicated as the percentage of luciferase expression levels of control cells which did not receive complexes treatment. To verify specific gene knockdown, identical experiments were performed for polypeptide/scr siRNA (scrambled siRNA) complexes.

2.9. Cell viability assay

HeLa-Luc cells were seeded in 96-well plates at 1×10^4 cells/well and cultured in serum-containing DMEM for 24 h. The medium was replaced by serum-free DMEM (100 μL/well) into which complexes were added at the siRNA concentrations and the polypeptide/siRNA weight ratios in accordance with the afore-mentioned transfection process. After incubation at 37 °C for 4 h, the medium was replaced by serum-containing DMEM and cells were further incubated for another 20 h. Cell viability was then evaluated by the MTT assay, and results were represented as percentage viability of control cells that did not receive complex treatment.

2.10. Statistical analysis

Statistical analysis was performed using Student's *t*-test and differences between test and control groups were judged to be significant at $^*p < 0.05$ and very significant at $^{**}p < 0.01$.

3. Results

3.1. Synthesis and characterization of PPABLG-SH

PPABLG-SH was synthesized via ring-opening polymerization (ROP) of VB-L-Glu-NCA initiated by HMDS and subsequent side-chain reductive amination by 2-aminoethanethiol and 1-(2-aminoethyl)piperidine (Fig. 1B and C) [22]. HMDS allowed a well-controlled ROP of VB-L-Glu-NCA, as demonstrated by the well-defined MW (degree of polymerization was 52 as calculated by GPC compared with the designed M/I of 50). The percentage of thiol groups on the PPABLG-SH before crosslinking was determined to be 4.7% (molar ratio) and 0.09% (molar ratio) after crosslinking using the Ellman's reagent, indicating that before crosslinking, an average of 2.4 thiol groups had been successfully conjugated to each polypeptide molecular chain and after crosslinking, most of the thiol groups were converted to disulfide bonds. The low incorporation efficiency of thiol groups was because they were incorporated by the reductive amination reaction between 2-aminoethanethiol hydrochloride and the benzaldehyde groups on polypeptide side chains. Before the reducing agent borane was added, 6.7 equiv. 1-(2-aminoethyl)piperidine was also added. Since the reaction between aldehyde and amine is reversible, two amines attached on polypeptide side chains will reach equilibrium. PPABLG-SH adopted typical α-helical structure as evidenced by the FTIR analysis showing the typical amide bands of α-helix at 1650 and 1553 cm^{-1}, and the

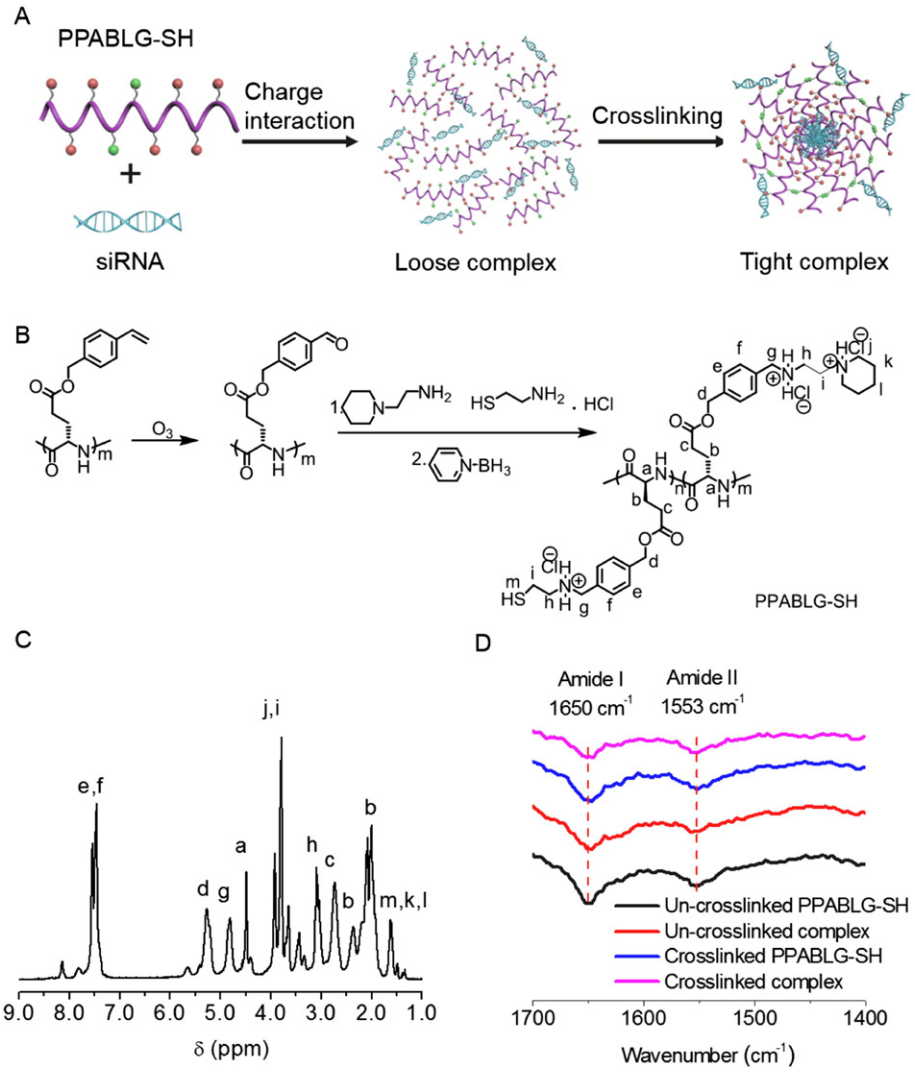

Fig. 1. (A) Schematic representation of PPABLG-SH/siRNA crosslinked complexes. (B) Reaction scheme of PPABLG-SH. (C) ^{1}H NMR spectrum of PPABLG-SH in TFA-*d*. (D) FTIR spectra of PPABLG-SH and PPABLG-SH/siRNA complexes before and after crosslinking.

