Supporting Information

An Artificial Control of Gene Silencing Activity Based on siRNA Conjugation with Polymeric Molecule Having Coil-Globule Transition Behavior

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Materials

N-isopropylacrylamide (NIPAAm), tributylphosphine (PBu₃), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxybenzotriazole monohydrate (HOBt·H₂O), and 1,2-bis(2-aminoethoxy)ethane were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). NIPAAm was recrystallized for two times from n-hexane before use. 2-(Dodecylthiocarbonothioylthio)-2-methylpropanoic acid (DDMAT) and Dulbecco’s modified eagle’s medium (DMEM) were purchased from Sigma Aldrich (St. Louis, MO, USA). 2,2’-Azobis(4-methoxy-2-4-dimethylvaleronitrile) (V-70), dichloromethane, 1,4-dioxane, N,N-dimethylformamide (DMF), tetrahydrofuran (THF), diethyl ether, triethylamine, and MagCapture™ microRNA Isolation Kit (Human Ago2) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka,
Japan). 1,4- Dioxane and DMF were purified by distillation under reduced pressure before use. Series of \( \alpha \)-methoxy-\( \omega \)-amino-poly(ethylene glycol) (PEG-NH\(_2\)) were purchased from NOF Co., Ltd. (Tokyo, Japan). DBCO-NHS ester was purchased from Click Chemistry Tools, Ltd. (Scottsdale, AZ). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (1 M, pH 7.3) was purchased from AMRESCO Inc. (Solon, OH). A luciferase-expressing human cervical cancer cell line, Hela-Luc, was purchased from Caliper LifeScience (Hopkinton, MA, USA). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Lipofectamine RNAiMAX was purchased from Invitrogen (Carlsbad, CA). Luciferase Assay System Kit was purchased from Promega Co. (Madison, WI). Series of RNA were synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Japan). The RNA sequences are as follows:

**siGL3** (azide-modified and TAMRA-labeled): 5’-(N\(_3\))-CUU ACG CUG AGU UCG AdTdT-3’ (sense strand), 5’-UCG AAG UAG UCA GCG UAA GdTdT(-TAMRA)-3’ (antisense strand).

**siScramble** (azide-modified) : 5’-(N\(_3\))- UUC UCC GAA CGU GUC ACG UdTdT-3’ (sense strand), 5’-ACG UGA CAC GUU CGG AGA AdTdT-3’ (antisense strand).

**Methods**

**Synthesis of PNIPAAm with trithiocarbonate terminus (PNIPAAm-CTA)**

PNIPAAm was synthesized via RAFT polymerization method\(^{1,2,3}\) as shown in Supporting Scheme 1. NIPAAm (3.3 g, 29.16 mmol), and DDMAT (22.87 mg, 0.063 mmol) were dissolved in DMF (10 mL) at room temperature for 10 min to obtain a homogenous mixture under an argon atmosphere. In a separate flask, V-70 (3.87 mg, 0.013 mmol) was diluted in 4 mL of DMF. Both of the mixtures were degassed via consecutive standard freeze-pump-thaw cycles for three times. The degassed mixtures were stirred for 5 min at 35 °C, then, 1 mL of V-70 solution was added into the NIPAAm solution to initiate RAFT polymerization. After the stirring at 35 °C for 12 h, the polymerization was stopped by cooling to room temperature and exposure to air. The reaction solution was diluted with arbitrary amount of THF and poured into excess amount of diethyl ether. The obtained crude precipitate was dissolved in THF.
and further precipitated into diethyl ether to completely remove unreacted monomer. The obtained precipitate was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da) and lyophilized to obtain trithiocarbonate-terminated polymer, PNIPAAm-CTA (2.5 g, yield 76%) as yellowish powder. PNIPAAm-CTA was characterized using size exclusion chromatography equipped with RI detector (RI 2075, JASCO, Tokyo, Japan) [column: superAW3000, superAW4000, and superAWL-guard column (TOSOH, Tokyo, Japan), eluent: DMF with 0.05 M LiCl at 40 °C, flow rate: 0.5 mL/min] and $^1$H NMR (AVANCE III 400, Bruker Corporation). Molecular weight and polydispersity index (Mw/Mn) of the polymer were calculated based on standard curve of poly(ethylene glycol). $^1$H-NMR: $D_n = 345$. GPC: Mn= 31,500 g/mol, Mw= 39,000 g/mol, (Mw/Mn) = 1.24. The chemical structure was confirmed by $^1$H NMR analysis in MeOD (Supporting Figure S1).

