Development of Excipient-Free Freeze-Dryable Unimolecular Hyperstar Polymers for Efficient siRNA Silencing

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Experimental Part

1. Materials

1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA), $CuBr_2$, ethidium bromide (EtBr) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO) with the highest purity and used as received unless otherwise stated. Dulbecco's Modified Eagle's Medium (DMEM), endothelial cell growth medium (EGM), trypsin-EDTA, and Fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD). Phosphate-buffered saline (PBS) was purchased from Cambrex Bio Science (Walkersville, MD). Monomer N,N-(dimethylamino)ethyl methacrylate (DMAEMA,

≥ 98%) was purified by passing through the column filled with basic aluminum oxide (Sorbent Technologies) to remove inhibitor. The purified monomers were stored at 4 ^{o}C and used up within a month. CuBr (98%, Aldrich) was purified using a modified literature procedure,[1] namely stirred with glacial acetic acid and copper wire at 40 ^{o}C overnight followed by filtration and rinse with 2-propanol and diethyl ether. Other chemicals were used without any further purification.

Polymerization of inimers using activators generated by electron transfer (AGET) atom transfer radical polymerization (ATRP) in microemulsion

A typical polymerization of AB* inimer consists of $[AB^*]_0/[CuBr_2]_0/[dNbpy]_0/[\text{sodium ascorbate}]_0 = 70/1/2/0.5$. In a disposable test tube dNbpy and $CuBr_2$ are complexed in 0.5 mL of DCM at 40 ^{o}C for 0.5 hour. Inimer is then added to the complexed mixture at room temperature before evaporating the DCM. The mixture is added dropwise to a water/Brij 98, 12g/1g solution over 30 minutes making sure the solution does not become hazy. During this time the temperature is slowly stepped up to 65 ^{o}C, do not leave at high temperature for more than 10 minutes before initiating. The mixture is then initiated with a sodium ascorbate/water solution. The reaction is stopped by exposure to air after one hour and the product was isolated by addition of THF into microemulsion, followed by centrifugation. The isolated polymer was further purified by dissolved in THF and then precipitated out in methanol to remove the surfactant. This hyperbranched polymer containing degradable ester linker groups and ca. 5,270 ATRP bromine initiating sites, characterized according to recent literature.[2] This hyperbranched polymer was used as a multifunctional macroinitiator (MI) for surface

initiated ATRP of an amine-containing functional monomer to produce a hyperstar polymer containing ester groups in the hyperbranched core and tertiary amine groups on the shell.

Source and information of modified and unmodified siRNAs.

All siRNAs used in these studies were chemically synthesized using silyl ethers to protect 5′-hydroxyls and acid-labile orthoesters to protect 2′-hydroxyls with 2′-ACE (2′-bis(acetoxyethoxy)-methyl ether) (Dharmacon, Lafayette, CO). After deprotection and purification, siRNA strands were annealed as described.[3] The unmodified SOD1 siRNA (U1) sequences (targeting open reading frame positions 288–308) are: sense 5′-CGAUGUGUCUAUUGAAGAUUC-3′, antisense 5′-AUCUUCAAUAGACACAUCGGC -3′. The chemically modified siRNA (R1) sequences are: sense 5′--$C^{S}G^{S}A^{S}U^{F}GU^{F}$GUCUAU UGAAG$^{S}A^{S}U^{FS}U^{FS}$C-3′, antisense 5′-$^{P}AU^{F}C^{F}U^{F}$UCAAUAGACAC$^{F}A^{S}U^{FS}C^{FS}G^{S}G^{S}$C. The superscript letters F and S represent 2′-O-F and HS-backbone modifications, respectively. P is a phosphate group. RNA labeling with various functional groups, purification, and characterization were accomplished according to established methods in our laboratory.[4,5]

2. Synthesis of cationic “hyperstar” polymers (HSP)

Typical procedures by using hyperbranched polymer **HB1** as MI are briefly described. The theoretical number of initiating sites per **HB1** polymer was assumed to be equal to the average number of inimer units (~ 5,270). A clean and dry 100 ml Schlenk flask was charged with 300 mg **HB1** (1.1 mmol Br), DMAEMA (25 ml, 0.15 mol), HMTETA (0.29 ml, 1.1 mmol), acetone (20.0 ml) and a stir bar. The flask was deoxygenated by five

freeze-pump-thaw cycles. During the final cycle, the flask was filled with nitrogen before CuBr (140 mg, 0.97 mmol) and $CuBr_2$ (24 mg, 0.11 mmol) were quickly added to the frozen mixture. The flask was sealed with a glass stopper then evacuated and back-filled with nitrogen five times before it was immersed in a water bath at 40 ^{o}C. Samples were withdrawn periodically for NMR measurement of monomer conversions and size exclusion chromatography (SEC) measurement of polymer molecular weights. The reaction was stopped after 6 hours at 27% DMAEMA conversion via exposure to air and dilution with acetone. The solution was filtered through a column filled with neutral alumina to remove the copper complex before the polymer was purified via dialysis against DI water (pH = 6.7) using Spectrum dialysis tubing (COMW 8000). The purified polymer was recovered as white solid powder (yield ~ 6 g) after lyophilizing to remove water (Figure S1).