helical structure of the thiolated polypeptide was maintained after the crosslinking and nanocomplex assembling (Fig. 1D). Such results thus indicated that the thiolated polypeptide might be able to maintain the helix-dependent membrane activity after crosslinking of the pendent thiol groups.

3.2. Formulation and characterization of PPABLG-SH/siRNA crosslinked complex

The formulation included a two-step process, charge–charge interaction followed by oxidative crosslinking (Fig. 1A). First, anionic siRNA

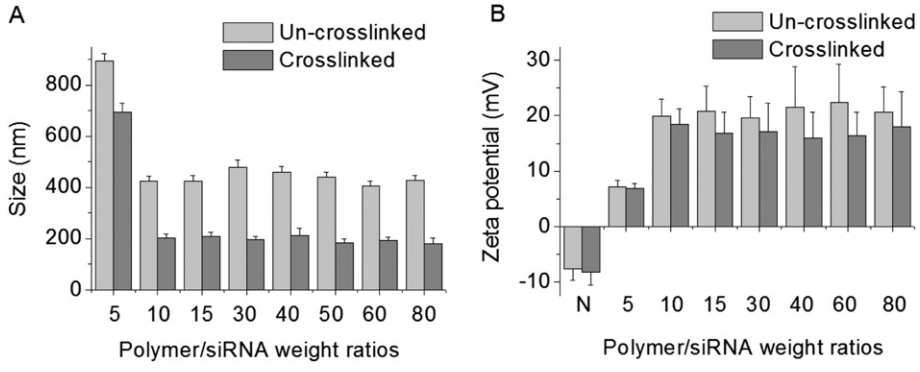

Fig. 2. Particle size (A) and zeta potential (B) of PPABLG-SH/siRNA complexes before and after crosslinking.

was added into the solution of α-helical cationic polypeptide PPABLG-SH. The charge-charge interactions between cationic polypeptide and anionic siRNA led to the formation of loose complexes with relatively large particle size (~500 nm, Fig. 2A) and positive zeta potential value (~20 mV, Fig 2B). To allow crosslinking of the PPABLG-SH/siRNA complex, the loose complex was incubated at 37 °C for 2 h in the glass vial filled with O_2 when the pendent thiol groups were oxidized to form intra- and intermolecular disulfide. Condensed nanocomplexes were thus yielded which possessed smaller size (~200 nm, Fig. 2A). All of the nanocomplexes showed PDI values lower than 0.3, indicating a relatively homogenous size distribution.

The agarose gel retardation assay revealed that non-thiolated PPABLG was unable to tightly condense siRNA as evidenced by the siRNA migration in electrophoresis even at high PPABLG/siRNA weight ratio up to 40 (Fig. 3B), according well with our previous findings [19]. In comparison, crosslinked complexes formed from the thiolated polypeptide, PPABLG-SH, led to retarded siRNA migration in the 4% agarose gel at the PPABLG-SH/siRNA weight ratio higher than 5, suggesting that siRNA could be effectively and individually entrapped by the helical, thiolated polypeptide after crosslinking of pendent thiols to form intra- and inter-molecular disulfide (Fig. 3A). Un-crosslinked complexes formed by PPABLG-SH as a control were unable to condense siRNA, which further substantiated the importance of crosslinking in yielding compact complexes (Fig. 3C). Such observation was further demonstrated by a quantitative EB exclusion assay, wherein crosslinked PPABLG-SH afforded markedly higher siRNA condensation efficiency (60%) than PPABLG (2%) and un-crosslinked PPABLG-SH (6%) (Fig. 3D).

The ability of crosslinked PPABLG-SH complexes to protect siRNA from nuclease digestion was then evaluated by incubating the crosslinked PPABLG-SH/siRNA complexes with 50% FBS that contained digestive RNase. After dissociating the encapsulated siRNA using heparin, we then evaluated the siRNA integrity via electrophoresis on a 4% agarose gel.

As shown in Fig. 4A, siRNA encapsulated in the crosslinked complexes remained intact upon the treatment of RNase compared with that encapsulated in the un-crosslinked complexes (Fig. 4B), indicating that crosslinked complex formed by PPABLG-SH was able to completely prevent the nucleolytic degradation of siRNA while complex formed by PPABLG revealed limited siRNA stability.