**Synthesis of PNIPAAm-TE**

Trithiocarbonate terminus of PNIPAAm-CTA was deprotected into thiol group, followed by a conversion into an unreactive thioether to circumvent unexpected side reactions in the following synthesis steps (Supporting Scheme S1). PNIPAAm-CTA (Mw: 39,000 g/mol) (0.41 g, 0.01 mmol) was dissolved in 1,4-dioxane (5 mL) and stirred for 6 h under an argon atmosphere in the presence of 2-ethanolamine (6.1 µL, 0.1 mmol) and tributylphosphine (2.5 µL, 0.01 mmol). Then, the reaction solution was poured into excess amount of diethyl ether, and the resulting precipitate was washed twice with diethyl ether, followed by drying under vacuum to obtain PNIPAAm-SH as white powder (0.3 g, 75%). The complete removal of dodecyl trithiocarbonate terminus was confirmed by $^1$H NMR analysis in MeOD, as suggested by the disappearance of methyl protons in dodecyl trithiocarbonate terminus at 0.88 ppm (data not shown). Subsequently, PNIPAAm-SH (0.3 g, 0.008 mmol), tributylphosphine (2.2 µL, 0.009 mmol), and maleimide (0.013 g, 0.008 mmol) were dissolved in 1,4-dioxane (5 mL), and the mixture was stirred at room temperature for 12 h. The unreacted maleimide and tributylphosphine were removed by precipitation into diethyl ether twice, and the precipitated polymer was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da). The dialyzed solution was lyophilized to obtain PNIPAAm-TE (0.27 g, 90%) as white powder.
Synthesis of DBCO-PNIPAAm-TE

Cyclooctyne moiety was introduced into the terminus of PNIPAAm-TE for the subsequent reaction with azide-siRNA (Supporting Scheme S2). PNIPAAm-TE (0.2 g, 0.005 mmol, Mw= 39,000 g/mol), 1,2-bis(2-aminoethoxy)ethane (71 µL, 0.5 mmol), EDC⋅HCl (0.009 g, 0.05 mmol), and HOBT⋅H₂O (0.007 g, 0.0.5 mmol) were dissolved in dichloromethane (2 mL). The reaction solution was stirred at room temperature for 6 h and was poured into excess amount of diethyl ether. The obtained precipitate was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da), followed by lyophilization to produce white powder. The crude product was further purified by ion exchange chromatography (CM-Sephadex C-50 and QAE-Sephadex A-50) to remove unreacted PNIPAAm-TE, and dialyzed against de-ionized water (molecular weight cut off: 3,500 Da). The dialyzed solution was lyophilized to obtain NH₂-PNIPAAm-TE as white powder (0.1 g, 50%). The primary amino group at the terminus was confirmed by ion-exchange HPLC using RI detector (RI-2031PLUS, JASCO)[column: SP-5PW (TOSOH), eluent: 2 mM PB pH 6.5, flow rate: 0.5 mL/min at room temperature] (data not shown). Next, NH₂-PNIPAAm-TE (50 mg, 1.28 µmol) and DBCO-NHS-ester (5.16 mg, 1.28 µmol) were dissolved in dichloromethane (2 mL). After an addition of catalytic amount of triethylamine, the reaction mixture was stirred at room temperature in a dark room for 12 h. Then, the reaction solution was dialyzed against methanol for 24 h, followed by dialysis against de-ionized water for 48 h (molecular weight cut off: 3,500 Da). The dialyzed solution was filtered through a 0.22 µm syringe driven filter and lyophilized to obtain DBCO-PNIPAAm-TE (41.3 mg, 82.6%) as white powder. A quantitative introduction of DBCO moiety into the polymer was confirmed by ¹H NMR analysis in D₂O, as calculated from the peak intensity ratio of phenyl protons in DBCO at 7.2-7.8 ppm (-C₆H₄) and PNIPAAm at 3.6 – 4.1 ppm (-CONHC(CH₃)₂) (Supporting Figure S2).