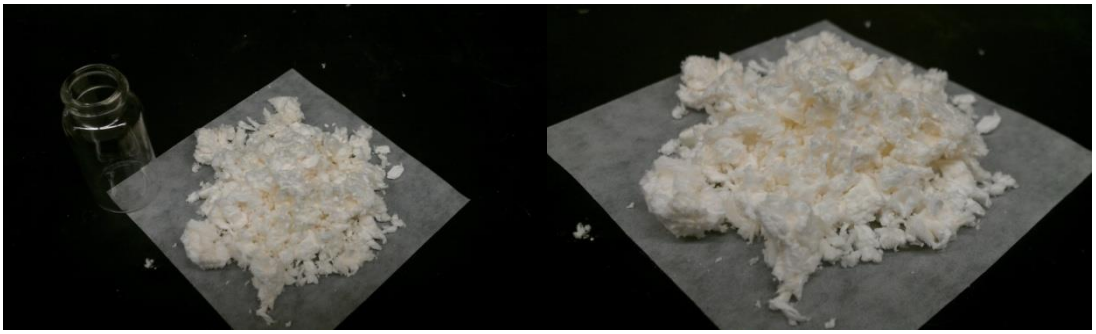


Figure S1. Digital pictures of the purified HSP after lyophilization.

3. Characterizations

Monomer conversions were determined from the concentration of unreacted monomers in the samples periodically removed from the reactions using a Bruker 400 MHz NMR spectrometer at 27 ^{o}C. After filtration through 0.45 μm filter, the polymer samples were separated by SEC with DMF as eluent. The DMF SEC used Polymer Standards Services

(PSS) columns (guard, 10^4, 10^3, and 10^2 Å GRAM 10 columns) at 55 ^oC with a flow rate = 1.00 ml/min and connected with a differential refractive index (RI) detector (Waters, 2410) using PSS WinGPC 7.5 software. The apparent molecular weights were calculated based on linear poly(methyl methacrylate) (PMMA) standards. The size and zeta potential distribution of the polymers and HSP/siRNA complexes were determined by dynamic light scattering (DLS) equipped with a Zetasizer Nano-ZS (He-Ne laser wavelength at 633nm) and an auto-titrator (Malvern Instruments, Malvern, UK). In particular, about 1 mL of polyplex solutions at various N/P ratios were prepared to a final concentration of 5 mg/mL plasmid DNA in 10% PBS (10 mM Hepes, 1 mM NaCl, pH 7.4) for the measurement of the zeta potentials and the hydrodynamic diameters.

Preparation of HSP/siRNA complexes

The HSP/siRNA complexes solution was prepared with 100 pmol of siRNA and polymers at different N/P ratios in 500 μl of Opti-MEM or other media noted and incubated for 30 min at room temperature.

5. Cell lines and culture

Human cervical cancer cells (HeLa) was grown in DMEM with 10% FBS at 37 °C in a 5% CO_2 humidified incubator. A plastic tissue culture CellBIND® flask (Corning) was used for Human Umbilical Vein Endothelial Cells (HUVECs)and a simple plastic tissue culture dish (Falcon) was used for other cell lines.

6. Cytotoxicity assay

To measure the cytotoxicity of the free polymers, HB (Ester), MTT assayswere performed.

Hela cells were seeded in 96-well CellBIND®plates at 15000 cells/well in 100μl DMEM media. All media contained 10% FBS and incubated overnight. After washing the cells with PBS, 100 μl of culture medium with various concentrations of HB was added to the wells in triplicate. For control wells, the same volume of pure culture medium was used. After 24 h of incubation, cell survival rate was measured by MTT cell proliferation assay. We added 15μlof filtered MTT solution (5mg/mL in PBS) to each well. After incubation at 37 °C for 4 h, the medium was removed from the wells and 100 μls top solution was added to dissolve any insoluble formazan crystals and incubated overnight at room temperature. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA) and the cell viability was calculated as a percentage of the untreated control cells.

7. Cell transfection

Cells were seeded at 250,000 cells/well in 6-well plates in 2 mL of DMEM containing 10% FBS and incubated at 37 °C for one day. Before transfection, the medium was exchanged with 1.5 mL of Opti-MEM. The polyplex solution was prepared with 100 pmol of siRNA and polymers at different N/P ratios in 500μl of Opti-MEM and incubated for 30 min at room temperature. After 4 h of polyplex treatment, the Hela cell medium was replaced with 2 mL of fresh DMEM medium containing 10% FBS and the cells were further incubated for 72 h. Next, the growth medium was removed and the cells were washed with PBS one time and lysed for 20 min atroom temperature with 500μl of M-PER (containing protease inhibitor) lysis buffer (Promega, Madison, WI). The protein contents were measured using a Micro BCA assay reagent kit (Pierce, Rockford, IL).