3.3. Uptake profile of the PPABLG-SH/siRNA crosslinked complex

As shown in Fig. 5A and B, crosslinked complexes remarkably promoted the internalization level of FAM–siRNA (over thousands fold vs. naked FAM–siRNA) which peaked at the PPABLG-SH/siRNA weight ratio of 20:1 and the siRNA concentration of 60 nM. Further increase in the siRNA concentration up to 80 nM and 100 nM did not lead to elevation in the intracellular internalization level. Compared with LPF as a commercial transfection reagent, a 2–3-fold higher uptake level was also noted, which could be attributed to the potent membrane activity of the helical PPABLG-SH to mediate intracellular cargo delivery. With the optimal formulation of crosslinked complexes (PPABLG-SH/siRNA weight ratio of 20), we further illustrated the intracellular uptake mechanism. The cell uptake study was first performed at 4 °C when energy-dependent endocytosis was totally inhibited. As shown in Fig. 5C, lowering the temperature led to ~70% inhibition of the cell uptake level, indicating that most of the complexes were internalized via energy-dependent endocytosis. Because endocytosis often leads to endosomal entrapment, it was indicated that the polypeptide nanocomplexes may be able to mediate effective endosomal escape. In addition to the endocytosis mechanism, we also probed the pore formation ability of the complexes by monitoring the uptake level of FITC–Tris, which is a hydrophilic and membrane-impermeable dye. As shown in Fig. 5D, crosslinked complexes significantly increased the FITC–Tris uptake level, indicating that the complexes had the capability to induce pore formation on cell membrane and accordingly facilitate direct penetration of siRNA cargos, another important mechanism mediated by cationic helical polypeptides. We further evaluated the capability of complexes to mediate endosomal escape via observation by CLSM. As shown in Fig. 5E, green fluorescence (FAM–siRNA) largely separated from red fluorescence (Lysotracker Red-stained endosomes/lysosomes), indicating effective endosomal

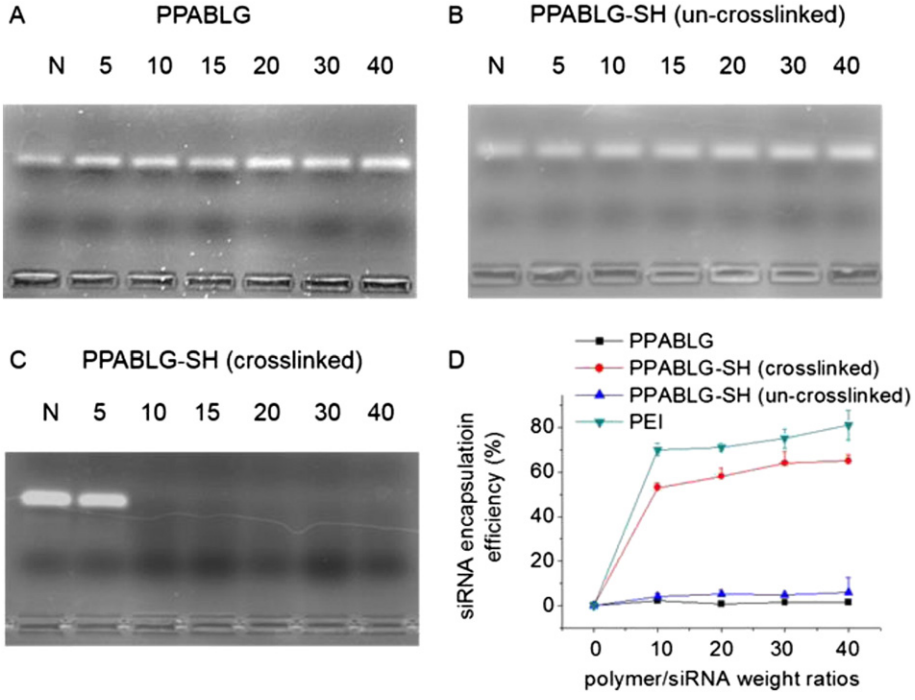

Fig. 3. (A) Gel retardation assay showing siRNA condensation by PPABLG (A), un-crosslinked PPABLG-SH (B), and crosslinked PPABLG-SH (C) at various polymer/siRNA weight ratios. N represents naked siRNA. PPABLG-SH was crosslinked at 37 °C for 2 h after polymer/siRNA complexes were formed. (D) Condensation efficiency of siRNA at various polymer/siRNA weight ratios as quantified by the EB exclusion assay (n = 3).

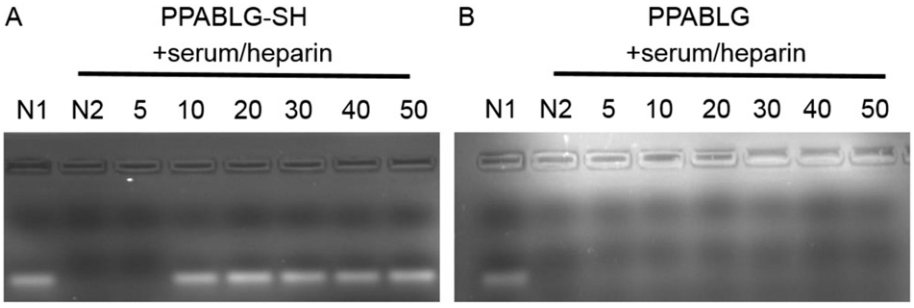

Fig. 4. Crosslinked PPABLG-SH complexes protect siRNA from nucleolytic degradation. Stability of siRNA in crosslinked PPABLG-SH complexes (A) or PPABLG complexes (B) following treatment with serum for 4 h. N1 represents naked siRNA without serum treatment and N2 represents naked siRNA treated with serum.

