

Dicer-Labile PEG Conjugates for siRNA Delivery

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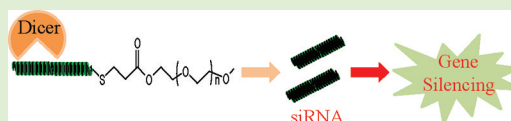
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Supporting Information

ABSTRACT: Poly(ethylene glycol) (PEG) conjugates of Dicer-substrate small interfering RNA (DsiRNA) have been prepared to investigate a new siRNA release strategy. 3'-sense or 5'-antisense thiol-modified, blunt-ended DsiRNAs, inhibiting enhanced green fluorescent protein (eGFP) expression, were covalently conjugated to PEG with varying molecular weights (2, 10, and 20 kg/mol) through a stable thioether bond using a Michael addition reaction. The DsiRNA conjugates with 2 kg/mol PEG (both 3'-sense or 5'-antisense strand conjugated) and the 10 kg/mol PEG conjugated to the 3'-sense strand of DsiRNA were efficiently cleaved by recombinant human Dicer to 21-mer siRNA, as determined by gel electrophoresis. Importantly, 2 and 10 kg/mol PEG conjugated to the 3'-sense strand of DsiRNA showed potent gene silencing activity in human neuroblastoma (SH-EP) cells, stably expressing eGFP, at both the mRNA and protein levels. Moreover, the 10 kg/mol PEG conjugates of the 3'-sense strand of DsiRNA were less immunogenic when compared with the unmodified DsiRNA, determined via an immune stimulation assay on human peripheral blood mononuclear cells.



INTRODUCTION

RNA interference (RNAi)^{1–4} provides a promising platform for treatment of gene-related diseases including cancer and viral infections. Among several triggers of RNAi, 21–23-nucleotide-long, 3'-overhanging, small interfering RNAs (siRNAs) have been widely utilized as one of the best established RNAi mediators.^{3–6} In recent years, dicer-substrate small interfering RNAs (DsiRNAs), 25–30 nucleotide-long RNA designs, have proved to have improved potency over conventional siRNAs in triggering RNAi in mammalian cells.^{7–16} DsiRNAs are cleaved by Dicer enzyme in the cytoplasm of cells into 21- to 23-nucleotide-long siRNA. It has been suggested that the involvement of Dicer enzyme in the formation of siRNA plays an important role in the formation of RNA-induced silencing complex (RISC) and consequently leads to highly efficient gene silencing.⁷ However, the therapeutic potency of both siRNA and DsiRNA is limited by their pharmacokinetic problems such as nonspecific distribution, rapid clearance, and immunogenicity.^{10,14–17} The use of DsiRNA increases the possibility of interferon (IFN) induction,^{18,19} although the IFN response to DsiRNA varies significantly among different cell types.²⁰ DsiRNA has improved nuclease-stability compared with siRNA.¹⁴ Both structures have a short in vivo half life.^{10,17,21}

Poly(ethylene glycol) (PEG) is a well-known, nonimmunogenic, long-circulating polymer. Covalent conjugation of PEG ("PEGylation") has been shown to improve the in vivo pharmacokinetics of protein drugs by reducing immunogenicity

and preventing rapid clearance.^{22–28} PEGylation has also been investigated for siRNA delivery in a number of studies and shown to improve the therapeutic efficacy.^{17,29–38} To trigger the RNAi mechanism by siRNA-PEG conjugates, PEG needs to be conjugated to siRNA via covalent bonds cleavable by a physiological stimulus. The most widely used bonds for reversible siRNA-PEG conjugates include disulfide bonds^{31,37,38} cleavable by reduced glutathione inside the cytoplasm and acid-labile bonds³⁴ cleavable at endosomal/lysosomal pH. Using such reversible bonds enables the release of siRNA from the polymer chain inside the cell.

In our present study, PEG conjugates of DsiRNA have been investigated for the first time as a new siRNA delivery strategy using polymeric carriers. PEG was conjugated to DsiRNA via a physiologically stable covalent bond. It was hypothesized that the release of siRNA from the PEG chain would occur in the cytoplasm via Dicer enzyme activity and the released siRNA would lead to efficient gene silencing. To investigate this novel strategy, 3'-sense or 5'-antisense thiol-modified, blunt-ended DsiRNAs, targeting against enhanced green fluorescent protein (eGFP) expression were covalently conjugated to PEG with varying molecular weights via a stable thioether bond using a mild and efficient synthetic approach (via Michael addition reaction).³⁹ The release of siRNA from

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the DsiRNA-PEG conjugates was investigated in vitro using recombinant human Dicer. The gene silencing efficiency of the conjugates was investigated using in vitro cultured human neuroblastoma SH-EP cells stably expressing eGFP and immunogenic properties in human peripheral mononuclear cells (PBMCs). The results have been presented below.

MATERIALS AND METHODS

Materials. Monomethoxy poly(ethylene glycol) (mPEG, 2 and 5 kg/mol), PEG (10 and 20 kg/mol), acryloyl chloride, acetyl chloride, and dithiothreitol (DTT) were purchased from Sigma-Aldrich. Triethylamine (TEA), dichloromethane, ethyl acetate, and acetonitrile were supplied from Ajax Finechem. mPEG of 10 and 20 kg/mol were synthesized via an acylation reaction of one of the hydroxyl end-group with acetyl chloride.

All 27-nucleotide-long, blunt-ended, nonmodified, double-stranded RNA oligonucleotides (DsiRNA) used in this study were previously reported by Kim et al. and are known to silence eGFP expression.⁷ The DsiRNA sequences used in the study are summarized in Table S1, Supporting Information (SI). They were synthesized and standard desalting-purified by Integrated DNA Technologies (IDT). The DsiRNAs were solubilized in HEPES (4-(2-hydroxy)piperazine-1-ethanesulfonic acid) buffer (pH 7.5) (IDT) to 500 μ M concentration following manufacturer's resuspension protocol. RNase free water was also purchased from IDT. Conventional 21-nucleotide siRNAs (Table S1, SI) were purchased from IDT or Qiagen.

Active fetal bovine serum (FBS) (sourced from Australia) and human serum (HS) utilized in stability experiments were purchased from JRH-Biosciences and Lonza, respectively. Human neuroblastoma SH-EP cells stably expressing eGFP (Children's Cancer Institute of Australia, Sydney)⁴⁰ were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% heat inactivated FBS (SAFC Biosciences). Cell culture flasks and plates were purchased from NUNC. Hank's balanced salt solution (HBSS) was purchased from SAFC Biosciences. Trypsin-EDTA was purchased from Thermo Scientific. Lipofectamine-2000 was purchased from Invitrogen. CellTiter-Blue cell viability assay reagents were purchased from Promega.

In addition to Tris/borate/EDTA (TBE) loading and running buffers (Biorad), 3% agarose or 15% polyacrylamide TBE-urea gels (Biorad) were used in gel electrophoresis experiments. GelRed (Biotium) was used for staining.

Recombinant human dicer and the enzymatic reaction reagents were purchased from Genlantis.

Power SYBR green PCR master mix used in PCR experiments was purchased from Applied Biosystems. β_2 -microglobulin Quantitect primer assay was purchased from Qiagen. RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) containing protease inhibitors used in Western Blot experiments was purchased from Sigma. BCA protein assay kit and Rabbit polyclonal antibody against eGFP were purchased from Pierce and Cell Signaling Technology, respectively. RPMI supplemented with 2 mM L-glutamine used in immune stimulation assay was purchased from Invitrogen. Quantitect reverse transcription kit and the IFIT-1 quantitect primer assay were purchased from Qiagen. CellTiter-Blue cell viability assay was purchased from Promega.

All other reagents were analytical grade and used as received.

