Supplementary Information

Tools of gene transfer applied for the delivery of non-nucleic acid polyanionic drugs

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Materials

All materials and chemical were purchased from Sigma-Aldrich, unless stated otherwise, and used as received without further purification. Trypsin/EDTA 0.25%/0.02% was purchased from Invitrogen. High-purity water was of MQ quality (18.2 MΩ/cm, Millipore MilliQ Direct 8).

Methods

Cell culture of RAW264.7 (murine monocyte macrophages) was cultured at 37°C with 5% CO_2, in full cell culture medium containing Dulbecco's Modified Eagle's Medium (DMEM, 10% FBS (Invitrogen, <5 EU/mL) and 1% P/S). Cells were passaged in 2-3 day cycles using cell scraper. For long term usage cells were cryopreserved within a nitrogen cryotank in cryomedia (DMEM, 20% FBS, 10% Dimethyl sulfoxide (DMSO), $1x10^6$ cells/vial). Following thawing, cells were cultured for at least one week before being used for experiments and were used up to passage 20.

Flow cytometry was performed using a BD Accuri® C6 flow cytometer using an excitation wavelength of 488 nm. In each case at least 1000 events were analyzed. Fluorescence measurements were conducted using an Enspire Perkin Elmer plate reader. Cells were visualized by Zeiss confocal laser scanning microscopy (LSM 710) and Zeiss Axio Observe Z1 microscopy.

NMR spectra were obtained with a Varian Mercury 400 NMR spectrometer on samples dissolved in deuterated chloroform, unless stated otherwise. Chemical shifts are reported in ppm from external tetramethylsilane. MS-ESI: Mass spectra were obtained with a Bruker maXis Impact. Gel Permeation Chromatography (GPC) was performed on a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector and a DAWN HELEOS 8 static light scattering detector, equipped with a HEMA-Bio Linear column with 10 μm particles, a length of 300 mm and an internal diameter of 8 mm from MZ-Analysentechnik providing an effective molecular weight range of 1000−1.000.000. The eluent was 0.1 μm filtered MilliQ water at a flow rate of 1 mL/min with 300 ppm sodium azide.

Synthesis of monomers

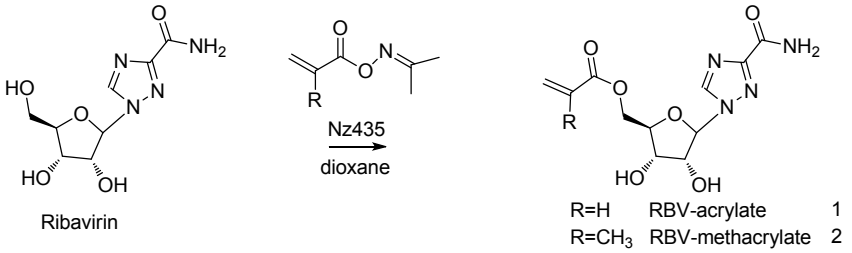

Figure SI 1 Synthesis of ribavirin (meth-) acrylate monomer, as reported in detail in publication[1]. Nz435: candida Antarctica lipase.

RBV (3.0 g, 12 mmol), enzyme beads (3.0 g), and a few crystals of di-tertbutyl-methylphenol were suspended in 150 mL dioxane. Acetone oxime (meth)-acrylate (6.75 g, 48 mmol) was added dropwise and the reaction was stirred at 50°C for 32 h. **1**: 500 mL MeOH was added and the mixture was filtered and reduced *in vacuo* followed by precipitation into diethyl ether. The precipitate is washed with 2x 50 mL pentane to yield RBV-5-O-acrylate as a white powder (1.2 g, 4.1 mmol). **2**: The reaction mixture was filtered and reduced *in vacuo* followed by precipitation into heptane. The precipitate was washed with 2x 50 mL ethyl acetate to yield RBV-5-O-methacrylate in a 50% yield as a white powder (1.9 g, 6.1 mmol).

Synthesis of Polymers

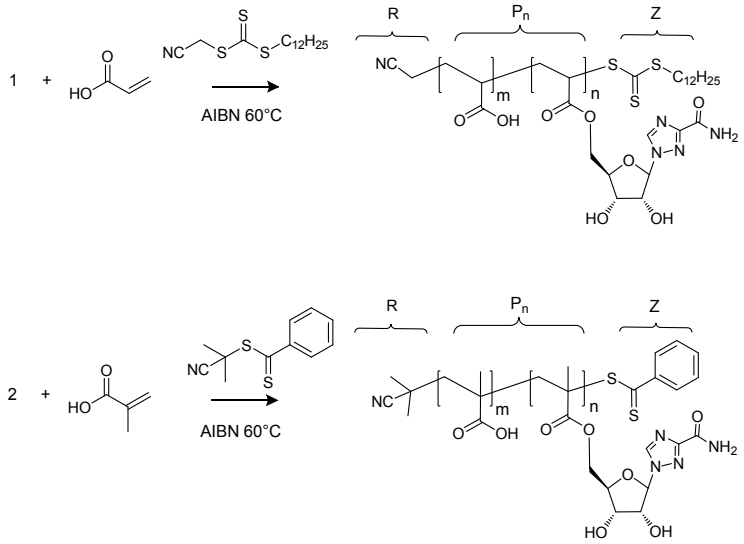

Figure SI 2 Polymerization of PAA and PMA under RAFT controlled conditions. Labeled macromolecular prodrugs were prepared analogously in presence of respective fluorescein. Pristine polymers without drug were prepared under same polymerization conditions. R and Z denote the RAFT agent rendered in the final structure.