8. Western blotting to determine target knockdown

Total protein was extracted by adding M-per lysis buffer (with protease inhibitor, Promega, Madison, WI) to cells. Protein concentration was determined by BCA ProteinAssay Kit (Pierce, Thermo Scientific) based on the manufacturer's instructions. To detect target protein, 35 μg total protein were separated on 15% SDS-polyacrylamide gels (Bio-Rad) and wet transferred to a Protran® (Whatman GmbH) nitrocellulose transfer membrane. The membrane was cut in half between 25 kD and 37 kD. The membrane below 25 kD was probed with sheep anti-SOD1 primary antibody and rabbit anti-sheep IgG secondary antibody. The upper half of the membrane was probed with anti-alpha Tubulin primary antibody and goat anti-mouse secondary antibody. The protein bands were visualized using Amersham ECL kit and image of the blot was taken with Fuji LAS 4000 Imager (GE Healthcare). Further analysis using ImageJ software was used to quantify band intensities. Each lane can be the measured independently for the intensity of the signal. To analyze protein knock down, a ratio was first calculated between the protein of interest (SOD1) and the control protein (α-tubulin) in each lane. Then the extent of SOD1 knockdown % for each experiment is normalized to the control group (without treatments) .

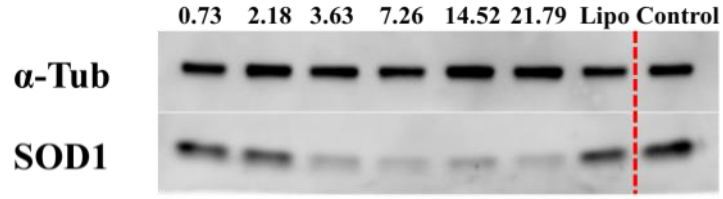
9. Lyophilization experiments

The polyplex solution was prepared with 100 pmol of siRNA and polymers at N/P ratios of 21.79 in 500 μl of Opti-MEM and incubated for 30 min at room temperature. The polyplex solution was frozen inliquid nitrogen for 30 min and then lyophilized for 24 h by using lyophilizer (LABCONCO Free Zone Plus 4.5), and then the powder polyplex was dissolved

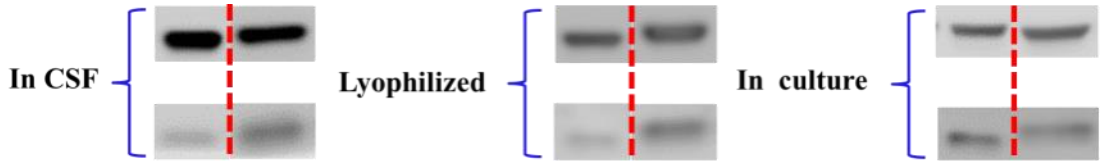
in the 1 ml Opti-MEM to do transfection.

10. Western blots for α-tublin and SOD1

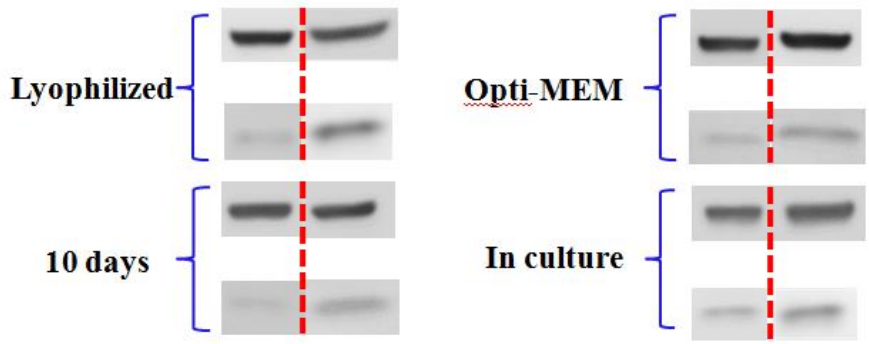
Western blots for α-tublin and SOD1 of HSP/siRNA complexes at different N/P ratios. Lipofectamine2000/siRNA complex was used as the positive control (Lipo) and cells without treatment was used as the negative control (Control). The HSP/siRNA was added to cells at 0.5, 1.5, 2.5, 5, 10 and 15 μg mL^{-1} with N/P molar ratios of 0.73, 2.18, 3.63, 7.26, 14.52 and 21.79, respectively.



Western blots for α-tublin and SOD1 of HSP/siRNA complexes using modified siRNA under different conditions (in CSF, lyophilized, and in a culture medium containing a 10% FBS) at an N/P molar ratio of 21.79. The top and bottom are α-Tub and SOD1, respectively. The left and right of the red dashed are experiment and control, respectively.



Western blots for α-tublin and SOD1 of HSP/siRNA complexes using unmodified siRNA after different treatments (lyophilized, 10 days at 37°C, and in culture) at an N/P molar ratio of 21.79. The top and bottom are α-Tub and SOD1, respectively. The left and right of the red dashed are experiment and control, respectively.



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