Methods. *Synthesis of PEG-acrylate ($M_n = 2$ and 5 kg/mol) (Scheme S1, Supporting Information).* In a 250 mL round-bottomed flask, mPEG (2 mmol) was dissolved in dichloromethane (CH_2Cl_2) (50 mL) and dried using a rotary evaporator. CH_2Cl_2 (50 mL) was then introduced and the solution was cooled in an ice bath. A mixture of acryloyl chloride (3 mmol) and TEA (3.3 mmol) was added dropwise. After 1 h, the resulting solution was warmed to room temperature and stirred overnight. The product was purified by dialysis and analyzed by ^1H NMR and aqueous GPC. ^1H NMR (300 MHz, CDCl_3 , δ): 3.37 (s, 3H, $-\text{O}-\text{CH}_3$), 3.64 (t, n \times 4H, $-\text{O}(\text{CH}_2\text{CH}_2)_n\text{O}-$), 4.30 (t, 2H, $-\text{CO}_2-\text{CH}_2-$), 5.82 (dd, 1H,

$\text{CH}_2=\text{CH}-$), 6.15 (q, 1H, $\text{CH}_2=\text{CH}-$), 6.41 (dd, 1H, $\text{CH}_2=\text{CH}-$). ^1H NMR spectra were recorded on a Bruker ACF300 (300 MHz) spectrometer. The end-group functionality of both PEG $M_{n,\text{GPC}} = 2$ (PDI = 1.03) and $M_{n,\text{GPC}} = 5$ (PDI = 1.05) was found to be close to 100%. Figure S1 in SI shows the NMR spectrum of PEG-acrylate ($M_n = 5$ kg/mol).

Synthesis of PEG-acrylate ($M_n = 10$ and 20 kg/mol). mPEG was first synthesized via an acylation reaction of one of the hydroxyl end groups of PEG (M_n 10 and 20 kg/mol) with acetyl chloride. A typical procedure is given for PEG (M_n 10 kg/mol): In a 100 mL round-bottomed flask, mPEG (2 g, 0.2 mmol) was dissolved in dichloromethane (CH_2Cl_2) (50 mL) and dried by azeotropic distillation to remove trace of water. CH_2Cl_2 (50 mL) was introduced, and the solution was cooled in an ice bath. A mixture of acryloyl chloride (0.22 mmol) and TEA (0.5 mmol) was added dropwise. After 1 h, the resulting solution was warmed to room temperature and stirred overnight. A solution of acryloyl chloride (0.3 mmol) was added dropwise. The product was purified by dialysis and analyzed using a Bruker ACF300 (300 MHz) spectrometer and by GPC. ^1H NMR (300 MHz, CDCl_3 , δ): 3.00 (s, 3H, $-\text{O}-(\text{CO})\text{CH}_3$), 3.64 (t, n \times 4H, $-\text{O}(\text{CH}_2\text{CH}_2)_n\text{O}-$), 4.10 (s, 3H, $\text{CH}_2-\text{O}-(\text{CO})\text{CH}_3$), 4.30 (t, 2H, $-\text{CO}_2-\text{CH}_2-$), 5.82 (dd, 1H, $\text{CH}_2=\text{CH}-$), 6.15 (q, 4H, $\text{CH}_2=\text{CH}-$), 6.41 (dd, 1H, $\text{CH}_2=\text{CH}-$). The end-group functionality was calculated by the ratio of intensity of signal at 4.30 and 4.20 and found to be 85 and 75% in the case of PEG $M_{n,\text{aqueousGPC}} = 10$ kg/mol (PDI = 1.04) and 20 kg/mol (PDI = 1.02), respectively.

DsiRNA Conjugation with PEG-Acrylate. A solution of TEA (14 nmol, 0.14×10^{-3} M), DTT (5 μ mol, 50×10^{-3} M), and 3'-sense or 5'-antisense thiol-modified DsiRNA (12 nmol, 0.12×10^{-3} M) in RNase-free water (100 μ L) was mixed for 15 min. DTT was removed by extractions with equal volumes of saturated ethyl acetate. The aqueous phase collected from extractions was mixed with a solution of PEG-acrylate (600 nmol, 6×10^{-3} M) in phosphate buffer (0.1 M, pH 8, 100 μ L) and shaken overnight at 37 $^\circ\text{C}$. The formation of conjugates was verified by agarose gel electrophoresis using Bio-Rad Ready Precast Gel System (Bio-Rad). The conjugation reaction mixtures and DsiRNAs were run on a precast 3% ethidium bromide-agarose gel at 100 V for 45 min using tris/borate/EDTA (TBE) loading and running buffers. Gels were visualized by Gene Genius Bio Imaging System (Syngene), then quantified using Image J software (National Institutes of Health, U.S.). The conjugates prepared in this study were coded as given in Table 1.

Table 1. Codes of the Synthesized Conjugates

thiol modification site of DsiRNA duplex	conjugated PEG number-average molecular weight (kg/mol)			
	2	5	10	20
3'-sense strand	3'-2k	3'-5k	3'-10k	3'-20k
5'-antisense strand	5'-2k	5'-5k	5'-10k	5'-20k

Gel permeation chromatography (GPC) was also used to verify the conjugation of DsiRNAs with different PEGs. GPC was performed using Shimadzu modular system comprising a DGU-12A solvent degasser, on LC-10AT pump, a CTO-10A column oven, and a RID-10A refractive index detector and a SPD-10A Shimadzu UV-vis detector (flow rate: 1 mL/min). The column system was equipped with a Polymer Laboratories 5.0 mm bead-size guard column (50 \times 7.8 mm²), followed by two PL aquagel MIXED-OH columns (8 μ m). Calibration was performed with PEO standards ranging from 0.106 to 909.5 kg/mol.

Conjugates were purified by high-performance liquid chromatography (HPLC) using a C18 column (300 \times 3.9 mm², 10 μ m, Phenomenex) equipped with a UV detector at 220 and 260 nm. Liquid chromatography was carried out under the following conditions: mobile phase A, 0.025 M phosphate buffer (pH 7); mobile phase B, acetonitrile; a gradient elution from 1 to 50% B in 30 min and then maintained at 50% B for 10 min; and flow rate 1.0 mL \cdot min⁻¹, column temperature: 25 $^\circ\text{C}$. Subsequently conjugates were dialyzed for 2 days

