SUPPORTING INFORMATION

Enhanced uptake and siRNA-mediated knockdown of a biologically relevant gene using Cyclodextrin Polyrotaxane

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S1. Chemicals for Synthesis of CD polyrotaxanes

α-CD and β-CD (Wacker-Chemie GmbH, München, Germany, pharmaceutical grade, 99%) were dried for 16 h at 70 °C under vacuum. For synthesizing Atto 647N labelled CD polyrotaxane, Atto 647N protein labeling kit was purchased from Jena Bioscience (Jena, Germany). Other reagents and solvents used for chemical synthesis of CD polyrotaxanes were obtained from Fisher Scientific GmbH (Schwerte, Germany) or Sigma-Aldrich (Taufkirchen, Germany) and used as received, except wherever special usage has been indicated.

S2. Materials and Methods

siRNA

Luciferase specific siRNA duplex was purchased from Integrated DNA Technologies (IA, USA). The sequences for this siRNA are sense, 5'-GGUUCCUGGAACAAUUGCUUUUACA-3' and anti-sense, 3'-UGUAAAAGCAAUUGUUCCAGGAACCAG-5.' This siRNA was used for investigating the knockdown efficiency of the nanoplexes in RAW 264.7 cells, after co-transfection with pGL3-Control Vector (Promega, WI, USA) containing the firefly luciferase gene.

S3. Determination of complexation of nucleotides using Agarose gel electrophoresis

Condensation of the nucleotides as a function of N/P ratios was assessed by analyzing their electrophoretic mobility from the nanoplexes in agarose gel and absence of intercalation of fluorescent ethidium bromide dye in the event of complete complexation of nucleotides with CD polyrotaxane. After incubation, the nanoplexes at various N/P ratios (along with 6x loading buffer) were loaded onto 0.5% agarose gel containing 1% ethidium bromide for visualization of the bands. Electrophoresis was conducted in TBE buffer (pH 7.4), for 60 min, at constant voltage of 50 V. For each run, unbound pDNA and siRNA were maintained as a control. Gel imaging was carried out using FUSION FX7™ Advance documentation system (PEQLAB

Biotechnologie GMBH, Erlangen, Germany) equipped with Acquisition Data Interface and Fusion-CAPT-Software for band integration and background correction.

S4. Cell viability: MTT assay

RAW 264.7 cells were cultivated at 37 °C, 85% relative humidity and 5% CO_2, in DMEM supplemented with 10% FCS. Cells were seeded into 96-well plates at a density of 20,000 cells per well in 200 µL of growth medium. Once confluent, they were incubated with the nanoplexes, at individual N/P ratios, for 24h in DMEM supplemented with FCS (final volume of 100 µL per well). Nanoplexes formulated with polyethylene imine (PEI), at the same N/P ratios (as polyrotaxane), were maintained as a control. The cellular treatment (supplementary information) was identical for both types of nanoplexes.

The cells were washed free of the nanoplexes, twice, with PBS (pH 7.4). Fresh medium containing 50 µL of MTT stock solution (5 mg/mL of MTT in PBS pH 7.4; MTT: Thiazolyl Blue Tetrazolium Bromide, M5655, Sigma-Aldrich, Taufkirchen, Germany) was added to each well and incubated for 4 h. The dye solution was then removed and the formazan crystals formed were solubilised in 200 µL of DMSO by shaking at room temperature. Absorbance of the solubilised formazan was measured in a plate reader at the wavelength of 550 nm. The cell viability was determined in comparison to similarly processed untreated cells (positive control, 100% cell viability) and cells treated with Triton X-100 (1% w/v; negative control, 0% cell viability).