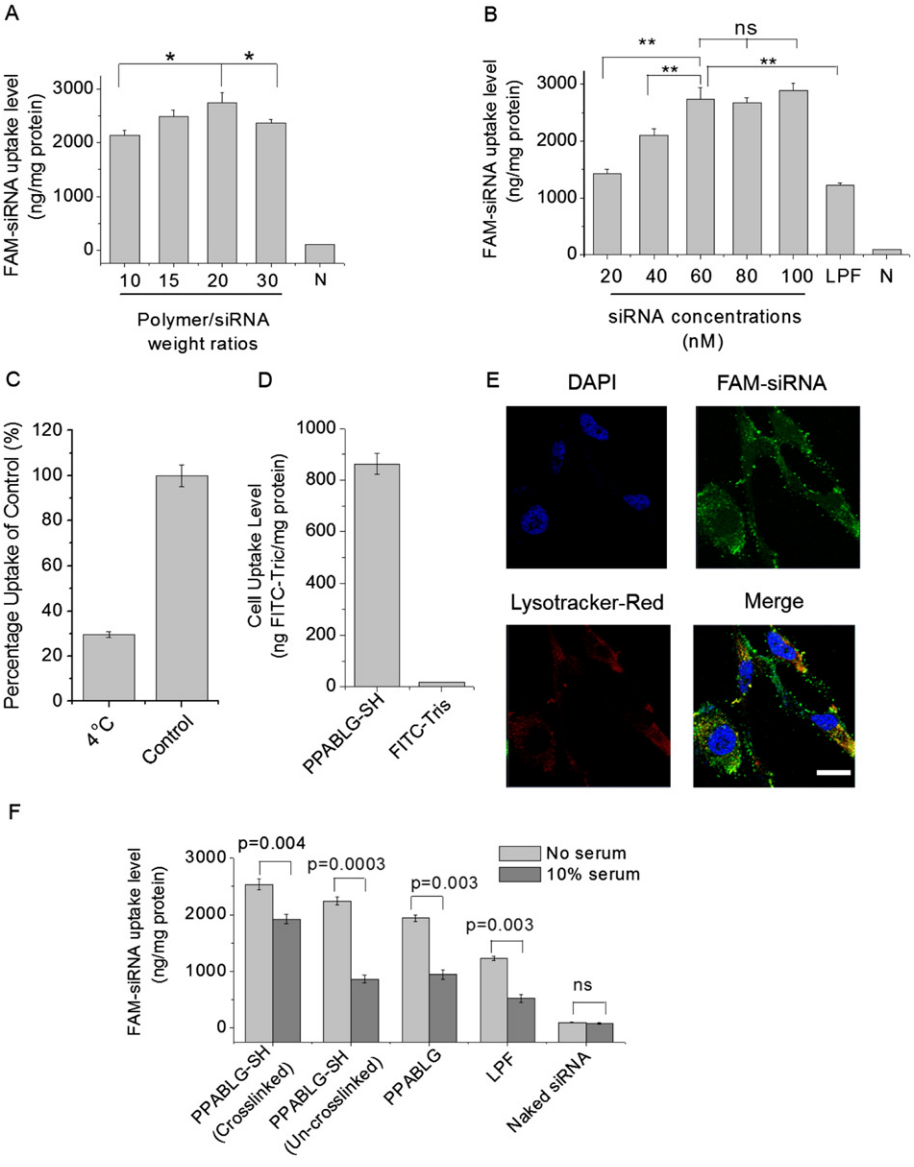

Fig. 5. (A) Uptake level of FAM–siRNA containing crosslinked PPABLG-SH complexes in HeLa-Luc cells following 4-h incubation at 37 °C at various polymer/siRNA weight ratios (siRNA concentration fixed at 60 nM) (n = 3). (B) Uptake level of FAM–siRNA-containing crosslinked PPABLG-SH complexes in HeLa-Luc cells following 4-h incubation at 37 °C at various siRNA concentrations (polymer/siRNA ratio fixed at 20) (n = 3). Naked FAM–siRNA (N) and LPF/siRNA complexes were used as controls at the siRNA concentration of 60 nM. (C) Uptake level of FAM–siRNA-containing crosslinked PPABLG-SH complexes in HeLa-Luc cells at 4 °C (n = 3). (D) FITC–Tris uptake level of HeLa-Luc cells following co-incubation with PPABLG-SH for 2 h at 37 °C (n = 3). (E) CLSM images of HeLa-Luc cells following treatment with PPABLG-SH/FAM–siRNA crosslinked complexes at 37 °C for 4 h (siRNA concentration of 60 nM; PPABLG-SH/siRNA weight ratio of 20) (bar = 20 μm). The nuclei were stained with DAPI, and the endosomes/lysosomes were stained with Lysotracker Red. (F) Uptake level of polymer/FAM–siRNA complexes in HeLa-Luc cells following 4-h incubation with or without serum at 37 °C (n = 3). siRNA concentration was fixed at 60 nM while the polymer/siRNA weight ratios was optimized at 40.

escape of the nanocomplexes. The green fluorescence was uniformly distributed in the cytoplasm, which again substantiated that the complexes were extensively internalized by HeLa-Luc cells post 4-h incubation and they had escaped out from endosomal entrapment.