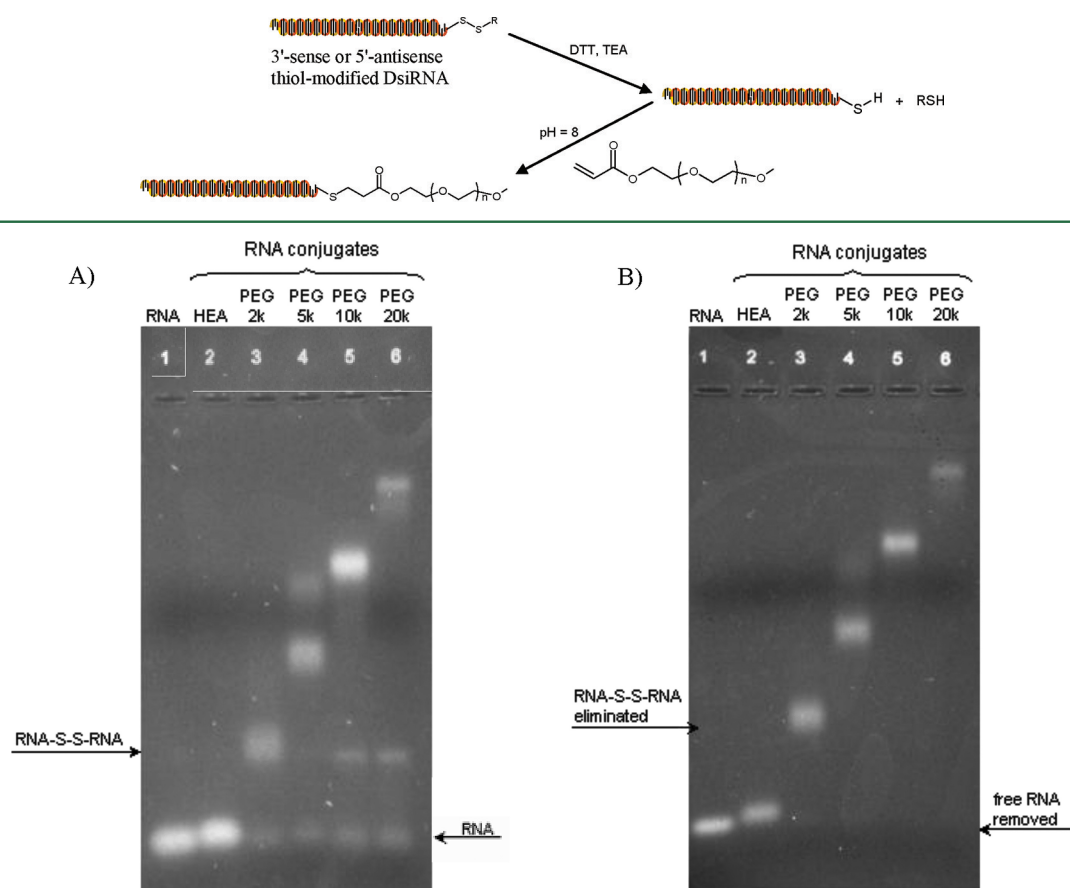
Scheme 1. Conjugation of PEG-Acrylate with Thiol-Modified DsiRNA via Michael Addition ($n = 45, 110, 225, 450$)

Figure 1. Agarose gel electrophoresis of 3'-sense thiol-modified-DsiRNA before and after conjugations. (A) Crude conjugate mixture. (B) HPLC-purified conjugates: line 1: 3'-sense thiol-modified DsiRNA (before conjugation), line 2: DsiRNA-HEA conjugate, and lines 3–6: DsiRNA-PEG conjugates (from 2 to 20 kg/mol PEG).

against water using a dialysis membrane (Spectrum, USA) with molecular weight cutoff (MWCO)-10 kg/mol for 2 and 5 kg/mol conjugates and MWCO-25 kg/mol for 10 and 20 kg/mol conjugates. Finally, all synthesized conjugates were freeze-dried and stored at -20°C . The concentration of the conjugates was determined by UV-spectroscopy at 260 nm using a double-beam Hitachi UV spectrometer (model no. U2800).

In addition, DsiRNA was also conjugated with hydroxyethyl acrylate (1 ethylene glycol unit) via the same method to compare more accurately the impact of the end-group modification. This conjugate was also purified in the same manner with the PEG conjugates.

Stability of Thioether Bond in DsiRNA-PEG Conjugates under Acidic Conditions. DsiRNA-PEG conjugate (2 nmol, M_n (PEG) = 5 kg/mol) was dissolved in acetic acid buffer (200 μL , at pH 4.5 or 5.5). The solution was shaken at 37°C . At predetermined time points, an aliquot was taken from this solution and frozen at -18°C . The stability of the bond between RNA and the polymer was analyzed by gel electrophoresis using 3% agarose or PAGE TBE urea gels.

In Vitro Human Dicer Activity by Recombinant Human Dicer. The human Dicer activity on the conjugates was tested following a protocol suggested by the supplier and also used by other groups.⁷ In brief, DsiRNA (50 pmol) was incubated with 1 or 2 units of recombinant human dicer at 37°C for 24 and 48 h. The reaction was performed in Dicer reaction buffer (10 μL , containing 24 mM HEPES, 200 mM NaCl, 40 μM EDTA, 1 mM ATP, 2.5 mM Mg_2Cl_2 , pH 8 for reaction with 1 unit of Dicer). All cofactors were proportionally scaled up when 2 units of Dicer were used; only the amount of reacted DsiRNA remained the same (50 pmol). At the end of incubation time, the Dicer activity was quenched by the addition of stop solution (2 μL , 10 mM EDTA). The samples (1 μL) were analyzed on a Small RNA

Lab-on-a-Chip (Agilent Technologies) using an Agilent 2100 Bioanalyzer (Agilent Technologies).

The same Dicer reaction conditions were applied to the conjugates. The conjugate samples were analyzed via PAGE using a 15% polyacrylamide TBE-urea gel at 180 V for 50 min and visualized by Gene Genius Bio imaging system. As a control marker, 21-mer siRNA was also loaded onto the gel. All dicing experiments were performed at least twice.

Cell Culture and Transfections. The human neuroblastoma cell line (SH-EP) that stably expresses eGFP was maintained as a monolayer in DMEM containing 10% FCS and 2 mM of L-glutamine. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2 . To transfect cells with the 3'-sense or 5'-antisense PEG conjugated DsiRNAs (27-mer) targeting eGFP, we plated cells at 1.5×10^5 for 16 h before transfection. The following day cells were transfected with the conjugates at differing concentrations ranging from 0.5 to 10 nM using lipofectamine 2000 following the manufacturer's instructions. Cells transfected with conventional 21-mer siRNA (100 nM) or unmodified 27-mer DsiRNA (0.2–10 nM) against eGFP were used as a positive control. Total RNA and cell lysates were collected 48–72 h post-treatment, and eGFP mRNA and protein levels were assessed using real-time PCR and Western blotting.

Measurement of eGFP mRNA Levels by Real-Time PCR. The expression of eGFP in SH-EP cells stably expressing eGFP was examined using real-time quantitative PCR. In brief, total RNA was collected and DNase-treated using the Qiagen RNeasy plus mini kit according to the manufacturer's instructions. Total RNA (0.5 μg) was reverse-transcribed. Real-time PCR was performed using the Power SYBR green PCR master mix according to the manufacturer's instructions using an Applied Biosystems 7500 real-time PCR system.

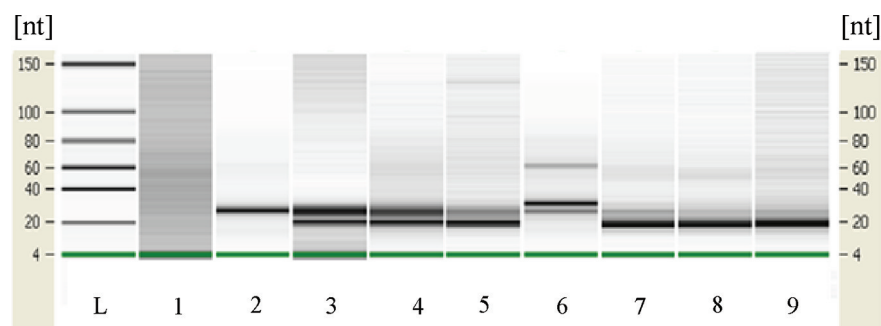


Figure 2. Bioanalyzer electroporation of 3'-sense- and 5'-antisense-thiol-modified DsiRNA (50 pmol) before and after incubation with 1 or 2 units of recombinant human Dicer for 24 or 48 h. Green line represents the smallest marker added as internal standard used to align sample with the ladder data. Lines: L, ladder; 1, blank; 2–5, 3'-sense-modified DsiRNA (2, before incubation with Dicer; 3, after incubation with 1 unit of Dicer for 24 h; 4, after incubation with 2 units of Dicer for 24 h; 5, after incubation with 2 units of Dicer for 48 h); 6–9, 5'-antisense-modified DsiRNA (6, before incubation with Dicer; 7, after incubation with 1 unit of Dicer for 24 h; 8, after incubation with 2 units of Dicer for 24 h; 9, after incubation with 2 units of Dicer for 48 h).