Synthesis of PEG-DBCO

PEG-DBCO was also synthesized according to Supporting Scheme S3. PEG-NH₂ for Mw= 40,000 g/mol (50 mg, 1.25 µmol) and DBCO-NHS ester (2.52 mg, 6.25 µmol) were dissolved in 2 mL of dichloromethane with catalytic amount of triethylamine for 12 h at room temperature in a dark room. Next, the reaction solution was
subsequently dialyzed against methanol for 24 h and de-ionized water for 48 h (molecular weight cut off: 3,500 Da). Then, the dialyzed solution was filtered through a 0.22 µm syringe driven filter and lyophilized to obtain PEG-DBCO (43 mg, 86 %) as white powder. Obtained white powder was analyzed by $^1$H NMR in DMSO-d$_6$ at 80 °C. The peak intensity ratio of phenyl proton in DBCO (-C$_6$H$_4$-, δ 7.2 – 7.6 ppm) and PEG protons (-C$_2$H$_4$O-, δ = 3.5 – 3.6 ppm) confirms a successful introduction of DBCO into the PEG terminus (Supporting Figure S3).

**Synthesis of siRNA-PNIPAAm-TE (PNIPAAm-siRNA)**

siRNA conjugation with DBCO-PNIPAAm-TE was performed according to Supporting Scheme S4, in a similar manner to the reported method.$^4$ Series of azide-siRNA solutions in 10 mM HEPES buffer (pH 7.3) (10 µL, 25 µM) and DBCO-PNIPAAm-maleimide solution in 10 mM HEPES buffer (pH 7.3) (4 µL, 50 µM) were mixed in a 1.5 mL microtube, and 10 mM HEPES buffer (pH 7.3) (64 µL) was added into the solutions. The reaction solutions were frozen at -20 °C overnight, and then, thawed at 4 °C for 1 h. The crude products were purified by ion-exchange HPLC to obtain series of siRNA-PNIPAAm-maleimide conjugates (PNIPAAm-siRNA) [Column: SuperQ-5PW (TOSOH), eluent: 10 mM HEPES pH 7.4 with gradient concentration of NaCl (from 0 mM to 1 M), flow rate: 0.8 mL/min, detector: UV absorbance at 260 nm] (Supporting Figure S4). In a similar manner, siRNA conjugated PNIPAAm with Mw of 9,500 g/mol and 22,000 g/mol were synthesized (data not shown).

**Synthesis of PEG-siRNA**

Series of PEG-siRNAs were also synthesized in a similar manner to the synthesis of PNIPAAm-siRNA (Supporting Scheme S5), except for the HPLC condition for purification. The solutions of series of siRNA azide in 10 mM HEPES buffer (pH 7.3) (10 µL, 25 µM) and solutions of PEG-DBCO in 10 mM HEPES buffer (pH 7.3) (4 µL, 50 µM) were mixed in a 1,5 microtube, and 10 mM HEPES buffer (pH 7.3) was added into the solutions. The reaction solutions were frozen at -20 °C for overnight and followed by thawing for 1 h at 4 °C. The crude products of PEG-siRNA were purified using ion-exchange HPLC equipped with UV absorbance at 260 nm. [Column: MonoQ 5/50GL (GE Healcare) at flow rate 2.0 mL/min in eluent 10 mM
HEPES pH 7.4 with gradient concentration of NaCl (from 0 mM to 1 M)] (Supporting Figure S5). In a similar manner, siRNA conjugated PEG with Mw of 10,000 g/mol and 20,000 g/mol were synthesized (data not shown).

