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Supporting Information

Naturally derived dextran-peptide vector for microRNA antagomir delivery

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1. Materials and Methods

1.1 Materials

Dextran (Dex10, Mw 9-11 kDa), glycidyl methacrylate (GMA), 4-(Dimethylamino)pyridine (DMAP), dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Chem-Impex International (Wood Dale, IL, USA). The peptide, NH_2-RRRRRHHHHHC-COOH (R5H5) was designed by us and synthesized at >95% purity by GenScript (Piscataway, NJ, USA). UltraPure™ Agarose-1000, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, non-essential amino acids, sodium pyruvate, GlutaMAX™ and Trypsin-EDTA, Lipofectamine® RNAiMAX reagent, nuclease-free water, ProLong® Gold Antifade Reagent, mirVana™ miRNA isoloation kit, TaqMan® microRNA reverse transcription kit, TaqMan® universal PCR master mix and Hoechst 433342 nucleic acid stain were all purchased from Life Technologies (Carlsbad, CA, USA). Label IT® siRNA Tracker Intracellular Localization kit (TM-Rhodamine) was purchased from Mirus (MirusBio, WI, USA). Antagomir-149 (mGmGmGmAmGmUmGmAmAmGmAmCmAmCmGmGmAmGmCmCmAmGmA) and the negative control (miRNA NC) (mAmAmUmGmCmUmCmGmUmAmUmCmGmUmGmAmUmAmCmGmUmGmC) were synthesized by Integrated DNA Technologies (Coralville, IA). Both Antagomir and miRNA NC are 2'O-Methyl RNA. The primers (Taqman® Assays) for miRNA-149, miRNA-NT and U6 snRNA were ordered from Life Technologies (Carlsbad, CA, USA). HepG2 cell line was purchased from ATCC (St. Cloud, MN, USA) and cultured in DMEM medium supplemented with 10% fetal bovine serum, non-essential amino acids, sodium pyruvate, GlutaMAX™ and penicillin-streptomycin at 37 ºC in a humidified atmosphere of 5% CO_2.

1.2 Preparation of dextran-peptide hybrid (Dex10-R5H5(40%))

The synthesis procedure was the same as the procedure in our previous work.[1] Firstly, a published procedure[2] was followed to synthesize methacrylate functionalized dextran (Dex-MA). Briefly, 500 mg of dextran was dissolved in 8 mL of DMSO in a stoppered round bottom flask (25 mL) under nitrogen atmosphere. Then 450 mg of DMAP was dissolved, followed by adding a calculated amount of GMA. The solution was stirred for 4 days at room temperature, and then an equimolar amount of concentrated HCl was added to neutralize DMAP and thus stop the reaction. The reaction mixture was dialyzed against deionized (DI) water at 4 ^{o}C for 4 days, and then lyophilized into white powder. Secondly, peptide R5H5 was conjugated to Dex-MA in DI water. Briefly, 25 mg of R5H5 was dissolved in 50 μL of DI water and 19.7 μL of TCEP (200mM) was added to reduce disulfide bonds that may form between two peptides. The mixture was incubated for 5 minutes at room temperature, followed by the addition of 1M NaOH (25 μL) to neutralize TCEP and trifluoroacetic acid (TFA) from peptide synthesis. In a separate 1.5 mL tube, a calculated amount of Dex-MA was added and then dissolved with DI water that was 3-fold weight of Dex-MA, followed by a sonication for 5 minutes to allow Dex-R5H5 to be dissolved completely. The Dex-MA solution was then added into 75 μL of the R5H5 peptide solution (33wt%) in water. Then 1M NaOH was used to adjust the pH of the mixture to 8.0. The mixture was incubated for 48 hours at room temperature under gentle shake. The final product, Dex10-R5H5(40%), was purified with a Zeba Spin Desalting Column (Thermo Fisher Scientific Inc., Rockford, IL, USA), and lyophilized into powder form and analyzed by ^{1}H-NMR. The degree of substitution (DS) of R5H5, which is defined as the number of R5H5 peptide chain per 100 dextran glucopyranose residues, was calculated by both the integration of the anomeric protons of dextran (5.0 ppm), and the integration of the protons from imidazole ring of histidine (6.9 and 7.7 ppm)