The primers for eGFP were as described previously, eGFP forward 5'-ATGGTGAGCAAGGGCGAGGA-3' and eGFP reverse 5'-ACTTGTGCCCGTTTACGTCGC-3'.⁴¹ All data were normalized to the housekeeping gene β_2 -microglobulin (β_2 -microglobulin Quantitect primer assay). All experiments were performed at least three times.

Measurement of eGFP Protein Levels by Western Blotting. Cell lysates were prepared from cells transfected with 3'-sense or 5'-antisense PEG conjugated DsiRNAs targeting eGFP by resuspending in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) containing protease inhibitors and incubated for 1 h on ice. Cell debris were cleared by centrifuging the lysates for 10 min at 13 000g at 4 °C. Supernatants were then collected and stored at -80 °C until analysis. Total protein in the lysates was determined using the BCA protein assay kit according to the manufacturer's instructions. Equal protein amounts (10 μ g) were loaded onto 10% SDS PAGE gels and transferred to nitrocellulose. eGFP protein expression was detected using a Rabbit polyclonal antibody against eGFP according to the manufacturer's instructions. Enhanced chemiluminescence (GE Healthcare) was used for detection. Densitometry was performed using ImageQuant software version 5.2 (Molecular Dynamics). Equal protein loading was confirmed by probing the membranes against the housekeeping protein GAPDH. All experiments were performed at least three times.

Fluorescence Microscopy Investigation of eGFP Inhibition. Round coverslips (13 mm) were first sterilized in 70% ethanol and washed twice with PBS and DMEM, then placed in wells of a 24-well microplate. SH-EP cells stably expressing eGFP were seeded at a concentration of 2×10^4 cells/mL in 500 μ L of media (DMEM with 10% FCS and 2 mM L-glutamine) per well and incubated overnight at 37 °C in a humidified atmosphere 5% CO₂. Cells were then transfected with nonmodified DsiRNAs or the PEG conjugated DsiRNAs (10 pmol, 500 μ L in DMEM with 10% FCS and 2 mM L-glutamine) using lipofectamine 2000 following the manufacturer's instructions. The coverslips were loaded onto microscope slides 72 h post-treatment and investigated using an Olympus Bx61 Fluorescent Microscope (Olympus, US). Each treatment was tested in three independent experiments. Cells (1 mL, 1×10^4 cells/mL) cultured without any treatment were used as the positive control.

Immune Stimulation Assays. To examine whether PEG-modified DsiRNAs were immunogenic, we obtained freshly collected blood from healthy human donors (HREC-09226). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-density gradient centrifugation and plated in a six-well plate containing RPMI supplemented with 10% FCS and 2 mM L-glutamine. For immunostimulation, PBMCs were treated with unmodified 27-mer DsiRNA (90nM) or 3' 10K-PEG-modified DsiRNA (90nM). PBMCs treated with 50 μ g [poly(I:C)] were used as a positive control. Total RNA was isolated using trizol as described previously 24 h post-

treatment.^{42,43} cDNA was reverse-transcribed, and DNase was treated using the Quantitect reverse transcription kit according to the manufactures instructions. Real-time PCR was performed as described above to measure the IFN-inducible gene, IFN-induced protein with tetratricopeptide repeats (IFIT-1) using the IFIT-1 quantitect primer assay. All data were normalized to the housekeeping gene β_2 -microglobulin (β_2 -microglobulin Quantitect primer assay). All experiments were performed using at least three separate PBMC isolations.

RESULTS AND DISCUSSION

Synthesis and Characterization of PEG-Conjugated Dicer Substrate siRNAs. 3'-Sense or 5'-antisense thiol-modified, 27-mer, blunt-ended DsiRNAs were conjugated with acrylate-modified PEG (PEG-acrylate) and hydroxyethylacrylate (HEA) via thioether bond, which is known to be physiologically stable.⁴⁴ The conjugation between DsiRNAs and PEG-acrylate was performed via a Michael addition reaction^{39,45} of thiol group of DsiRNA to the acrylic bond of PEG (Scheme 1). The conjugates prepared with PEGs having varying molecular weights are listed in Table 1. The formation of conjugates was first verified using gel electrophoresis. A representative gel electrophoregram of the conjugation reaction mixtures of 3'-sense DsiRNA and PEG-acrylates is shown in Figure 1A. The shift of the DsiRNA band to higher molecular weights according to the molecular weight of the conjugated PEG was clear on the gels. The presence of unconjugated DsiRNA was also observed on the gel electrophoregrams. Integrating the intensity of the unconjugated DsiRNA bands using Image J software, the percent conjugation yields [(conjugated DsiRNA: total DsiRNA used in the reaction) \times 100] were determined to be between 56 and 76%. A small amount of DsiRNA dimers (DsiRNA-S-S-DsiRNA structure) was also observed on the gel. The formation of dimers to some degree can be expected during the conjugation reaction after the removal of DTT. Finally, gel electrophoregrams also presented bands (in Figure 1A line 4) of secondary products generated from the bis-hydroxylPEG impurities, attributed to DsiRNA-PEG-DsiRNA conjugates. During the synthesis of PEG-acrylate, both of PEG's end-group hydroxyl groups may be functionalized with an acrylate yielding bisfunctional PEG-acrylate, which may result in the conjugation of two RNAs to one PEG chain. The conjugation yields of 5'-antisense-modified DsiRNA with PEG-acrylates, determined in the same manner, showed also similar results to those of 3'-sense-modified DsiRNA (data not shown). The conjugations were also verified

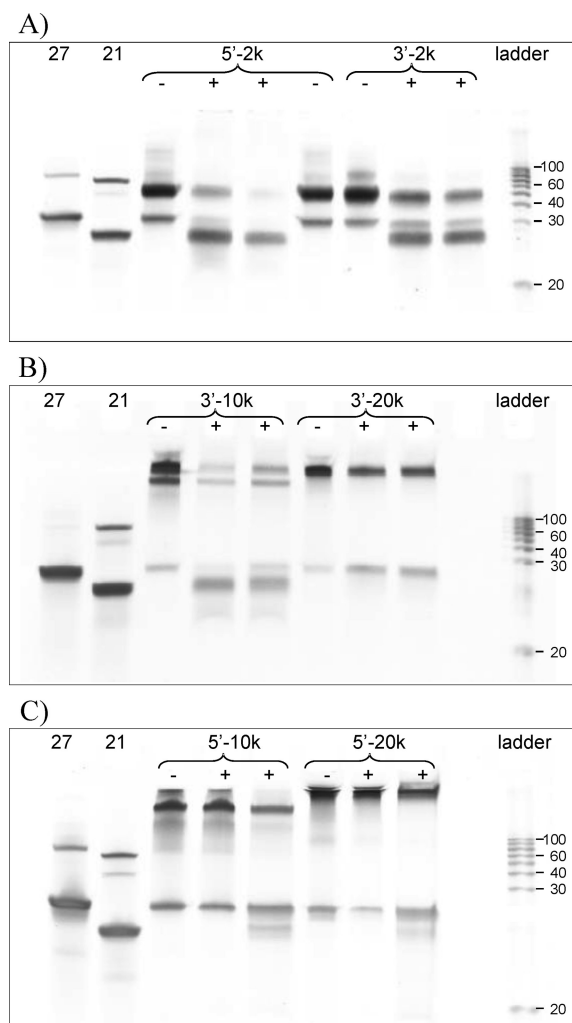


Figure 3. Dicer activity on PEG-conjugated DsiRNAs. (A) 5'-antisense DsiRNA-PEG (2 kg/mol) and 3'-sense DsiRNA-PEG (2 kg/mol) conjugates, (B) 3'-sense DsiRNA-PEG (10 and 20 kg/mol) conjugates, and (C) 5'-antisense DsiRNA-PEG (10 and 20 kg/mol) conjugates before and after incubation with Dicer. (–) represents untreated samples; (+) represents samples after incubation with 2 units of Dicer for 48 h. 27 and 21 represent unmodified 27-mer DsiRNA and 21-mer siRNA, respectively, as controls. Each sample contains 50 pmol RNA. Gel running conditions: 15% PAGE TBE urea gel at 180 V for 50 min.

by aqueous gel permeation chromatography (GPC) (Figure S2, SI). Chromatograms of 3'-sense-modified DsiRNA and 5'-antisense-modified DsiRNA clearly showed the shift of the RNA signal to the lower retention times after the conjugation according to the molecular weight of the PEG-acrylate attached.

