Electronic Supplementary Material (ESI) for Nanoscale.

PSMA-Specific Degradable Dextran for Multiplexed Immunotargeted siRNA Therapeutics against Prostate Cancer

Zhihang Chen, Balaji Krishnamachary, Yelena Mironchik, Sangeeta Ray Banerjee, Martin G. Pomper, Zaver M. Bhujwalla

The Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine

Materials and General Experimental Methods

All organic chemicals and solvents of analytical grade were obtained from Aldrich (St. Louis, MO), Sigma (Milwaukee, WI) and Alfa Aesar (Ward Hill, MA) unless otherwise specified. Fetal bovine serum, penicillin, streptomycin were from Invitrogen (Carlsbad, CA). Amicon ultra-15 centrifugal filter tubes (10,000 MW cutoff) were from Millipore (Bedford, MA). The PC3-PIP and PC3-Flu human prostate cancer cells were kindly provided by Dr. M. G. Pomper. PD-L1 and CD46 siRNA were synthesized by Dharmacon (Lafayette, CO). ^{1}H, ^{13}C NMR spectra were recorded on a Bruker Avance III 500 MHz NMR spectrometer (Bruker BioSpin Corporation, Billerica, MA), and chemical shifts were reported in ppm relative to tetramethylsilane.

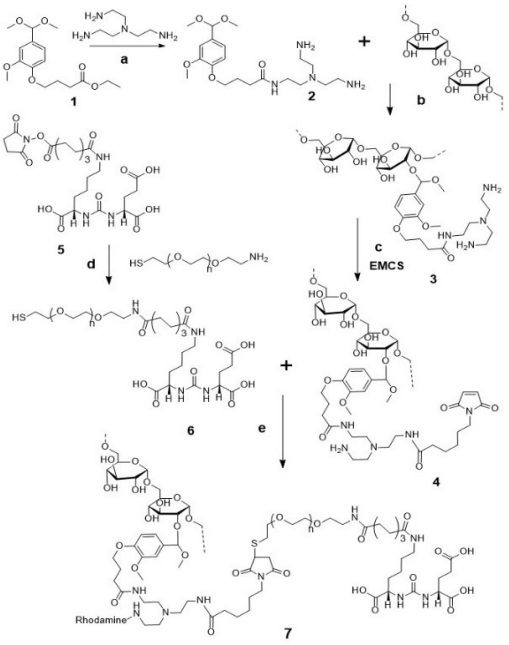

Figure S1. Synthesis procedure

Compound 2: Ethyl 4-(4-formyl-3-methoxy)-phenyl butyrate dimethyl acetal [1] 400 mg was mixed with tris(2-aminoethyl)amine 1.5 ml. This mixture was stirred at 100 ºC under nitrogen atmosphere for 24 h. After reaction, tris(2-aminoethyl)amine was removed by distilling at 100 ºC under high vacuum. Yellow oil raw product was further purified by basic

aluminum oxide column chromatography (elution: CH_2Cl_2/ N $(CH_3CH_2)_3$ = 95/5). ^{1}H NMR (500 MHz, CDCl3): δ 6.99−6.95 (m, 2H, Ar), 6.86 (d, 1H, J = 8.3 Hz, Ar*H*), 5.31 (s, 1H, C*H*$(OCH_3)_2$), 4.05 (t, 2H, J = 7.6 Hz, OCH_2), 3.87 (s, 3H, ArOC*H*$_3$), 3.32 (s, 6H, CH(OC*H*$_3)_2$), 3.28 (t, 2H, J = 7.4 CONHC*H*$_2$CH$_2$), 2.72 (t, 4H, J = 5.9, N(CH_2C*H*$_2$NH$_2)_2$), 2.55 (t, 2H, J = 6.0, CONHCH$_2$C*H*$_2$), 2.52 (t, 4H, J = 6.1, N(C*H*$_2$CH$_2$NH$_2)_2$), 2.41 (t, 2H, J = 7.2 Hz, C*H*$_2$CO), 2.14 (dddd, 2H, J = 7.2, 7.2, 6.4, 6.4 Hz, CH_2C*H*$_2$CH$_2$). ^{13}C NMR (500 MHz, $CDCl_3$): δ 172.56, 149.17, 148.39, 131.00, 119.39, 112.42, 109.92, 103.25, 67.94, 57.70, 57.26, 55.92, 53.19, 52.83, 39.89, 38.25, 32.85, 25.44. MALDI HRMS calculated for C20H36N4NaO5 (M + Na): 435.2578, found: 435.25861