1.3 Preparation of Dex10-R5H5(40%)/miRNA complexes

Dex10-R5H5(40%) was dissolved in sterile DI water to make a stock solution at 1 mg/mL and further diluted to PBS to various concentrations, which depend on how much Dex10-R5H5(40%) will be needed to form complexes at the desired N/P ratios. The miRNA stock solution was prepared in nuclease-free

water at the final concentration of 100 μM and then diluted to 1 μM in PBS. The Dex10-R5H5(40%)/miRNA complexes at various N/P ratios were formed by mixing equal volume of Dex-R5H5 at different concentrations and miRNA solutions at the concentration of 1 μM, and then incubated for 20 minutes at room temperature to allow a complete electrostatic interaction between peptide and nucleic acid molecules. Here, the N/P ratio is a molar ratio of arginine residue (N) in the peptide to phosphate group (P) in the miRNA molecule.

1.4 Size and zeta-potential measurements

Dex10-R5H5(40%)/antagomir-149 complexes were prepared as described above at different N/P ratios. The size and zeta-potential of the Dex10-R5H5(40%)/antagomir-149 complexes were determined by a Malvern Nano-ZS Zetasizer (UK). The size measurements were performed in disposable sizing cuvettes at a laser wavelength of 633 nm and a scattering angle of 173 °, and the zeta-potential measurements were performed in disposable zeta-potential cells. Before each measurement, Dex-R5H5 complex solution was diluted 10 times with PBS. Each sample was measured for 3 times at 25 ^{o}C.

1.5 Gel retardation assay

Dex10-R5H5(40%)/antagomir-149 complex solutions were prepared at N/P ratios from 0 to 10. An aliquot (20 pmol miRNA) of each solution was mixed with loading dye and loaded onto a 4 % agarose gel. The loaded gel was exposed to 120 V for 40 min in 0.5 × TBE buffer. Then the gel was stained in ethidium bromide (0.5 μg/mL) for 30 minutes and destained with water for 15 minutes. The gel was visualized and documented with a UVP BioDoc-It imaging system (Upland, CA, USA).

1.6 Intracellular localization of Dex10-R5H5(40%)/antagomir-149 complexes

Intracellular localization of Dex10-R5H5(40%)/antagomir-149 complexes in HepG2 cells was studied by an Olympus FLUOVIEW FV1000 confocal laser scanning microscopy (CLSM) (Japan). Antagomir-149 was firstly labeled with TM-Rhodamine following a standard staining procedure as described in the

manual of Label IT® siRNA Tracker Intracellular Localization kit. HepG2 cells were seeded on a Nalgene Lab-Tek™ II 8-well chamber slide (Waltham, MA, USA) at a density of 20, 000 cells/well and cultured for 24 hours. Then the medium was replaced by 180 μL of fresh medium, and 20 μL of the complex solutions containing 20 pmol Rhodamine-labeled antagomir-149 was subsequently added into each well. The complex solution was Dex-R5H5 or RNAiMAX complexed with antagomir-149. Dex-R5H5/antagomir-149 complexes were formed at the N/P ratios of 1. 2 and 4, while RNAiMAX/ antagomir-149 complexes were formed according to manufacturer's instruction. Then the medium was removed and cells were washed with cold PBS three times after 6-hour incubation. 3.7% paraformaldehyde was applied to cells for 30 minutes to fix cells. Then the cells were washed with PBS three times again, and incubated in 100 μL of Hoechst 33342 at 2 μg/mL for 30 minutes. For microscopy imaging, the chamber slide was mounted with Prolong Gold Antifade solution and covered with a glass slip. The slide was then examined by CLSM with excitation wavelengths at 405 and 559 nm for Hoechst 33342 and TM-Rhodamine, respectively, as well as differential interference contrast (DIC) microscopy. The images were recorded and processed with Olympus FV10-ASW software.