The conjugates were then purified by HPLC, followed by dialysis to remove the unconjugated DsiRNA and disulfide dimers. Figure 1B shows the representative gel electrophoregram of the 3'-sense DsiRNA-PEG conjugates after the purification process. It is clear from the gel that the free DsiRNA and dimers were removed completely after the purification.

The release of siRNA from the DsiRNA-PEG conjugate is intended to be triggered only by the action of the Dicer enzyme. Therefore, the bond between DsiRNA and PEG should be stable until the conjugates meet the Dicer enzyme inside the cytoplasm. It is known that the ester bond between

DsiRNA and PEG can hydrolyze at acidic pH values. To investigate the stability of the conjugates at slightly low pH similar than those found in intracellular acidic vesicles such as endosomes and lysosome, the 5'-antisense-modified DsiRNA-PEG conjugate (M_n PEG = 5 kg/mol) was incubated at two different pH values (4.5 and 5.5) at 37 °C for 48 h. The samples were then analyzed by gel electrophoresis to see the presence of RNA released from the polymer. No free RNA was detected, indicating that the thioether and ester bonds were stable at acidic pH environment (Figure S3, SI).

It is well known that PEGylation improves the nuclease resistance and serum stability of siRNA.^{31–33,35,38} DsiRNA designs have higher nuclease resistance and serum stability when compared with siRNA.^{7,14–16} An in vitro serum stability assay that was performed by incubating nonmodified DsiRNA or PEGylated DsiRNA in 90% FBS for up to 48 h showed that PEGylated DsiRNA (either 3'-sense or 5'-antisense conjugates) has serum stabilities similar to nonmodified DsiRNA. Both PEGylated and nonmodified DsiRNA could keep its structural integrity up to 12 h in 90% serum (Figure S4, SI).

Dicer Cleaves 21-mer siRNA from PEG-Conjugated Dicer Substrate siRNAs. Ideally, conjugation of PEG should not alter the conversion of DsiRNA by Dicer enzyme to 21-mer siRNA. To test this hypothesis, we first examined Dicer cleavage of the nonconjugated, 3'- and 5'-thiol-modified DsiRNAs in vitro using Bioanalyzer, a microfluidic-based instrument providing electrophoretic separation on microchips instead of gels. The results are shown in Figure 2. The complete cleavage of 3'-sense thiol-modified DsiRNA to 21-mer RNA was attained when 50 pmol DsiRNA was incubated with 2 units of Dicer enzyme for 48 h (Lane 5, Figure 2). The complete cleavage of 5'-antisense thiol modified DsiRNA (50 pmol) by 2 units of Dicer occurred in 24 h; however, the data revealed that the cleavage of 5'-antisense-thiol-modified DsiRNA yielded 19-mer instead of 21-mer siRNA (Lane 9, Figure 2).

Considering this finding, the DsiRNA-PEG conjugates (50 pmol) were incubated with 2 units of Dicer for 48 h and analyzed by gel electrophoresis to investigate the cleavability of the conjugates to 21-mer siRNA (Figure 3). The products cleaved from the conjugates were compared with the unmodified 27-mer DsiRNA and unmodified 21-mer siRNA. All conjugates except 5'-10k, 5'-20k, and 3'-20k conjugates showed some degree of cleavage by Dicer. It was observed from the band intensities in Figure 3A,B that the 2k conjugates (both 3'-2k and 5'-2k conjugates) and 3'-10k conjugate were relatively well-cleaved, leading to the formation of an RNA band at the same level as the 21-mer siRNA band. Cleavage of 3'-20k did not produce 21-mer RNA (Figure 3 B). Similarly, 5'-10k and 5'-20k conjugates yielded an insignificant quantity of RNA traces shorter than 27-mer (Figure 3C). It should be noted that a small amount of free (unconjugated) 27-mer DsiRNA (5'- or 3'-thiol modified) in all conjugate samples was present as nonpurified samples was deliberately used to utilize the free DsiRNA in each sample as an internal control. Hence especially for 5'-10k and 5'-20k conjugates, the formation of the shorter RNA sequences after the incubation with Dicer was attributed to the cleavage of the free (unconjugated) 27-mer DsiRNAs by the enzyme. In 3'-20k and 5'-20k conjugate samples, the increased intensity of the 27-mer RNA bands after the incubation of the conjugates with Dicer indicated that the Dicer activity yielded mostly 27-mer DsiRNA or more probably RNA oligomer–PEG conjugates that contain RNA segment shorter than 27-mer. No