**Agarose gel electrophoresis**

The obtained siRNA-conjugates with PNIPAAm and PEG polymers were further analyzed by agarose gel electrophoresis. Unconjugated siRNA, PNIPAAm-siRNA, and PEG-siRNA (100 ng of siRNA for each sample) were loaded onto agarose gel (2.5% agarose, 1 × TBE) including SYBR safe (Thermo Fisher Scientific Inc., Waltham, MA, USA) and treated at 100 V for 60 min. Next, the band from siRNA and its polymer conjugates were visualized using ChemiDoc XRS Plus Image Lab System (BIO-RAD, California, USA).

**Light scattering analysis**

Light scattering analysis was performed using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) at a detection angle of 173 °. The scattering light intensities of the solutions of siRNA and its polymer conjugates in 10 mM HEPES buffer (pH 7.3) (siRNA concentration at 5 µM, 30 µL) were measured at various temperatures from 20 to 40 °C. The results are presented as mean value of count rate in kilo counts per second (kcps) and standard deviation obtained from three times repetitions.

**Hydrodynamic diameter measured by fluorescence correlation spectroscopy (FCS)**

FCS analysis was performed using a LSM710 confocal laser scanning microscope (CLSM, Carl Zeiss, Oberlochen, Germany) equipped with a Confocor3 module and C-Aprochromat 40 × water immersion objective. An argon laser (514 nm) was used for excitation and a 560 – 600 nm band pass filter for emission. Solutions of TAMRA-labeled siRNA and its polymer conjugates in 10 mM HEPES buffer (pH 7.3) were put into an 8-well chamber (Nalge Nunc International, Rochester, NY, USA) for measurements (siRNA concentration was 100 nM). The measurements were performed for ten times repetitions at ambient temperature and 37 °C with a sampling time for 10 s. The diffusion coefficients of TAMRA-labeled siRNA and its polymer
conjugates were calculated according to the manufacture’s protocol and rhodamine6G as a reference, and the obtained diffusion coefficients were further converted into hydrodynamic diameter based on Stokes-Einstein equation.

**Endogenous luciferase gene silencing analysis**

Hela-Luc cells were seeded on a 96-well plate at a density of 2,500 cells per well in 100 µL of DMEM containing 10% FBS, followed by 24 h incubation at 37 °C. Lipoplexes of siRNA and its polymer conjugates were prepared with Lipofectamine RNAiMAX according to the manufacturer’s protocol, and were applied to each well at the final siRNA concentration of 0.1 nM, 1 nM, 5 nM, 10 nM, and 20 nM. After another incubation at 37 °C and 30 °C for 48 h, the cells were washed with 100 µL of PBS and lysed with 20 µL of cell culture lysis buffer (Promega, Fitchburg, WI, USA). Luminescence intensities of cell lysates were measured using Luciferase Assay System (Promega) and a luminometer (Glomax 96, Promega). The relative luminescence unit (RLU) value was calculated from the obtained luminescence intensity as a relative value to non-treated wells. The results are presented as mean and standard error of the mean obtained from six samples.

**Cellular uptake assay**

Hela-luc cells were seeded onto 12-well plates at a density of 50,000 cells per well in 1 mL of DMEM containing 10% FBS and incubated for 24 h. Next, the culture medium was replaced with fresh culture medium, and lipoplexes prepared from TAMRA-labeled siRNA and its polymer conjugates were applied to each well at the final siRNA concentration of 10 nM. The cells were further incubated at 37 °C and 30 °C for 24 and 48 hours. After the incubation, the cells were washed two times with PBS and collected by trypsinization. Then, the fluorescence intensity of TAMRA from the collected cells was measured by flow cytometric analysis using a Guava easyCyte 6-2L (Merck Millipore, Germany). The results are expressed as mean and standard deviation obtained from three samples.