Compound 3: 30 mg of dextran (40 kDa) was dissolved in 2 ml anhydrous DMSO containing 2 g 4Å active molecular sieves. Then p-toluenesulfonic acid monohydrate (270 mg, 4 *eq* of compound **2**) was added to this mixture. This mixture was stirred at room temperature under nitrogen atmosphere for 4 h to remove water in the solution. Meanwhile 150 mg compound **2** (2 *eq* of glucose unit) was dissolved in anhydrous DMSO (1 mL) with 1 g 4Å active molecular sieves, and the solution was stirred at room temperature under nitrogen atmosphere for 4 h. Then compound **2** solution was transferred into dextran solution containing a 4Å active molecular sieve, and this mixture was stirred at 65 ^{o}C under nitrogen atmosphere for 16 h. This reaction was stopped by adding 100 µl triethylamine, and this DMSO solution was dropped into ethyl acetate to precipitate the light-yellow product. The suspension was centrifuged to obtain a solid precipitate that was purified thrice by precipitating in ethyl acetate. After drying, 55.3 mg product was obtained. ^{1}H NMR (500 MHz, D2O) δ 7.15−6.9 (m, 0.5 × 3H, Ar), 5.32 (m, 0.51 × 1H, acetal-H), 4.92 (m, 1H, glucose-H_1), 4.37−3.32 (m, 6H, glucose-H_{2-6}; 0.5 × 2H, ArOC*H*$_2$; 0.5 × 3H, ArOC*H*$_3$; 0.5 × 2H, C*H*$_2$NHCO; 0.5 × 3H, acetal-OC*H*$_3$), 2.41-2.83 (br. app. s, 0.5 × 4H, NHCH$_2$C*H*$_2$NH2; 0.5 × 4H, NHC*H*$_2$CH$_2$NH2; 0.5 × 2H,

CONHCH$_2$C*H*$_2$NH,), 2.35 (br. app. s, 0.5 × 2H, C*H*$_2$CO), 2.05 (br. app. s, 0.5 × 2H, ArOCH$_2$C*H*$_2$).

Compound 4: 50 mg of compound 3 was dissolved in 2.0 ml PBS buffer (pH 7.4), then 100 µl DMSO containing 3 mg EMCS (N-ε-malemidocaproyl-oxysuccinimide ester) was added to this buffer. After stirring at room temperature for 2 h, this dextran product was purified by centrifugal filter tubes (Amicon ultra-15, 10,000 MW cutoff). After repeating this purification twice and lyophilization, 49.1 mg solid was harvested. This product was applied to the next reaction without further purification and characterization.

Compound 6: 20mg SH-PEG-NH$_2$ was dissolved in 1 mL PBS buffer (pH 7.4), then 100 µl DMSO containing 6 mg compound 5 was added to this buffer. After stirring at room temperature for 2 h, this product was applied to the next reaction without further purification and characterization.

Compound 7: 45 mg of compound 4 was dissolved in 2.0 ml PBS buffer (pH 7.4), then mixed with the reaction solution of compound 6 from the previous step. After stirring at room temperature for 2 h, 100 µl DMSO containing 1 mg rhodamine 6G NHS ester was added to this reaction solution. After stirring at room temperature for 2 h, this dextran product was purified by centrifugal filter tubes (Amicon ultra-15, 10,000 MW cutoff). After repeating purification 2 more times and lyophilization, 64.1 mg purple solid was harvested. Colorimetric assay of rhodamine 6G at 530 nm indicated that there were 1.2 rhodamine molecules in one dextran molecule. ^{1}H NMR (500 MHz, D2O) δ 7.15−6.9 (m, 0.5 × 3H, Ar), 5.32 (m, 0.51 × 1H, acetal-H), 4.92 (m, 1H, glucose-H$_1$), 4.37−3.32 (m, 12H, glucose-H$_{2-6}$; 0.5 × 2H, ArOC*H*$_2$; 0.5 × 3H, ArOC*H*$_3$; 6H, PEG-CH$_2$; 0.5 × 2H, C*H*$_2$NHCO; 0.5 × 3H, acetal-OC*H*$_3$), 2.41-2.83 (br. app. s, 0.5 × 4H, NHCH$_2$C*H*$_2$NH2; 0.5 × 4H, NHC*H*$_2$CH$_2$NH2;

0.5 × 2H, $CONHCH_2CH_2NH$,), 2.35 (br. app. s, 0.5 × 2H, CH_2CO), 2.05 (br. app. s, 0.5 × 2H, $ArOCH_2CH_2$). Signals from protons in the PSMA targeting moieties and rhodamine were too weak to be detected. Calculations indicated approximately 8.3 PEG molecules per dextran molecule.