S5. FACS

RAW 264.7 cells were seeded in a 24 well plate at a density of 50, 000 cells per well in 1 ml of the growth medium. Once confluent, they were incubated with nanoplexes dispersed in Krebs-Ringer's solution (KRB; pH 7.4), for a period of 4 h. Following the incubation period, the nanoplex solution was replaced by DMEM supplemented with FCS and the cells were incubated for further 18 h. The cells were then washed twice with PBS, were collected and centrifuged at 500×g and the resulting cell pellette was finally suspended in 500 µL of cell medium. A minimum of 10,000 individual cells per sample were analyzed using FACScan fluorescence-activated cell sorter (Becton-Dickinson, Heidelberg, Germany). The percentage of cell-associated fluorescence was determined using CellQuest software (Beckton-Dickinson, Heidelberg, Germany).

S6. Confocal laser scanning microscope (CLSM)

RAW 264.7 cells were seeded in 24 well imaging plate FC, with Fluorocarbon Film Bottom (PAA Laboratories GmbH, Pasching, Austria), at a density of 50, 000 cells per well, in 1 ml of the growth medium. Once confluent, they were incubated with the individual inhibitors in DMEM supplemented with FCS, for a period of 3h. Following this the cells were incubated with the nanoplexes in KRB, along with the inhibitors, for a further duration of 4 h. Subsequently, the nanoplex solution was replaced by inhibitor solutions in DMEM supplemented with FCS and the cells were incubated for further 18 h before subjecting them to fixation and staining.

The cells were washed twice with PBS, incubated for 15 min with 25 μg/mL of FITC-WGA (Emission: 515 nm, Excitation: 495 nm) in an incubator followed by two further washings with PBS and fixation with 4% paraformaldehyde in PBS for 10 min at room temperature. Once the cell membranes were stained fluorescent green by this dye, the cell nuclei were further stained with DAPI (Emmission: 461 nm, Excitation: 374 nm) by additional incubation with this dye for 15 min at room temperature. Finally, the cells were washed twice with PBS, mounted in the fluorophor protector FluorSafe® reagent (Calbiochem, San Diego, CA, USA) and observed by CLSM.

The imaging was performed employing a CLSM (LSM 510; Zeiss, Jena, Germany) equipped with an argon/neon laser and a 63x water immersion objective. The excitation wavelengths used were 488 nm for FITC-WGA, 360 nm for DAPI and 543 nm for Cy-3 siRNA containing nanoplexes and the fluorescence signals were collected after the band pass filters 500/530 nm for FITC-WGA, 390/465 nm for DAPI and 560/615 nm for the Cy-3 siRNA containing nanoplexes, respectively. The location of red nanoparticles with respect to the green cell membranes and blue nuclei was determined by acquiring 3D images using a stepper motor and image processing with the aid of Volocity® (Improvision, Tübingen, Germany) software.

S7. Biological efficacy of nanoplexes in cellular assays

***S7.1 In vitro* gene silencing: Luciferase knockdown assay**

In vitro gene silencing efficacy of the nanoplexes was investigated in RAW 264.7 cells, plated in a 24 well plate at a density of 50, 000 cells per well, in 1 ml of the growth medium. After incubating overnight, on the day of transfection, luciferase gene was transiently expressed in the cells using jetPRIME™: DNA and siRNA co-transfection reagent from Polyplus-transfection™ (PEQLAB Biotechnologie GMBH, Erlangen, Germany), as per the manufacturer's protocol. The medium in each well

was replaced by fresh medium, without serum, containing pGL3-Control Vector (1 µg per well along with jetPRIME™ reagent) and incubated for a period of 3h. The medium containing pGL3 was then replaced by serum-free medium containing anti-Luc siRNA complexed nanoplexes (100 pmol per well) and incubated for further 4h. Untreated cells, cells treated with naked siRNA and siRNA formulated with jetPRIME™ reagent, with the same siRNA concentration in each case, were employed as controls during this study. The medium containing either formulated or non-formulated siRNA was then removed and the cells were incubated with normal growth medium, for a further period of 65 h (total transfection period of 72 h), with intermittent replacement of medium every 24 h. Luciferase gene expression was measured using commercial Luciferase assay kit (Promega, WI, USA) and Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany), as per the manufacturer's protocol. Protein concentration in the cell lysates was determined BCA assay as described by the manufacturer (Bicinchinoninic Acid Kit for Protein Determination, Sigma, Cat. No. BCA1-1KT). The transfection experiments were conducted in triplicates and transfection efficiency was expressed as mean light units per mg of cell protein. These values were further used for determining percent Luciferase gene knockdown by the nanoplexes in comparison to the controls. Knockdown of luciferase gene was also verified by Real-Time PCR.