1.7 MiRNA-149 quantification

The expression level of miRNA-149 in HepG2 cell line was analyzed by TaqMan MicroRNA Assay. Cells were plated (400000 cells per well) on 6-well plates and cultured for 24 hours. Then old medium was replaced by 1.8 mL of fresh medium and 200 μL of various drug formulations, including Dex-R5H5 with miRNA149 or NC microRNA at N/P ratios of 2 and 4, and RNAiMAX with miRNA-149 or NC miRNA. After 48-hour incubation, total RNA was isolated using a mirVana™ miRNA isolation kit, followed by reversing transcribe RNA by a Taqman® Reverse Transcription kit with 10 ng of total RNA. In the step of reverse transcription, an excessive amount of primer (5X) was added following the procedure for the double-stranded template to eliminate the effect of antagomir. Then TaqMan microRNA assay reaction mixture was prepared according to the provided protocol, and Real-time PCR amplification was carried out by a Bio-Rad CFX96 Real-Time Detection System (Hercules, CA, USA). The PCR data

was analyzed using qbase+ software (Belgium) and the commonly used 2-ΔΔCT method[3]. U6 snRNA was used as an internal standard, and all miRNA expression levels were normalized to control (no treatment).

1.8 Determination of Cytotoxicity

The cytotoxicity of Dex10-R5H5(40%)/antagomir-149 complexes was evaluated against HepG2 cells using Vybrant® MTT Cell Proliferation Assay. Briefly, cells were seeding in 96 well-plates at the density of 20000 cells per well. After 24-hour incubation, the old medium was replaced with 90 μL of fresh medium and 10 μL of various formulations of drugs, including Dex-R5H5 with antagomir-149 or miRNA-NC at N/P ratios of 2 and 4. After incubation at 37 ºC for 48 hours, the medium was replaced with 100 μL of fresh medium and 10 μL of MTT stock solution (5 mg/mL in PBS), and the cells were incubated for 4 hours at 37 ºC. Then, the medium was removed and 150 μL of DMSO was added to each well to dissolve the purple formazan crystals. The absorbance was measured at 570 nm using a Tecan Infinite 200 microplate reader (Switzerland). The cytotoxicity test was performed in 8 replicates for each sample. The cells without any treatment were used as a control (100% cell viability), and the cell viability was expressed as percentage of the control.

1.9 Statistical Analysis

Data was analyzed using single-factor analysis of variance (ANOVA), and expressed as mean ± standard deviation. The comparison among groups was performed using Student's t-test. A p value less than 0.05 was considered statistically significant.

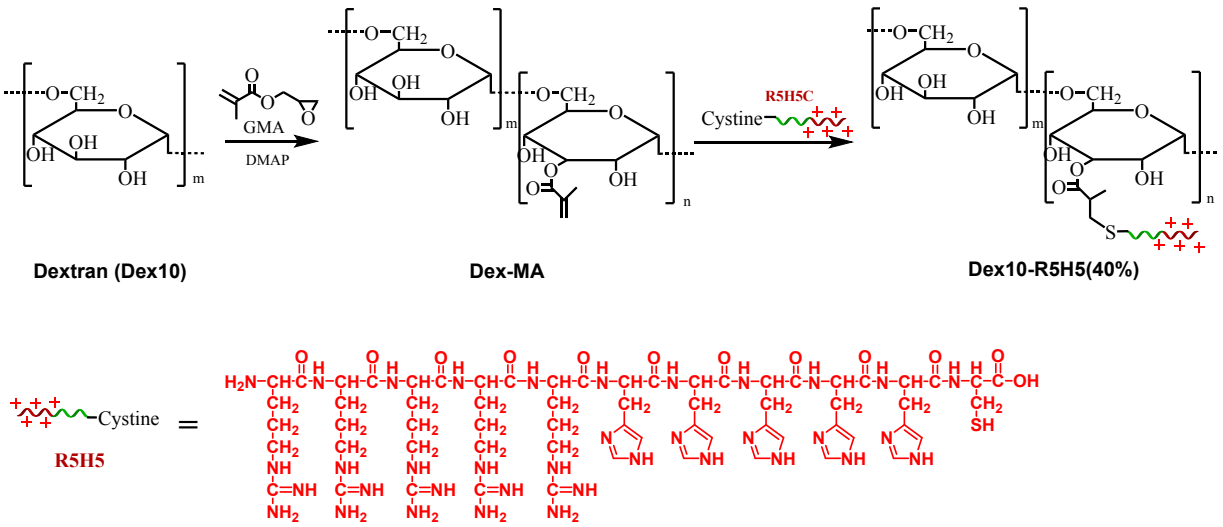

Figure S1. Synthetic route and chemical structure of Dex10-R5H5(40%).