Determination of size distribution and zeta potential of siRNA/compound 7 nanoplex

The hydrodynamic radius and size distribution of siRNA/compound 7 were determined by dynamic light scattering. Dextran-siRNA nanoplex for size distribution measurement was prepared at N/P ratios of 15 by adding compound 7 in 600 µl PBS pH=7.4 buffer to a solution of siRNA (400 µL, in DI water, 50 µg/ml), followed by vortexing for 10 s and incubating for 20 min at room temperature. The DLS measurements were performed in triplicate. Dextran-siRNA nanoplex (N/P = 15) for measurement of zeta potential was prepared by adding compound 7 in 600 µL DI water to a solution of siRNA (400 µL, in DI waters, 50 µg/mL), followed by vortexing for 10 s and incubating for 10 min at room temperature. The average zeta potential of natural dextran alone, compound 4 and dextran-siRNA nanoplex (N/P = 15) in DI water solution were measured with a Zetasizer Nano ZS instrument (Malvern) equipped with a clear standard zeta capillary electrophoresis cell cuvette from 20 acquisitions with a concentration of approximately 0.5 mg/mL. The zeta potential measurement of dextran-siRNA nanoplex was performed immediately after the complex was produced. The measurements were performed in triplicate.

Rhodamine absorbance at pH 5.5 and pH 7.4 buffer at different time points.

Compound 7 (2 mg) was dissolved in 2 ml buffer (pH 5.5 or pH 7.4) and incubated for the required time. After incubation, the free cleaved amine molecules with rhodamine were

removed by molecular weight cutoff centrifugation (Amicon ultra-15, 10,000 MW cutoff). Four further centrifugations in DI water (containing trace amount of triethylamine to prevent the further degradation) were performed to remove free rhodamine completely. After lyophilization, compound 7 was weighed and prepared as a 1mg/mL solution for absorbance measurements.

The Stability of Encapsulated siRNA in Fetal Bovine Serum (FBS)

A nuclease stability assay was conducted to determine the stability of siRNA encapsulated in the siRNA nanoplex. The nanoplex was incubated for 2, 8 and 24 h in 70% fresh serum at 37 °C. At the end of incubation, all encapsulated siRNA was displaced from the nanoplex by adding 4% SDS and loaded to wells (1 μg/well siRNA) containing 4% agarose gel.

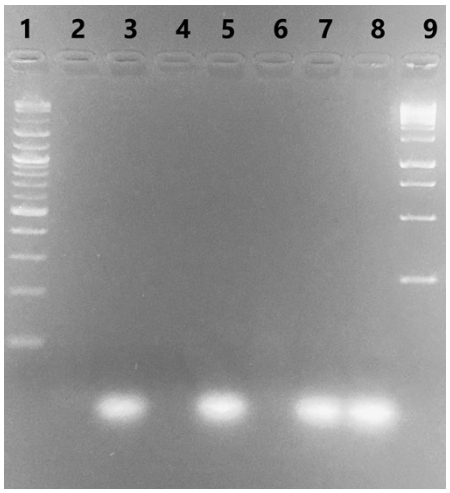

Figure S2: Stability of encapsulated siRNA in FBS. (Lane1 and 9: marker; lane 2: siRNA alone treated with FBS for 24 h; lane 3: siRNA nanoplex treated with FBS for 24 h; lane 4: siRNA alone treated with FBS for 8 h; lane 5: siRNA nanoplex treated with FBS for 8 h; lane 6: siRNA alone treated with FBS for 2 h; lane 7: siRNA nanoplex treated with FBS for 2 h; lane 8: siRNA control).

Cell culture

Human prostate cancer PC3-PIP and PC3-Flu cells were kindly provided by Dr. M. G. Pomper. Fetal bovine serum, penicillin, and streptomycin were from Invitrogen (Carlsbad, CA). Cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum in a humidified incubator at 37°C/5% CO_2. Cells were seeded at a density of 400,000 cells per dish in a 6 cm dish (for RT-PCR experiments) or at 20,000 cells per well in a 4-well slide chamber (for confocal laser scanning fluorescence microscopy studies) 24 h prior to the transfection experiment.