S7.2 Real-Time PCR (RT-PCR) to study luciferase knockdown

RT-PCR experiments were conducted according to a reported procedure [Leonard F, Collnot EM, Lehr CM. A three-dimensional coculture of enterocytes, monocytes and dendritic cells to model inflamed intestinal mucosa *in vitro*. Mol Pharm 2010; 7:2103-2119].

After transfection, RNA was extracted from the cells of various treatment groups using Qiagen RNeasy Mini Kit (Qiagen Inc., CA, USA). The Qiagen QuantiTect Reverse Transcriptase Kit (Qiagen Inc., CA, USA) was then employed for the synthesis of double stranded cDNA from 1 µg of mRNA and the product was employed for analysis by RT-PCR. PCR analysis was performed using Biorad CFX96 real time rotary analyzer (Bio-Rad Laboratories GmbH, Munich, Germany) using the following cycling protocol: initial denaturation of 1 min at 95 °C and 39 cycles of 95 °C for 10 s, 65 °C for 30 s and 72 °C for 30 s, a final amplification at 65 °C for 1 min, followed by a melt curve analysis (from 60°C to 98°C in 0.5°C steps for 5 s). DyNAmo™ Flash SYBR® Green qPCR Kit (Finnzymes, Fisher Scientific GmbH,

Schwerte, Germany) was employed during the experiment. Volume of template DNA equivalent to a concentration of 1 µg was amplified in the reaction mixture containing 1 µL of 10 µM primers, 10 µL 2 x DyNAmo Flash SYBR Green Master Mix and sufficient RNAse free water, so that the final reaction volume was 20 µL. ß-actin was included as an internal standard for each set of RNA samples analyzed and non-template controls, replacing cDNA with water, were maintained to control DNA contamination of the reagents. Standard curves were created for the quantification using CFX Manager™ software. Sequences of primer pairs used were: ß-actin: Primer sense sequence 5'-TGC GTG ACA TTA AGG AGA AG-3', Primer antisense sequence 5'-GTC AGG CAG CTC GTA GCT CT-3'; Luciferase (Eurofins MWG GmbH, Ebersberg, Germany): Primer sense sequence 5'-TGG GCT CAC TGA GAC TAC ATC A-3', Primer antisense sequence 5'-CGC GCC CGG TTT ATC ATC-3'.

S8. Results and Discussion

S8.1 Determination of complexation of nucleotides using Agarose gel electrophoresis

The nucleotide condensation as a function of N/P ratio was visualized by agarose gel electrophoresis assay relying on the ability of fluorescent ethidium bromide dye, included in the gel, to intercalate between the bps of any unbound nucleic acid. Adequate binding of nucleotides is an essential prerequisite for efficient gene silencing as unstable nanoplexes would fail to provide sufficient protection to the complexed siRNA against degradation by serum nucleases [Nguyen J, Reul R, Roesler S, Dayyoub E, Schmehl T, Gessler T, Seeger W, Kissel TH. Amine-modified poly (vinyl alcohol)s as non-viral vectors for siRNA delivery: effects of the degree of amine substitution on physicochemical properties and knockdown efficiency. Pharm Res 2010; 27:2670-2682]. The results have been depicted in Figure A.

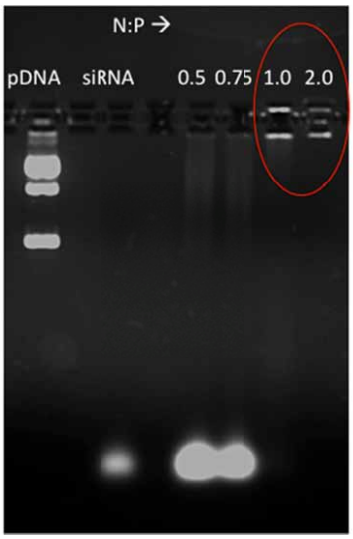

Figure A. Condensation of CD polyrotaxane with nucleotides at various N/P ratios as visualized by agarose gel electrophoresis assay. Complete binging of the nucleotides is observed at the N/P ratio of 2.0.