**Cellular viability assay**

Cells were seeded at a density 2,500 cells per well in 96-well plates and incubated at 37 °C for 24 h. Lipoplexes of siRNA and its polymer conjugates were prepared in a
similar manner to that for gene silencing assay, and were applied to each well at the final siRNA concentration of 0.1 nM, 1 nM, 5 nM, and 10 nM. The cells were further incubated at 37 °C and 30 °C for 48 h. Next, 10 µL of Cell Counting Kit-8 (DOJINDO laboratories, Kumamoto, Japan) solution as following the manufacturer’s protocol. The cellular viability was analyzed by measuring absorbance at 450 nm in each well using a microplate reader (iMark, BIO-RAD). The cellular viability in each well was calculated from the obtained values as a percentage to untreated control wells. The results were expressed as mean and standard error of the mean obtained from six samples.

**Counting of Ago2-associated asRNA**

Hela-Luc cells were seeded on a cell culture flask at a density of 1,250,000 cells in 10 mL of DMEM containing 10% FBS, followed by incubation at 37 °C for 24 h. Lipoplexes prepared from TAMRA-labeled siRNA, or its polymer conjugates were applied to cells in a flask at the final siRNA concentration of 100 nM. After another incubation at 37 °C or 30 °C for 48 h, the culture medium was removed and the cells were rinsed twice with PBS, then, collected by trypsinization. The Ago2-associated TAMRA-labeled asRNA was extracted from the collected cells using MagCapture microRNA Isolation Kit (Wako) according to the manufacture’s protocol. The fluorescence intensity of the extracted RNA solution was measured by a spectrofluorometer (JASCO, Tokyo, Japan). The amount of collected TAMRA-labeled asRNA and populations of TAMRA-labeled asRNA per cell were calculated based on a standard curve of TAMRA-labeled asRNA. The results were presented as mean and standard error of the mean obtained from three samples.
Supporting Schemes

Supporting Scheme S1. Synthetic procedure of PNIPAAm-TE.

Supporting Scheme S2. Synthetic procedure of DBCO-PNIPAAm-TE.
Supporting Scheme S3. Synthetic procedure of PEG-DBCO.

Supporting Scheme S4. Synthetic procedure of PNIPAAm-siRNA.

Supporting Scheme S5. Synthetic procedure of PEG-siRNA.
Supporting Tables

Supporting Table S1. The hydrodynamic diameters (nm) of TAMRA-labeled unconjugated-siRNA, TAMRA-labeled PNIPAAm-siRNA, and TAMRA-labeled PEG-siRNA (100 nM siRNA, 10 mM HEPES pH 7.4), at ambient temperature or 37 °C, determined by FCS analysis. Results were shown as mean and standard deviation obtained from ten measurements.

<table>
<thead>
<tr>
<th></th>
<th>Unconjugated-siRNA</th>
<th>PNIPAAm-siRNA</th>
<th>PEG-siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>r. t.</td>
<td>4.14 ± 0.44</td>
<td>7.61 ± 0.23</td>
<td>8.83 ± 0.39</td>
</tr>
<tr>
<td>37 °C</td>
<td>4.10 ± 0.28</td>
<td>6.50 ± 0.21</td>
<td>8.83 ± 0.29</td>
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</tbody>
</table>

Supporting Table S2. The list of hydrodynamic diameters (nm) of TAMRA-labeled unconjugated-siRNA and TAMRA-labeled siRNA conjugated polymers with various molecular weights (100 nM siRNA, 10 mM HEPES pH 7.4), at ambient temperature, determined by FCS analysis. Results were shown as mean and standard deviation obtained from ten measurements.