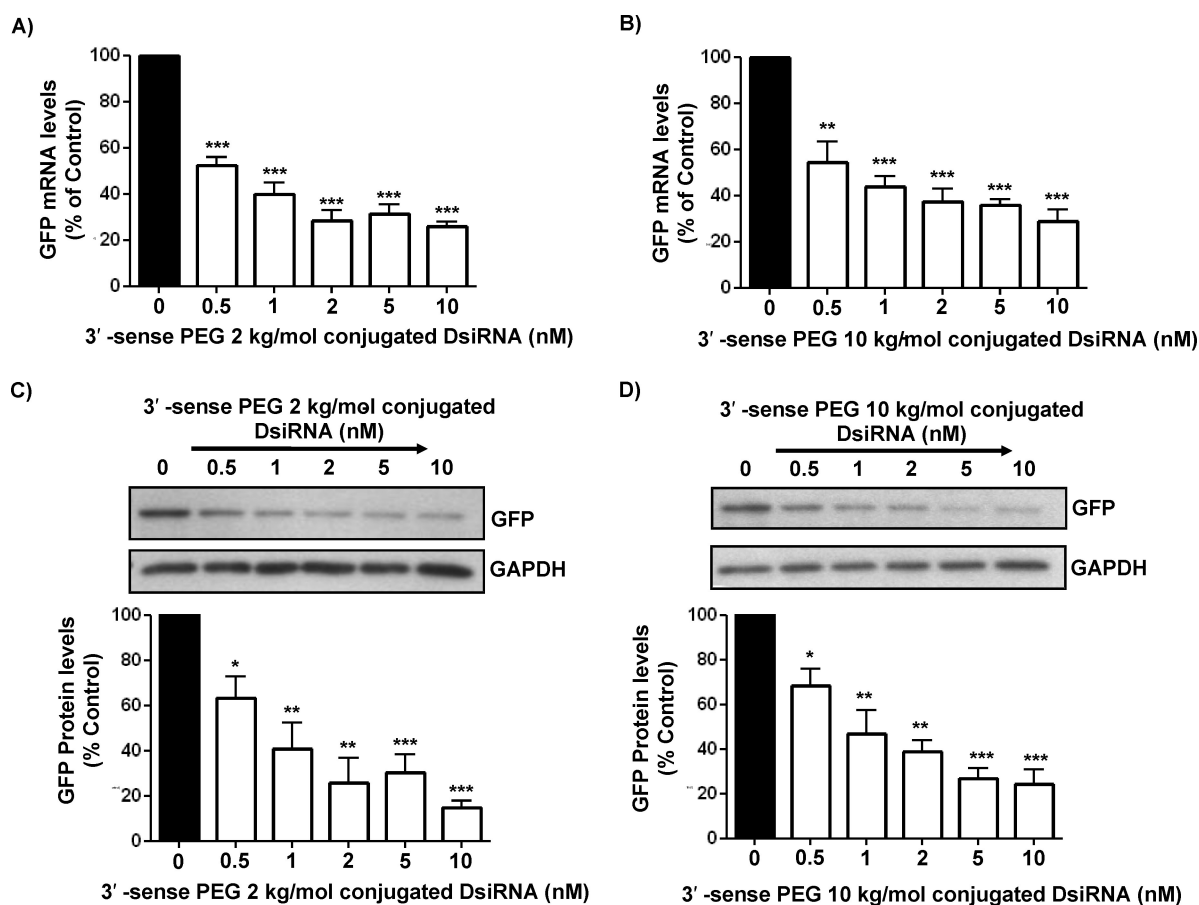


Figure 4. Efficacy of 3'-sense PEG conjugated DsiRNAs targeting eGFP. Representative graphs showing eGFP mRNA levels in SH-EP cells stably expressing eGFP treated with increasing concentrations (0.5–10 nM) of 3'-sense PEG (A) 2 kg/mol and (B) 10 kg/mol conjugated DsiRNAs targeting eGFP. Data was expressed as a percentage of control. All data were normalized to the housekeeping gene β_2 -microglobulin. ($n = 3$ to 4 separate experiments, *** $p < 0.0001$). (C,D) Representative Western blots and densitometry demonstrating the effect of increasing concentrations (0.5–10 nM) of 3'-sense PEG 2 or 10 kg/mol conjugated DsiRNA in silencing eGFP protein expression. GAPDH was used as loading control. All data were normalized to the housekeeping protein GAPDH ($n = 3$ to 4 separate experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

attempt was made to analyze these products because the Dicer-triggered release of 21-mer siRNA from the conjugates was the primary interest in this study.

In conclusion, in this study, it was hypothesized that the Dicer activity on the conjugates would generate the 21-mer siRNA species. This hypothesis was generally supported by the results obtained. In particular, 3'-2k and 3'-10k could be cleaved clearly to 21-mer after reaction with dicer. Cleavage profile and efficiency were affected by the conjugation site and the molecular weight of PEG conjugated to the DsiRNA. Consistent with the cleavage of unconjugated 5'-antisense thiol-modified DsiRNA, which produced 19-mer instead of 21-mer, cleavage of 5'-10k and 5'-20k conjugates by Dicer was less site specific and perhaps random in the presence of conjugated polymer when compared with the cleavage of 3'-sense-modified DsiRNA and its conjugates. The differences in the Dicer cleavability between the 3'-modified and 5'-modified DsiRNAs and conjugates may be a result of the strand selection process and binding of DsiRNA to the dicer enzyme. Indeed, 3'-overhanging DsiRNA designs instead of blunt-ended DsiRNA are more predictable and have a limited dicing pattern most probably because of the defined 3'-PAZ binding site.⁸ 3'-20k conjugates could have been site-specifically cleaved by Dicer, but it is probable that the larger size of the conjugated polymer have sterically prevented the interaction of Dicer with DsiRNA.

As a control experiment, Dicer activity on a mixture of (unconjugated) PEG and (unconjugated) DsiRNA was also tested under the same conditions to examine if the presence of PEG affected the Dicer enzyme activity. A mixture of unconjugated 3'-sense or 5'-antisense-modified DsiRNA and free PEG having varying molecular weights (at 1:1 mol ratio) were incubated with Dicer enzyme. The mixtures were then analyzed by gel electrophoresis (Figure S5, SI). The results indicated that presence of PEG (regardless of its molecular weight) did not inhibit the dicing activity of the enzyme because the Dicer enzyme exhibited similar cleavage efficiency with DsiRNA/polymer mixtures as it did with the unconjugated DsiRNA samples without added PEG.

PEG-Conjugated Dicer Substrate siRNAs Efficiently Silence eGFP. Human neuroblastoma (SH-EP) cells stably expressing eGFP were transfected with the 3'-sense- or 5'-antisense-modified DsiRNA and their (HPLC-purified) conjugates with different sizes of PEG (2, 10, and 20 kg/mol) using lipofectamine 2000 as a well-established transfection agent. Cells treated with different sizes of PEG conjugated to the 3'-sense strand of DsiRNA showed potent gene silencing activity at both the mRNA and protein levels (Figure 4). Moreover, the 3'-2k and 3'-10k conjugates (3'-sense strand DsiRNA conjugates with 2 kg/mol and 10 kg/mol PEG) displayed similar patterns of gene silencing (Figure 4). For example, cells

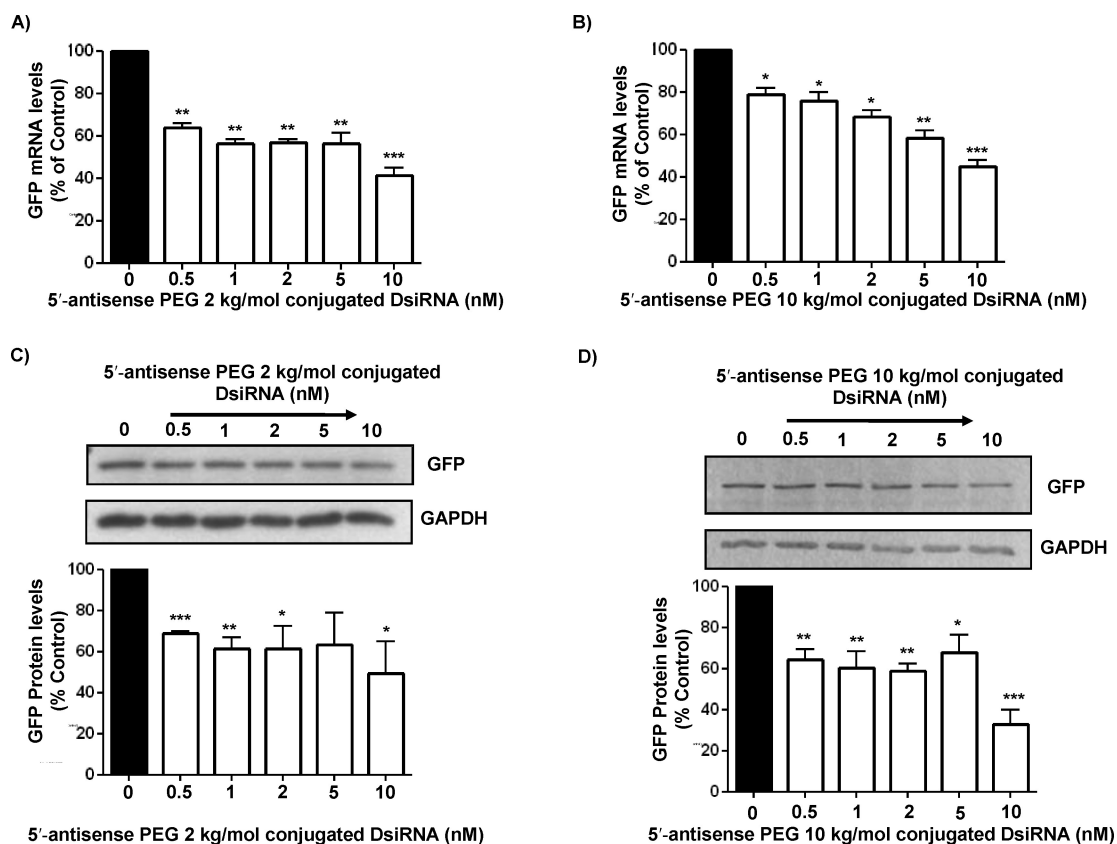


Figure 5. Efficacy of 5'-antisense PEG conjugated DsiRNAs targeting eGFP. Representative graph showing eGFP mRNA levels in SH-EP cells stably expressing eGFP treated with increasing concentrations (0.5–10 nM) of 5'-antisense PEG (A) 2 kg/mol and (B) 10 kg/mol conjugated DsiRNAs targeting eGFP. Data were expressed as a percentage of control. All data were normalized to the housekeeping gene β_2 -microglobulin. ($n = 3$ to 4 separate experiments, ** $p < 0.01$ and *** $p < 0.0001$). (C,D) Representative Western blots and densitometry demonstrating the effect of increasing concentrations (0.5–10 nM) of 5'-antisense PEG 2 or 10 kg/mol conjugated DsiRNA in silencing eGFP protein expression. GAPDH was used as loading control. All data were normalized to the housekeeping protein GAPDH ($n = 3$ to 4 separate experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