When used in vivo, interference raised by serum often serves as an obstacle against the efficiency of non-viral gene delivery vectors [24]. As such, the ability of crosslinked PPABLG-SH complexes to mediate intracellular delivery of siRNA was further probed in the presence of serum. Because of the interference raised by serum, the highest cell uptake level was noted at the optimal PPABLG-SH/siRNA ratio of 40, which represented a 75% uptake level of that under serum-free condition (Fig. 5F). In comparison, a dramatic reduction in the cell uptake level was observed for both the PPABLG/siRNA complexes (63%) and the un-crosslinked PPABLG-SH/siRNA complexes (54%) in the presence of serum, which could be attributed to the aggregation and destabilization of the loose complexes upon non-specific adsorption of negatively charged serum proteins onto the positively charged complexes. Such discrepancy between the crosslinked and un-crosslinked complexes thus indicated that crosslinking of the polypeptide via disulfide bonding endowed the complexes with improved resistance against serum by allowing stable and tight nanostructures with lower surface charges.

3.4. Redox-responsive siRNA release profiles

The release profile of crosslinked complexes was evaluated as a function of glutathione (GSH) concentration. GSH is an intracellular reducing reagent that is wildly present in the cytosol at the concentration of around 10 mM and rarely present in the extracellular environment (with a low concentration of around 4.5 μM). As shown in Fig. 6A, at the GSH concentration of 10 mM, the encapsulated siRNA was completely released within 60 min, while at the GSH concentration of 4.5 μM and 0 mM, only a small portion of siRNA (3%–5%) was released within 120 min. As shown in Fig. 6B, in the presence of 10 mM GSH, the complex size increased dramatically from 200 nm to 500 nm within 20 min, indicating that the cleavage of the disulfide bond led to the transformation from tight complexes to loose complexes. The size further increased to over 1000 nm within 60 min, indicating that thorough disruption of the complex structure, which was in accordance with the complete siRNA release within 60 min. These results demonstrated that the crosslinked PPABLG-SH/siRNA complexes were stable in the extracellular environment while could rapidly release the siRNA cargo under reductive conditions in the intracellular environment.

3.5. Crosslinked complexes induce effective gene silencing with low cytotoxicity

In consistence with their cell uptake level, maximal gene silencing efficiency was noted for the crosslinked PPABLG-SH complexes at the siRNA concentration of 60 nM and weight ratio of 20 (Fig. 7A), which was comparable to that of LPF as one of most widely used commercial reagent. Further increase in the siRNA concentration and polypeptide/siRNA weight ratio did not lead to further improvement in the gene knockdown efficiency. As shown in Fig. 7B, crosslinked complexes mediated gene knockdown efficiency of as high as 60% while un-crosslinked complexes and PPABLG/siRNA complexes only led to gene knockdown efficiency of 40%. Compared to their un-crosslinked counterpart and the complexes formed with PPABLG, the crosslinked complexes exhibited strongest ability in terms of mediating gene knockdown (Fig. 7B), which again substantiated the significance of disulfide crosslinking towards stabilized siRNA encapsulation as well as redox-triggered intracellular siRNA release that ultimately contributed to improved RNAi efficiency. As a control, complexes containing scr siRNA did not induce appreciable luciferase knockdown, which indicated that the observed gene knockdown was due to the sequence-specific RNA interference rather than material toxicity.

Cytotoxicity assessment using the MTT assay further revealed that after 24-h treatment, the crosslinked PPABLG-SH complexes showed minimal cytotoxicity (viability higher than 85%) at the siRNA concentration of 60 nM used for the previous transfection studies. This result further ensured the safety of the crosslinked complexes when applied as siRNA delivery vectors (Fig. 7C).

4. Discussion

Synthetic non-viral vectors, especially cationic polymers and lipids, have been widely developed and applied as siRNA delivery vectors. However, stability of the nanocomplexes formed merely through electrostatic interactions remains low in the biological milieu mainly due to the smaller size and linearity of siRNA when compared with plasmid DNA, which thus serves as a major obstacle for in vivo application [5]. The poor stability of the nanocomplexes is a major impediment especially when cationic helical polypeptides are used as siRNA vectors because the polypeptide adopts a linear and rigid rod-like structure which further weakens the interaction with siRNA. Our previous study [19] showed that siRNA cannot be independently condensed by the cationic, helical polypeptide even though they can form loose complexes and the free polypeptide can also facilitate the intracellular delivery of siRNA by generating pores on cell membranes. Moreover, since siRNA could not be tightly condensed by the polypeptide, it is vulnerable to enzymatic hydrolysis by ubiquitous nucleases in the body when the formulation is subjected to use for in vivo RNAi.