Imaging studies of compound 7/siRNA nanoplex cell uptake

PC3-PIP and PC3-Flu cells on the glass slide of a 4-well slide chamber were incubated for 24 h. For ZJ-43 treatment, 100 μg/mL ZJ-43 was added to the medium for 1 h incubation. Then compound 7/siRNA nanoplex (concentration of compound 7: 1 μg/ml; N/P = 15) was added for 2 h incubation. After incubation, the transfection mixture was removed, and cells were washed twice with fresh medium. Fluorescence microscopy images of live cells were generated on a Zeiss LSM 700 META confocal laser-scanning microscope (Carl Zeiss, Inc. Oberkochen, Germany).

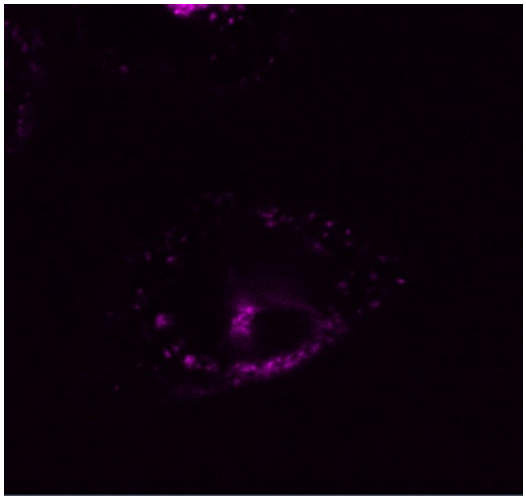

Figure S3: Localization of nanoplex in PC3-PIP cells identified in a magnified microscopy image obtained × 40.

Cell viability

Concentration dependent cytotoxicity of PSMA-dextran alone and PSMA-dextran with non-coded siRNA in PC3-PIP cells after 72 h incubation as measured by the CCK8 assay.

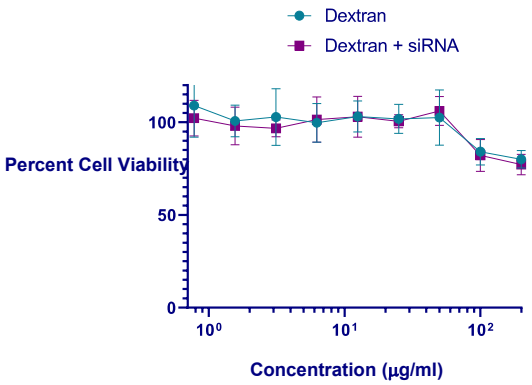

Figure S4: Percent cell viability following treatment in PC3-PIP cancer cells. Values represent Mean ± SD, n = 6.

Quantification of relative fluorescence intensity of dextran in treated cells

Cells in 6 cm dishes were treated with siRNA/compound 7 nanoplex under different conditions. After incubation, cells were harvested, and washed for quantification of fluorescence intensity. Quantification of fluorescence intensity of dextran uptake was performed by Aurora flow cytometry (Cytek, Bethesda, MD).

RNA isolation, quantitative reverse transcription-PCR (qRT-PCR) and immunoblot analysis.

RNA was isolated following a standard protocol. Briefly, the PC3 -PIP and PC3-FLU cells grown in 60 mm petri dish (0.35 x10^6 cells density per dish) were transfected with siRNA against either PD-L1 or CD46. Twenty-four hours post transfection, cells in the petri dish were washed with PBS and 350µl of RLT buffer was added to lyse the cells. Cells were scraped and later passed through a QIAshredder. Later, lysed cells collected in a collection tube were further processed for isolating RNA using RNeasy mini kit column (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1000 ng of RNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed using Syber green (Bio-Rad) and gene specific primers. The expression of target RNA relative to the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) was calculated based on the threshold cycle (Ct) as $R = 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{HPRT1}$ and $\Delta(\Delta Ct) = \Delta Ct_{transfected} - \Delta Ct_{control}$. Flow cytometry was also applied to evaluate PD-L1 and CD46 expression in cells.