treated with 10 nM of the 3'-2k and 3'-10k conjugates were able to silence eGFP mRNA levels by $74 \pm 2\%$ and $71 \pm 5\%$, respectively, when compared with control cells. Importantly, the activity of the PEG-modified DsiRNA was not too dissimilar to cells treated with unmodified 21-mer siRNA (50 nM) or 27-mer (1 nM) DsiRNA (Figure S6, SI). The reduced gene silencing activity of PEG-modified DsiRNA with respect to the unmodified 27-mer DsiRNA was attributed to the incomplete cleavage of PEG-conjugated DsiRNA by dicer to 21-mer siRNA sequence silencing eGFP gene, in accord with the results presented in Figure 3. Cells incubated with DsiRNA-conjugated with the 20 kg/mol PEG on the 3'-sense strand showed reduced gene silencing activity when compared with the 2 and 10 kg/mol PEG dicer conjugates [gene silencing activity, 3'-20k (10 nM) = $34 \pm 2\%$; and 5-20k (10 nM) = $48 \pm 3\%$] (Figure S7, SI). The decrease in gene silencing activity for the 20 kg/mol PEG-modified DsiRNAs when compared with the activity of 2 and 10 kg/mol PEG conjugates may be due to the larger hydrodynamic volume of PEG, thus partially inhibiting the ability of the dicer enzyme to interact with modified DsiRNA.

To characterize fully the effect of PEG conjugated to DsiRNA, we also examined different sizes of PEG (2, 10, and 20 kg/mol) conjugated to the 5'-antisense strand of the DsiRNA. Cells treated with the 5'-antisense PEG conjugates displayed reduced activity when compared with the 3'-sense

modified DsiRNA, with a maximum gene silencing of $58 \pm 3\%$ for the 2 kg/mol conjugated PEG (10 nM) and $55 \pm 3\%$ for the 10 kg/mol conjugated PEG conjugated DsiRNA (10 nM) (Figure 5). The reduced gene silencing activity of 5'-antisense conjugates when compared with the 3'-sense conjugates was attributed to the poor cleavability of these conjugates by Dicer to 21-mer siRNAs. There was a difference in the amount of eGFP knockdown between the 5'-20k and 3'-20k conjugates (Figure S7, SI). However, this decrease was very small with a % knockdown difference between the two conjugates ranging from 5.4 to 11.7% over the different concentrations of DsiRNA. Moreover, the difference at the mRNA expression between the two conjugates did not correlate at the protein level. Taken together, the results demonstrate that different sizes of PEG are able to be conjugated to DsiRNA with approximately 10-fold inhibition in its gene silencing activity.

Gene silencing activity of the 3'-sense- or 5'-antisense-modified DsiRNA and their conjugates with different sizes of PEG (2, 10, and 20 kg/mol) was also visualized via fluorescence microscopy (Figures 6 and 7). Both the nonmodified DsiRNA (10 nM) and 3'- and 5'-thiol-modified DsiRNAs (10 nM) caused significant reduction in fluorescence of SH-EP cells stably expressing eGFP. Transfection with 3'-thiol-modified DsiRNA was the most efficient in silencing eGFP expression. No significant difference in the silencing effect of the unmodified DsiRNA and 5'-thiol modified

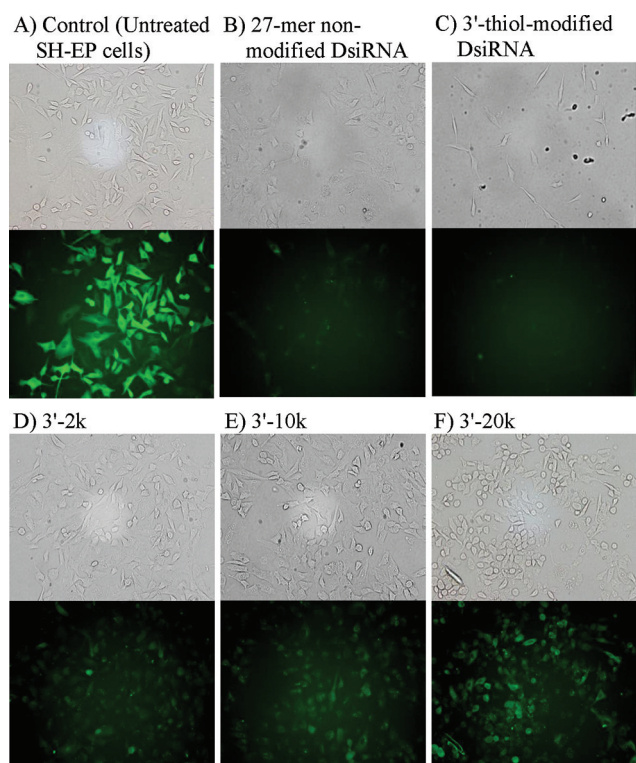


Figure 6. Representative phase contrast and fluorescence microscope images of SH-EP cells stably expressing eGFP before and after transfection with 3'-sense-thiol-modified DsiRNA and DsiRNA-PEG conjugates. (A) Untreated cells; (B) cells treated with 10 nM of nonmodified 27-mer DsiRNA; (C) cells treated with 3'-sense-thiol-modified DsiRNA (10nM); (D) cells treated with 3'-sense-thiol-modified DsiRNA conjugates of 2 kg/mol PEG(10nM); (E) cells treated with 3'-sense-thiol-modified DsiRNA conjugates of 10 kg/mol PEG (10nM); and (F) cells treated with 3'-sense-thiol-modified DsiRNA conjugates of 20 kg/mol PEG (10nM). Cells (1×10^4 cells/mL) were transfected with a final concentration of 10nM in 1 mL of total well volume. Results shown are the representative images, $n = 9$.

DsiRNA was observed. The DsiRNA-PEG conjugates (10 nM) showed slightly less activity compared with the unmodified DsiRNA and 3'- and 5'-thiol-modified DsiRNAs; nevertheless, they significantly reduced the fluorescence of SH-EP cells (Figures 6 and 7). The fluorescent microscope results were in accord with the mRNA reduction and protein inhibition results (Figures 4 and 5). The increase in the molecular weight of PEG conjugated to DsiRNAs resulted in less efficient fluorescence reduction. The 2 kg/mol conjugates (both 3'-2 and 5'-2 kg/mol) were the most efficient at reducing the fluorescence of SH-EP cells, followed by 10 and 20 kg/mol conjugates. Here it should be noted that the cytotoxicity of the 3'-sense conjugates with varying molecular weights do not significantly differ from each other and the 3'-sense thiol-modified DsiRNA (Figure S8, SI). Similar to the thiol-modified DsiRNAs, PEG conjugation to the 3'-sense-strand of DsiRNAs displayed better fluorescent reduction effect when compared with the PEG conjugation at the 5'-antisense-strand.