Recently there have been multiple reports in developing stabilized polymer/siRNA complexes by either increasing the positive charge density of polymers or the negative charge density of siRNA. Increasing positive charge density of polymers can indeed improve the interactions between polymer and siRNA, which however, will generate undesired charge-associated toxicity at the meantime [27]. Alternatively, increasing the molecular weight and negative charge density of siRNA by

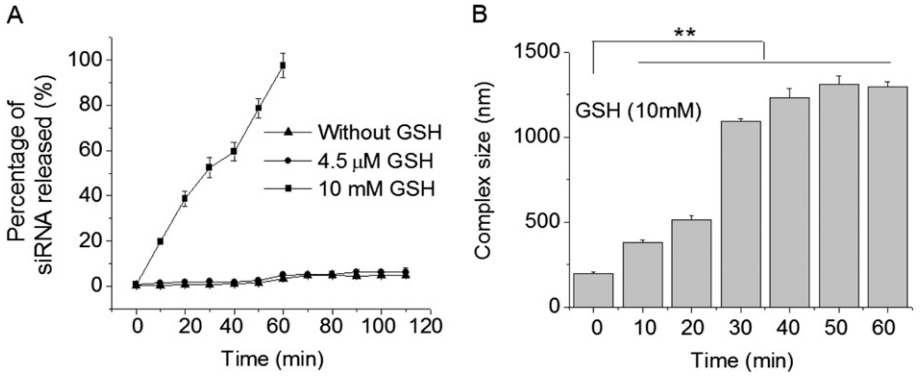

Fig. 6. (A) siRNA release from complexes upon treatment with GSH of various concentrations (n = 3). (B) Particle size of PPABLG-SH/siRNA upon treatment with GSH (10 mM).

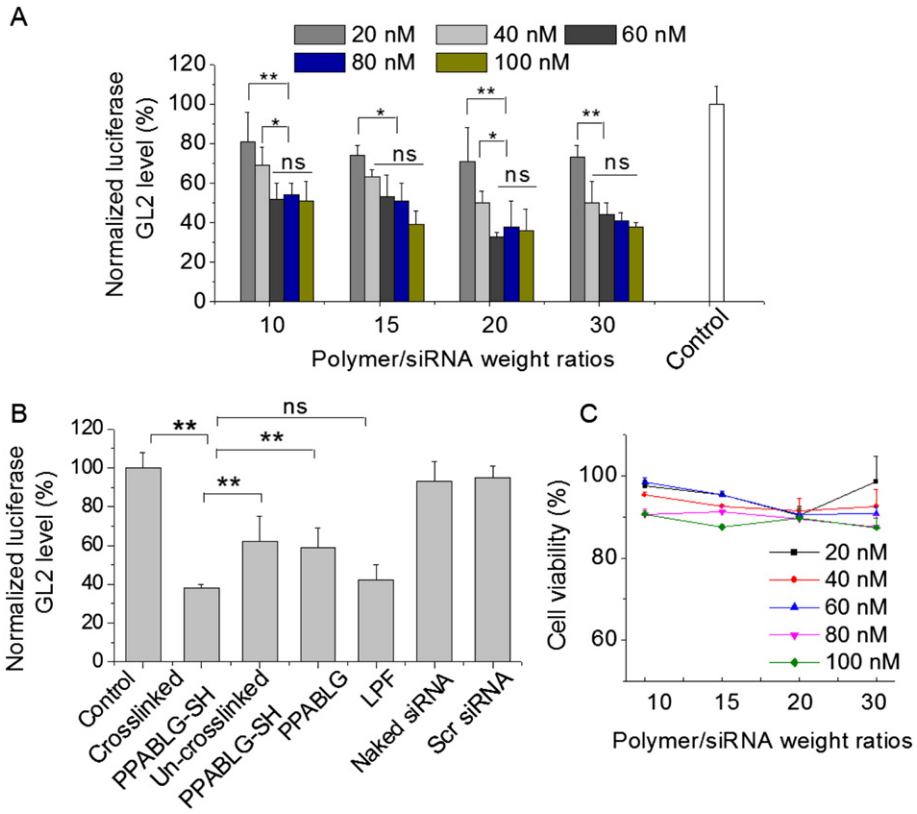

Fig. 7. (A) Normalized luciferase GL2 level of HeLa-Luc cells following incubation with PPABLG-SH/siRNA crosslinked complexes at various siRNA concentrations and PPABLG-SH/siRNA weight ratios (n = 3). (B) Normalized luciferase GL2 level of HeLa-Luc cells following incubation with different polymer/siRNA complexes. Scr siRNA represents crosslinked PPABLG-SH complexes containing scrambled siRNA. siRNA concentration was fixed at 60 nM and the PPABLG-SH/siRNA weight ratio was fixed at 20 (n = 3). (C) Cytotoxicity of PPABLG-SH/siRNA crosslinked complexes in HeLa-Luc cells (n = 3).

polymerizing the siRNA stands affords an effective strategy to improve complexation [28,29]. Park et al. reported that polymerized siRNAs (poly-siRNA) could be successfully prepared using a disulfide linkage at the 5′ end of each siRNA strand and could be complexed by the cationic carriers to form stable complexes [30]. Despite the success in stabilizing the complexes with polycations, the polymerized siRNA suffers from potential risk in stimulating IFN-α response and thus safety remains as a serious concern for this strategy [29,30]. Additional strategies also include chemical conjugation of siRNA to the delivery vector via cleavable disulfide bonding [31] and the co-condensation with ionic crosslinking agents such as sodium triphosphate (TPP) [32]. The incorporation of another polyanion that provides additional interaction forces with polycations to co-encapsulate the siRNA cargo is one of the most commonly used strategies to stabilize the cationic polymer/siRNA complexes. However, the introduction of the third component renders the complexes with excessive complexity which makes the optimization of the nanostructure difficult [33].