Complete quenching of nucleotide fluorescence was observed at the N/P ratio of 2.0, indicating complete complexation of nucleic acids by CD polyrotaxane. At lower N/P ratios, ethidium bromide intercalated between the bps of free nucleotide, which was visualized as fluorescent bands due to their electrophoetic mobility in the agarose gel. Thus, N/P ratio of 2.0 was considered as a suitable working ratio for satisfactory nanoplex formation.

S8.2 Luciferase silencing

The efficiency of a cationic polymer in siRNA delivery ultimately depends on its ability to release the siRNA in cellular cytosol, after mediating its escape from endosomal degradation. Several cationic polymers like PEI, despite their ability to enhance the cellular uptake and endosomal escape of condensed siRNA, exhibit low knockdown efficiency due to very strong complexation and insufficient siRNA release [Hossain S, Stanislaus A, Chua MJ, Tada S, Tagawa Y, Chowdhury EH, Akaike T. Carbonate apatite-facilitated intracellularly delivered siRNA for efficient knockdown of functional genes. J Control Release 2010;147:101-118; Mao S, Neu M, Germershaus O, Merkel O, Sitterberg J, Bakowsky U, Kissel T. Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethylene imine)-graft-poly(ethylene glycol) block copolymer/siRNA polyplexes. Bioconjug Chem 2006;17:1209-1218].

The CD polyrotaxane nanoplexes were initially evaluated for their efficiency to silence a transiently expressed reporter gene to establish the proof of principle of its biological efficacy. RAW 264.7 cells were sequentially transfected with pGL3 vector to express the luciferase gene mRNA and protein and then with anti-Luc siRNA (100 pmol per well) loaded nanoplexes to cleave the expressed mRNA. The results of this assay have been depicted in Figure B and C.

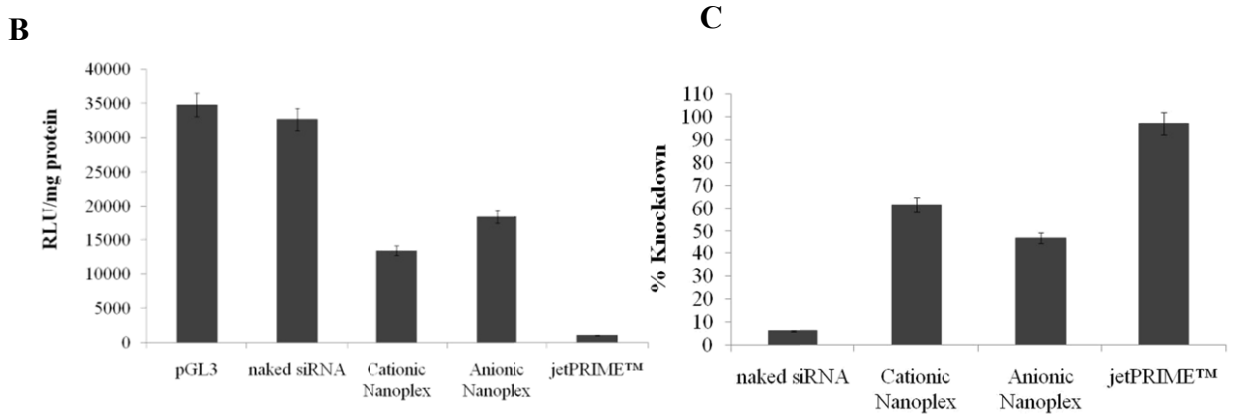

Figure C. Luciferase knockdown assay by Luciferase assay kit. Results expressed as mean light units per mg of cell protein. D. Percent Luciferase gene knockdown by the nanoplexes in comparison to the controls; Order jetPRIME™>cationic nanoplexes>anionic nanoplexes>naked siRNA