PEG-Conjugated Dicer Substrate siRNAs Are Less Immunostimulatory Compared with Unmodified Dicer Substrate siRNAs. To examine whether conjugating PEG to DsiRNA could affect the immunogenic potential of DsiRNA, we treated human PBMCs with unmodified 27-mer DsiRNA or its conjugate with 10 kg/mol PEG. Data obtained from the in

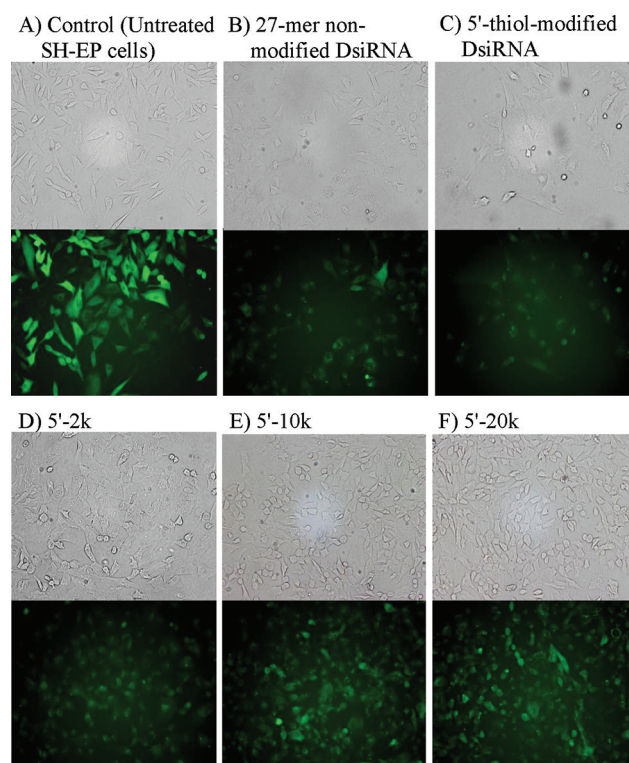


Figure 7. Representative phase contrast and fluorescence microscope images of SH-EP cells stably expressing eGFP before and after transfection with 5'-antisense-thiol-modified DsiRNA and DsiRNA-PEG conjugates. (A) Untreated cells; (B) cells treated with 10nM of nonmodified 27-mer DsiRNA; (C) cells treated with 5'-antisense-thiol-modified DsiRNA (10nM); (D) cells treated with 5'-antisense-thiol-modified DsiRNA conjugates of 2 kg/mol PEG(10nM); (E) cells treated with 5'-antisense-thiol-modified DsiRNA conjugates of 10 kg/mol PEG (10nM); and (F) cells treated with 5'-antisense-thiol-modified DsiRNA conjugates of 20 kg/mol PEG (10nM). Cells (1×10^4 cells/mL) were transfected with a final concentration of 10nM in 1 mL of total well volume. Results shown are the representative images, $n = 9$.

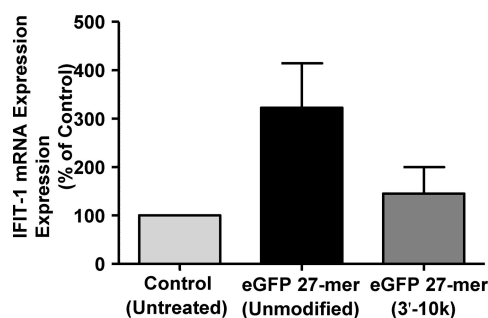


Figure 8. Immunostimulatory response of unmodified or 3'-sense PEG conjugated DsiRNA. A representative graph showing IFIT-1 mRNA expression in human PBMCs treated with unmodified eGFP DsiRNA (90 nM) or 3'-10 kg/mol PEG-modified eGFP DsiRNA (90 nM). Data were expressed as a percentage of control. All data were normalized to the housekeeping gene β_2 -microglobulin ($n = 3$ individual PBMC isolations).

vitro dicer cleavage assay and gene silencing experiments showed that there was very little difference between both the 3'-2k and 3'-10k conjugates. This indicates that the transfection efficiencies of both conjugates were very similar. As a proof-of-principle, we wanted to determine whether PEG conjugated to

DsiRNA could potentially shield any immune-stimulating effect commonly observed with DsiRNA. Because the shielding effect of 10 kDa PEG is higher than that of 2 kDa PEG and most PEGylated therapeutics use PEGs with molecular weights larger than 5 kDa, we used the 3'-10k conjugates for these experiments. To assess immunogenicity, we measured the mRNA levels of the IFN-inducible gene IFIT1. This gene is increased upon IFN activation or directly by dsRNA receptors including Toll-like receptor-3 (TLR-3). Therefore, its induction is considered to be indicative of a siRNA-mediated immune response.⁴⁶ It should be noted here that the concentration chosen for immunogenicity experiments was 90 nM, which was the minimum concentration of unmodified DsiRNA required to elicit a reproducible immune response in human peripheral blood mononuclear cells collected from individual patient donors. Our results demonstrate that unmodified DsiRNA increased IFIT1 mRNA levels when compared with control (untreated) cells (Figure 8). Interestingly, our PEG-modified DsiRNA was less immunogenic when compared with the same unmodified DsiRNA (Figure 8). Importantly, PBMCs treated with poly(I:C) (a known immune activator) showed a significant increase in IFIT-1 levels, confirming that the isolated PBMCs were responsive to immune stimulation (Figure S9, SI). The reduced immunogenicity observed with the PEG conjugate was attributed to the shielding effect of PEG since fluorescence microscopy results (Figures 6 and 7) showed that there was very little difference in the level of eGFP knockdown between the 3'-10k conjugate and the unmodified DsiRNA (note that all of the samples were used at the same concentration, 10 nM), suggesting that the transfection efficiency of the two were similar. However, detailed investigations on the immunogenicity of the conjugates should be performed in future studies.

CONCLUSIONS

In this study, 3'-sense or 5'-antisense thiol-modified, blunt-ended DsiRNAs targeting eGFP gene were conjugated with acrylate-modified PEGs having varying molecular weights via a physiologically stable bond. The recombinant Human Dicer was able to efficiently release 21-mer siRNA from 2k conjugates (both 3'-2k and 5'-2k conjugates) and 3'-10k conjugate. Cells treated with 2 and 10 kg/mol PEG conjugated to the 3'-sense strand of DsiRNA showed potent gene silencing activity at both the mRNA and protein levels. PEG conjugates of 5'-antisense strand of DsiRNA showed less gene silencing activity compared with the 3'-sense strand DsiRNA conjugates, in accord with Dicer cleavage profile of the 5'-conjugates. Interestingly, 10 kg/mol PEG conjugates of the 3'-sense strand of DsiRNA were less immunogenic when compared with the same unmodified DsiRNA.

In summary, this study presents a new strategy for siRNA release from polymeric delivery vehicles. Whereas PEG was utilized in this study for conjugation to DsiRNA and proved to reduce the immunogenicity of DsiRNA, it is also possible to utilize varying functional polymers such as endosomal disruptive polymers, actively targeting or fusogenic peptides to deliver siRNA into the cells using this new release strategy.

ASSOCIATED CONTENT

Supporting Information

eGFPS1 inhibiting RNA sequences used in this study, ¹H NMR spectrum of PEG-acrylate ($M_n = 5$ kg/mol) in CDCl₃, aqueous GPC chromatogram of 5'-antisense conjugates, stability of 5'-

antisense DsiRNA conjugated with PEG 5 kg/mol (5'-5k) in an acidic environment, representative blots of DsiRNA and conjugates after incubation in 90% FBS for up to 48 h, dicer enzyme activity on mixtures of free PEG with the unconjugated DsiRNAs, the efficacy of unmodified siRNA targeting eGFP, the efficacy of 3'-sense or 5'-antisense PEG 20 kg/mol conjugated DsiRNAs, cytotoxic effect of 3'-sense modified DsiRNA and its conjugates (10 nM), and immunostimulatory response of freshly isolated human PBMCs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

#Equal contribution

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