Compared with these reported approaches, we developed a two-step formulation strategy to allow helical polypeptide-mediated tight encapsulation of siRNA and stabilization of polypeptide/siRNA complexes. Thiol groups were introduced to the side chains of a smart polypeptide PPABLG that has been demonstrated to possess excellent membrane activity. Loose complexes were first formed via charge interactions between oppositely charged polypeptide and siRNA, and the tight and stable complexes were subsequently formed upon crosslinking of the thiol groups to form intra- as well as intermolecular disulfide. This formulation strategy could be applied to the delivery of most kinds of siRNA except those modified by thiol groups. The achieved disulfide bonds not only features augmented siRNA binding and stabilized nanostructure, but also allows redox-responsive siRNA release in the cytosol. As such, integrity of the siRNA cargo against nuclease degradation was markedly improved, and the stability of the nanocomplexes against serum was also greatly enhanced, which collectively contributed to the notably enhanced RNAi efficiency. When there have been large body of works reported utilizing additional polyanionic component or increasing the positive charge density of cationic polymers to achieve stable formulation with siRNA, this two-step formulation strategy reported in this paper offers an alternative, potentially move controllable, pathway to achieve stable siRNA delivery nanocomplexes. There are several advantages of using this disulfide cross-linking strategy to achieve stably formulated siRNA delivery complex over multi-component, charge complex. The rigid rod-like structures of the helical polypeptide are well maintained following the formulation process, thus its excellent membrane activity is largely preserved for successful siRNA delivery. In addition, the release of siRNA from the complex is better controlled through GSH triggered reduction of the disulfide bond. When the disulfide bonds are cleaved, the nanostructure hold tightly via disulfide cross-linking becomes unstable and siRNA becomes more susceptible to release. This potentially provides a means for active control over siRNA release, in contrast to other siRNA delivery vehicles based on charge interactions with uncontrollable siRNA release profiles and unknown siRNA release mechanism.

5. Conclusion

In this study, we developed a cationic, helical, and thiolated polypeptide which can form stable nanocomplexes with siRNA through a two-step formulation process, the charge interaction between polypeptide and siRNA and post crosslinking of the pendent thiol groups on polypeptide side chains. The crosslinked nanocomplexes feature tight encapsulation of siRNA to maintain stable nanostructures at the extracellular compartment while they facilitated siRNA release in the cytosol

upon redox-triggered disulfide cleavage. More importantly, formation of the crosslinked nanocomplexes did not compromise the helical secondary structure of the polypeptide, thus its excellent membrane activity was well maintained to mediate effective cellular internalization as well as endosomal escape of the siRNA cargo. This crosslinked polypeptide formulation would thus serve as a promising approach to address several critical challenges in non-viral siRNA delivery.