Figures D clearly depict that the knockdown of luciferase gene by naked anti-Luc siRNA was only about 3%, as against 61% by nanoplexes with a net positive charge and 46% by nanoplexes with a net negative charge. Poor knockdown by naked siRNA may be attributed to its insufficient intracellular uptake or degradation during culture conditions. The assay also proves efficiency of CD polyrotaxane to overcome cellular barriers to siRNA delivery namely enhancement of the stability of the associated siRNA, its effective internalization and efficient release into cellular cytoplasm following its escape from the endosome. Also luciferase knockdown was greater for cationic nanoplexes as compared to the anionic ones, which may be attributed to higher cellular uptake and hence higher transfection efficiency of the former, due to stronger interaction with the negative cell membranes.

The results were further verified by analyzing luciferase mRNA levels using RT-PCR. The results have been presented in Figure D.

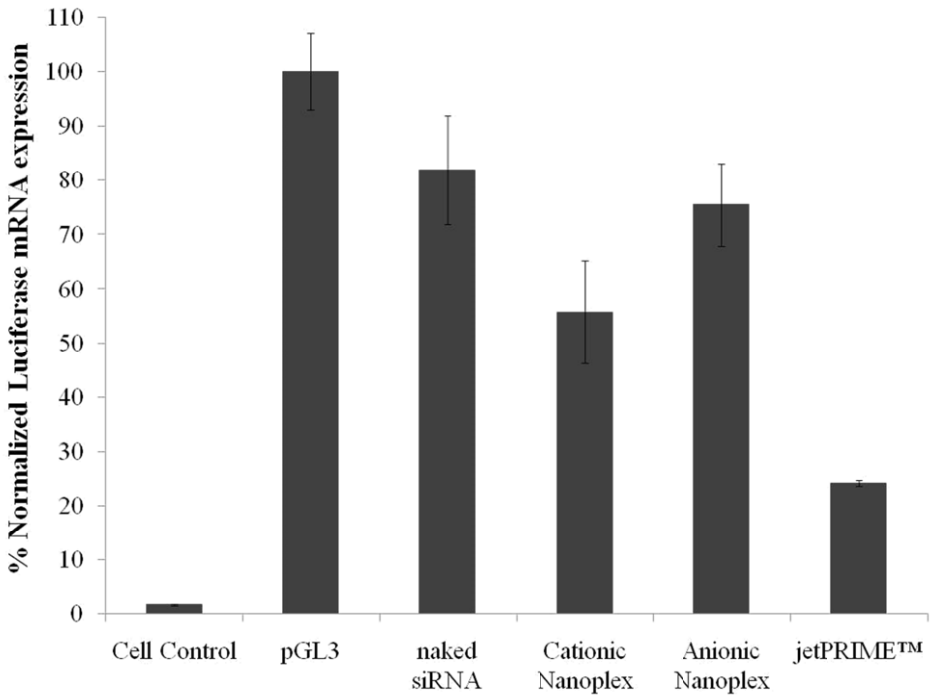

Figure D. Luciferase gene silencing monitored using RT-PCR. Luciferase mRNA levels were normalized to ß-actin mRNA expression. The results were obtained after transfection of RAW 264.7 cells with nanoplexes formulated using anti-Luc siRNA (100 pmol) for 72 h.

Reduction in luciferase mRNA expression, upon single treatment of cells with 100 pmol of anti-Luc siRNA formulated with CD polyrotaxane (nanoplexes) has been depicted in Figure D. The results corroborated well with those obtained with Luciferase assay kit. The knockdown efficiency by cationic nanoplexes, in terms of the mRNA levels as compared with those of pGL3 control, was almost 60%, which was in compliance with observations of luciferase assay kit. Similarly decrease in luciferase mRNA levels, after treatment with naked siRNA, was negligible and the mRNA levels after treatment with anionic nanoplexes were higher as compared to those after treatment with cationic nanoplexes.