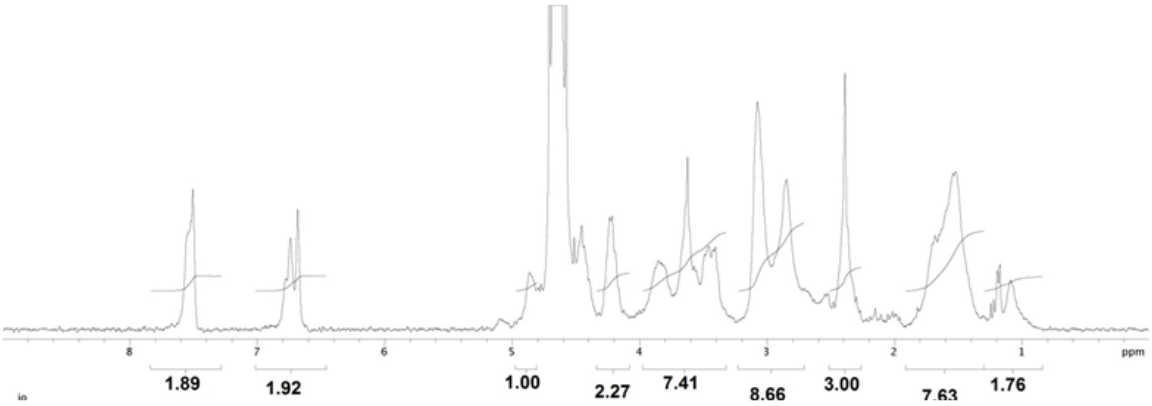

Figure S2. 300MHz ^{1}H NMR spectrum of Dex10-R5H5(40%) in D_2O.

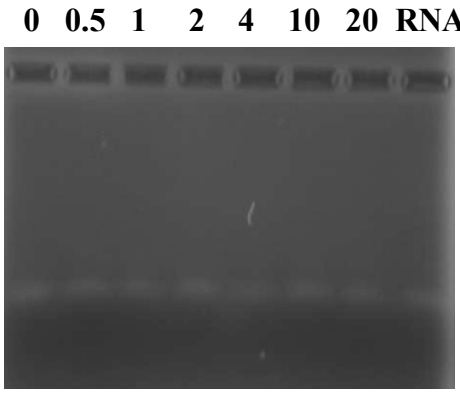

Figure S3. Electrophoretic mobility of Dex10-R5H5(40%)/antagomir-149 at different N/P ratios and RNAiMAX at designed ratio following its protocol.

Reference

1. Tang, Q.; Cao, B.; Lei, X.; Sun, B. B.; Zhang, Y. Q.; Cheng, G., Dextran-Peptide Hybrid for Efficient Gene Delivery. *Langmuir* **2014,** *30* (18), 5202-5208.

2. (a) vanDijkWolthuis, W. N. E.; KettenesvandenBosch, J. J.; vanderKerkvanHoof, A.; Hennink, W. E., Reaction of dextran with glycidyl methacrylate: An unexpected transesterification. *Macromolecules* **1997,** *30* (11), 3411-3413; (b) Cao, B.; Li, L. L.; Wu, H. Y.; Qiong, T.; Sun, B. B.; Dong, H.; Zhe, J.; Cheng, G., Zwitteration of dextran: a facile route to integrate antifouling, switchability and optical transparency into natural polymers. *Chem Commun* **2014,** *50* (24), 3234 - 3237.

3. Livak, K. J.; Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **2001,** *25* (4), 402-408.