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References

[1] G.J. Hannon, RNA interference, Nature 418 (2002) 244–251.
[2] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Webe, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[3] M.T. McManus, P.A. Sharp, Gene silencing in mammals by small interfering RNAs, Nat. Rev. Genet. 3 (2002) 737–747.
[4] M.C. Siomi, Short interfering RNA-mediated gene silencing; towards successful application in human patients, Adv. Drug Deliv. Rev. 61 (2009) 668–671.
[5] M. Ghildiyal, P.D. Zamore, Small silencing RNAs: an expanding universe, Nat. Rev. Genet. 10 (2009) 94–108.
[6] M. Oishi, Y. Nagasaki, K. Itaka, N. Nishiyama, K. Kataoka, Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells, J. Am. Chem. Soc. 127 (2005) 1624–1625.
[7] B. Ballarin-Gonzalez, K.A. Howard, Polycation-based nanoparticle delivery of RNAi therapeutics: adverse effects and solutions, Adv. Drug Deliv. Rev. 64 (2012) 1717–1729.
[8] J.M. Vargason, G. Szittya, J. Burgyan, T.M. Tanaka Hall, Size selective recognition of siRNA by an RNA silencing suppressor, Cell 115 (2003) 799–811.
[9] M.E. Davis, The first targeted delivery of siRNA in humans via a self-assembling, Mol. Pharm. 6 (2008) 659–668.
[10] H.J. Kim, A. Ishii, K. Miyata, Y. Lee, S. Wu, M. Oba, N. Nishiyama, K. Kataoka, Introduction of stearoyl moieties into a biocompatible cationic polyaspartamide derivative, PAsp(DET), with endosomal escaping function for enhanced siRNA-mediated gene knockdown, J. Control. Release 145 (2010) 141–148.
[11] K. Itaka, N. Kanayama, N. Nishiyama, W.D. Jang, Y. Yamasaki, K. Nakamura, H. Kawaguchi, K. Kataoka, Supramolecular nanocarrier of siRNA from PEG-based block catiomer carrying diamine side chain with distinctive pKa directed to enhance intracellular gene silencing, J. Am. Chem. Soc. 126 (2004) 13612–13613.
[12] E. Song, P. Zhu, S.K. Lee, D. Chowdhury, S. Kussman, D.M. Dykxhoorn, Y. Feng, D. Palliser, D.B. Weiner, P. Shankar, W.A. Marasco, J. Lieberman, Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, Nat. Biotechnol. 23 (2005) 709–717.
[13] S. Matsumoto, R.J. Christie, N. Nishiyama, K. Miyata, A. Ishii, M. Oba, H. Koyama, Y. Yamasaki, K. Kataoka, Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery, Biomacromolecules 10 (2009) 119–127.
[14] L. Crombez, G. Aldrian-Herrada, K. Konate, Q.N. Nguyen, G.K. McMaster, R. Brasseur, F. Heitz, G. Divita, A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells, Mol. Ther. 17 (2009) 95–103.
[15] A. Akinc, A. Zumbuehl, M. Goldberg, E.S. Leshchiner, V. Busini, N. Hossain, S.A. Bacallado, D.N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J.R. Dorkin, K. Narayanannair Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K.G. Rajeev, D.W. Sah, J. Soutschek, I. Toudjarska, H.P. Vornlocher, T.S. Zimmermann, R. Langer, D.G. Anderson, A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, Nat. Biotechnol. 26 (2008) 561–569.
[16] N. Zheng, L. Yin, Z. Song, L. Ma, H. Tang, N.P. Gabrielson, H. Lu, J. Cheng, Maximizing gene delivery efficiencies of cationic helical polypeptides via balanced membrane penetration and cellular targeting, Biomaterials 35 (2014) 1302–1314.
[17] L. Yin, Z. Song, K.H. Kim, N. Zheng, H. Tang, H. Lu, N. Gabrielson, J. Cheng, Reconfiguring the architectures of cationic helical polypeptides to control nonviral gene delivery, Biomaterials 34 (2013) 2340–2349.
[18] L. Yin, Z. Song, Q. Qu, K.H. Kim, N. Zheng, C. Yao, I. Chaudhury, H. Tang, N.P. Gabrielson, F.M. Uckun, J. Cheng, Supramolecular self-assembled nanoparticles mediate oral delivery of therapeutic TNF-alpha siRNA against systemic inflammation, Angew. Chem. Int. Ed. 52 (2013) 5757–5761.
[19] N.P. Gabrielson, H. Lu, L. Yin, K.H. Kim, J. Cheng, A cell-penetrating helical polymer for siRNA delivery to mammalian cells, Mol. Ther. 20 (2012) 1599–1609.
[20] N.P. Gabrielson, H. Lu, L. Yin, D. Li, F. Wang, J. Cheng, Reactive and bioactive cationic alpha-helical polypeptide template for nonviral gene delivery, Angew. Chem. Int. Ed. 51 (2012) 1143–1147.
[21] R. Bartz, H. Fan, J. Zhang, N. Innocent, C. Cherrin, S.C. Beck, Y. Pei, A. Momose, V. Jadhav, D.M. Tellers, F. Meng, L.S. Crocker, L. Sepp-Lorenzino, S.F. Barnett, Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape, Biochem. J. 435 (2011) 475–487.
[22] H. Lu, J. Wang, Y.G. Bai, J.W. Lang, S.Y. Liu, Y. Lin, J.J. Cheng, Ionic polypeptides with unusual helical stability, Nat. Commun. 2 (2011) 206.
[23] M.K. Marschutz, A. Bernkop-Schnurch, Thiolated polymers self-crosslinking properties of thiolated 450 kDa poly(acrylic acid) and their influence on mucoadhesion, Eur. J. Pharm. Sci. 15 (2002) 387–394.
[24] T. Sato, T. Ishii, Y. Okahata, In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency, Biomaterials 22 (2001) 2075–2080.
[25] P. Vader, L.J. van der Aa, J.F. Engbersen, G. Storm, R.M. Schiffelers, A method for quantifying cellular uptake of fluorescently labeled siRNA, J. Control. Release 148 (2010) 106–109.
[26] C. Zhang, N. Tang, X. Liu, W. Liang, W. Xu, V.P. Torchilin, siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene, J. Control. Release 112 (2006) 229–239.
[27] M. Breunig, C. Hozsa, U. Lungwitz, K. Watanabe, I. Umeda, H. Kato, A. Goepferich, Mechanistic investigation of poly(ethylene imine)-based siRNA delivery: disulfide bonds boost intracellular release of the cargo, J. Control. Release 130 (2008) 57–63.
[28] R. Namgung, W.J. Kim, A highly entangled polymeric nanoconstruct assembled by siRNA and its reduction-triggered siRNA release for gene silencing, Small 8 (2012) 3209–3219.
[29] S.J. Lee, M.S. Huh, S.Y. Lee, S. Min, I.C. Kwon, Tumor homing poly siRNA glycol chitosan self cross linked, Angew. Chem. Int. Ed. 51 (2012) 7203–7207.
[30] H. Mok, S.H. Lee, J.W. Park, T.G. Park, Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing.pdf, Nat. Mater. 9 (2010) 272–278.
[31] D.B. Rozema, D.L. Lewis, D.H. Wakefield, S.C. Wong, J.J. Klein, P.L. Roesch, S.L. Bertin, T.W. Reppen, Q. Chu, A.V. Blokhin, J.E. Hagstrom, J.A. Wolff, Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 12982–12987.
[32] T. Rojanarata, P. Opanasopit, S. Techaarpornkul, T. Ngawhirunpat, U. Ruktanonchai, Chitosan–thiamine pyrophosphate as a novel carrier for siRNA delivery, Pharm. Res. 25 (2008) 2807–2814.
[33] H. Ragelle, G. Vandermeulen, V. Preat, Chitosan-based siRNA delivery systems, J. Control. Release 172 (2013) 207–218.