<table>
<thead>
<tr>
<th>Hydrodynamic diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconjugated-siRNA</td>
</tr>
<tr>
<td>PNIPAAm-siRNA (10K)</td>
</tr>
<tr>
<td>PNIPAAm-siRNA (20K)</td>
</tr>
<tr>
<td>PNIPAAm-siRNA (40K)</td>
</tr>
<tr>
<td>PEG-siRNA (10K)</td>
</tr>
<tr>
<td>PEG-siRNA (20K)</td>
</tr>
<tr>
<td>PEG-siRNA (40K)</td>
</tr>
</tbody>
</table>
Supporting Table S3. The number of Ago2-associated asRNA per cell after the treatment with TAMRA-labeled unconjugated siRNA, TAMRA-labeled PNIPAAm-siRNA, and TAMRA-labeled PEG-siRNA, at 30 °C or 37 °C. The Ago2-associated asRNA was collected from the treated HeLa-Luc cells by MagCapture microRNA Isolation Kit according to manufacturer’s protocol. Results were shown as mean and standard error of the mean obtained from three samples.

<table>
<thead>
<tr>
<th></th>
<th>Unconjugated-siRNA</th>
<th>PNIPAAm-siRNA</th>
<th>PEG-siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 °C</td>
<td>190,000 ± 42,000</td>
<td>69,000 ± 10,000</td>
<td>11,000 ± 6,500</td>
</tr>
<tr>
<td>37 °C</td>
<td>340,000 ± 20,000</td>
<td>190,000 ± 13,000</td>
<td>7,000 ± 3,000</td>
</tr>
</tbody>
</table>

Supporting Figures

Supporting Figure S1. $^1$H NMR spectrum of PNIPAAM-CTA in MeOD.
Supporting Figure S2. $^1$H NMR spectrum of DBCO-PNIPAAm-TE in D$_2$O.

Supporting Figure S3. $^1$H NMR spectrum of PEG-DBCO in DMSO-d$_6$. 
**Supporting Figure S4.** Ion exchange chromatography chart of the reaction solution of DBCO-PNIPAAm-TE and azide-siRNA (UV detection at 260 nm).

**Supporting Figure S5.** Ion exchange chromatography chart of the reaction solution of PEG-DBCO and azide-siRNA (UV detection at 260 nm).
Supporting Figure S6. Agarose gel electrophoresis of unconjugated-siRNA, PNIPAAm-siRNA, and PEG-siRNA.

Supporting Figure S7. Gene silencing efficacies of unconjugated siRNA and its conjugates (molecular weight of the conjugated polymer: 10,000 g/mol) at 37 °C (a) and 30 °C (b) for HeLa-Luc cells. HeLa-Luc cells were treated with siRNA series using Lipofectamine RNAiMAX at indicated siRNA concentrations for 48 hours. Luciferase expressions were measured by luminometer. Results were shown as mean and standard error of the mean obtained from six samples.
Supporting Figure S8. Gene silencing efficacies of unconjugated siRNA and its conjugates (molecular weight of the conjugated polymer: 20,000 g/mol) at 37 °C (a) and 30 °C (b) for HeLa-Luc cells. HeLa-Luc cells were treated with siRNA series using Lipofectamine RNAiMAX at indicated siRNA concentrations for 48 hours. Luciferase expressions were measured by luminometer. Results were shown as mean and standard error of the mean obtained from six samples.

Supporting Figure S9. Cell viability after the treatment of HeLa-Luc cells with unconjugated siRNA or siRNA conjugates at 30 °C (a) or 37 °C (b). 48 hours after the treatment at the indicated siRNA concentration, the cell viability was measured by CCK-8 kit. Results were shown as mean and standard error of the mean obtained from six samples.
Supporting Figure S10. Cellular uptake efficacies of unconjugated siRNA or siRNA conjugates for HeLa-Luc cells. 24 hours or 48 hours after the treatment of TAMRA-labeled siRNA series (10 nM of siRNA concentration) at 30 °C (a) or 37 °C (b), fluorescence intensities from the cells were measured by flow cytometry. Results were shown as mean and standard deviation obtained from three samples.
References