For immunoblot analysis, protein was isolated from cells using RIPA (Radio Immuno Precipitation Assay) buffer fortified with various protease and phosphatases inhibitors following standard protocol. Total protein (80 µg for PD-L1 and 40µg for CD46) was resolved on a polyacrylamide gel (PAGE) and transferred to a nitrocellulose membrane overnight at 4^0C. The membrane was then probed for PD-L1 using Rabbit polyclonal antibody against human PD-L1 (GeneTex, Irvine, CA, Catalog number- GTX104763, 1: 1000 dilution), and CD46 using a rabbit monoclonal antibody against CD46 (Cell Signaling Technology, Danvers, MA, Catalog number-13241, Clone D6N7H, 1: 1000 dilution). As a loading control, monoclonal antibody against GAPDH (SIGMA-ALDRICH, St. Louis, MO, Catalog number-G8795, 1:50,000 dilution) was also used to probe these immunoblots. Primary antibody was washed and appropriate secondary antibodies conjugated with HRP

(GE Healthcare, Piscataway, NJ) were added for 1 h at room temperature. After washing the secondary antibody, signal was detected using SuperSignal ™ West pico Plus chemiluminescence substrate (Thermo Scientific, Rockford, IL).

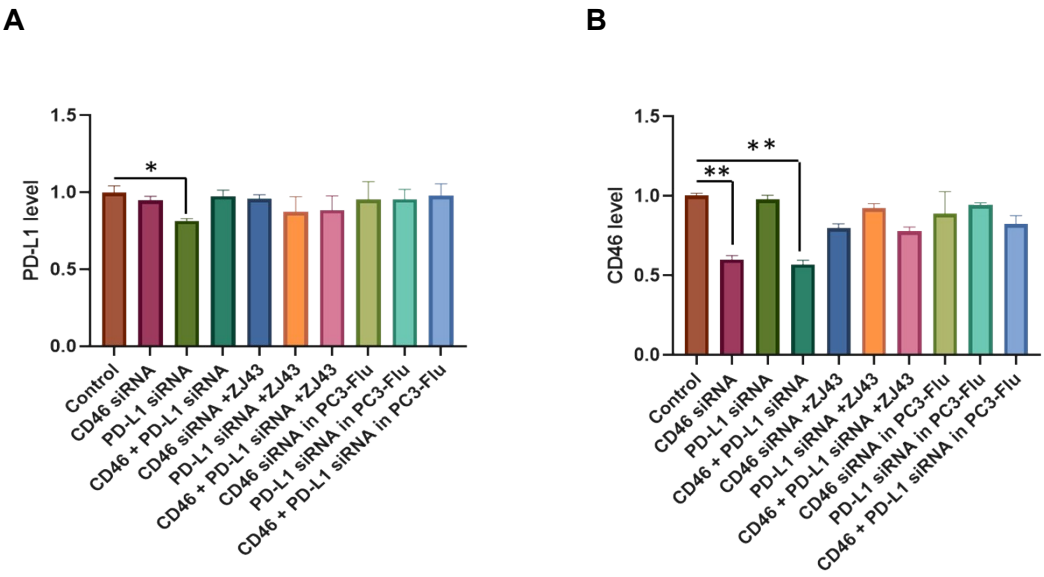

Figure S5: (A) PD-L1 and **(B)** CD46 levels evaluated by flow cytometry in PC3-PIP (PSMA positive) and PC3-Flu (PSMA-negative) cells following different siRNA treatments. siRNA concentration: 100 nM; ZJ43 concentration: 100µg/mL; cells were treated with siRNA-dextran for 24 h, PD-L1 and CD46 levels were normalized to untreated cells. *P < 0.05, **P < 0.005, n = 3.

siRNA sequence: siRNA sequences used in the study are listed here. Underlined nucleotides correspond to the overhang.

Gene Name	Gene ID	siRNA Sequence	Target sequence	Target position
Human CD46	NM_172351	CUAUGGAGCUCAUUGGUAAUU	CTATGGAGCTCATTGGTAA	Nucleotides 287- 305
Human CD46	NM_172351	AGAUAUCAGGAUUUGGAAAUU	AGATATCAGGATTTGGAAA	Nucleotides 872- 890
Human CD46	NM_172351	GGAAGGAAUACUUGACAGUUU	GGAAGGAATACTTGACAGT	Nucleotides 1074- 1092
Human CD274	NM_014143.4	CCUACUGGCAUUUGCUGAACGCAUU	CCTACTGGCATTTGCTGAACGCATT	Nucleotides 101- 125