All macromolecular prodrugs were analyzed for their drug content by ^{1}H-NMR, obtained with a Varian Mercury 400 NMR spectrometer on samples dissolved in deuterated chloroform, unless stated otherwise. Comparing peaks from the polymer backbone with peaks from the drug molecule. Characterization of molar mass was and polydispersity were carried out using Gel Permeation Chromatography (GPC) performed on a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector and a DAWN HELEOS 8 LS detector to determine absolute molar mass, and a SPD-M20A PDA detector.

Poly(acrylic acid) copolymers. A Schlenk tube was charged with cyanomethyl dodecyl trithiocarbonate (RAFT agent, 12.5 mg, 0.039 mmol), AIBN (azo-bis-isobutyronitrile, initiator, 1.6 mg, 0.01 mmol), 1 eq. fluorescein-acrylate (15.2 mg, 0.039 mmol), RBV-acrylate (206 mg, 0.703 mmol), acrylic acid (1.00 g, 13.8 mmol) and 3 mL dimethylformamide. The mixture was degassed by three freeze-pump-thaw cycles and polymerized for 7.5 h at 60°C. The monomers conversion was determined by NMR to be 68%. The reaction mixture was precipitated into diethylether, re-dissolved in methanol, and precipitated into diethyl ether. Polymer without RBV was prepared by the same procedure.

Polyplex and Lipoplex formation. Polyplexes were prepared by adding polymer (0.1 g/L, 5 μL) into polyethylene imine (PEI, 0.1 g/L, 10 μL) while mixed, allowed initial condensation for 20 sec. followed by addition of PBS to a final volume of 50 μL. The samples were incubated for 30 min. allowing stabilization of complexes. Lipoplexes were prepared by addition of 1 μL of polymer solution (0.1 g/L, 5 μL) to a solution of lipofectamin (10x dilution of the commercial stock in PBS, 10 μL) followed by addition of PBS to a final volume of 50 μL. The samples were incubated for 30 min. allowing stabilization of complexes. Complexes in PBS (10 μL) were added to the cells, resulting in a final concentration of 10 μg/mL or 1 μg/mL polymer.

Cellular Uptake. RAW264.7 cells (20 000 cells/ well, 100 μL) were seeded a clear sterile 96-well multiwell plate in full DMEM medium and allowed complete attachment (24 h). Media was replenished (90 μL) and substrates (10 μL) were added in different concentrations and incubated for 24 h (unless indicated otherwise). Cells were harvested by trypsinization. First, the cells were washed with PBS (100 μL), harvested by addition of Trypsin/EDTA (0.25%, 20 μL, 3-5 min.) followed by dilution in cold PBS (200 μL), and then collected into 1.5 mL Eppendorf tubes on ice. Cells were analyzed using flow cytometry. In each case at least 1000 events were analyzed. A standard cut off was applied to all samples (threshold 80 000). Auto-fluorescence of cells was subtracted in all presented results.

Visualization of Cells. For visualization of uptake and morphology RAW264.7 cells (100 000 cells/well, 1 mL) were seeded directly into clear sterile 12-well multiwall plates equipped with round glass slides (ø 16 mm), sterilized prior to cell seeding in 1 mL PBS using UV-sterilization. After 24 h incubation for cellular attachment, the glass slides were transferred to a fresh clear sterile 12-well multiwall plate. Media (900 μL) was replenished added and substrates (100 μL) were added. Following 24 h incubation, cells were washed in warm Hank's Balanced Salt Solution (2x 1000 μL), fixed by addition of 4% paraformaldehyde solution (500 μL, 10 min.), then washed with cold PBS (2x 500 μL). The nuclei were stained with 4',6-diamino-2-phylindole (DAPI, 1 μg/mL, 500 μL, 10 min.), followed by wash in PBS (2x, 500 μL) to remove excess staining reagent. Then the glass slides with cells were mounted in onto cover glass slides, using Vectashield mounting medium, sealed and visualized using either Zeiss confocal laser scanning microscopy (LSM 710) or Zeiss Axio Observe Z1 microscopy.

Intracellular Activity. For NO inhibition experiments RAW264.7 cells were seeded (20 000 cells/well, 100 μL) in clear sterile 96-well multiplates. After 4 h allowing cell attachment, polymers and L-N^G-nitroarginine methyl ester (L-NAME, 1 mM) were added. Following incubation (24 h) media was renewed and cells were stimulated through the addition of lipopolysaccharide (LPS, E. coli 026:B6, 1 μg/mL). After additional incubation (24 h)

relative nitric oxide levels were determined by measuring nitrite levels through the Griess assay in phenol red-free DMEM. In short, 50 µL media was transferred to a new clear 96-well multiplate and 50 µL sulfanilic acid (10 g/L, 5% phosphoric acid) were added. After incubation (5 min.) 50 µL N-1-napthylethylenediamine dihydrochloride (1 g/L) was added. The nitrite levels were quantified against a freshly-prepared sodium nitrite standard curve (100 mM sodium nitrite dissolved in water) in a separate multiplate. The nitrite standard solution was diluted 1000-times in cell media and serial diluted 2-fold, with a final volume of 50 µL. Absorbance was measured at 548 nm using EnSpire Multimode Plate Reader and all values were blank corrected. Each experiment was normalized against the negative control consisting of LPS stimulated cells without the addition of any reagent/polymer, obtaining relative nitric oxide levels. Viability of the cells was measured by quantifying metabolic activity through the PrestoBlue assay (Invitrogen). In parallel the media in the original cell containing multiplate was replenished (100 µL) and prestoBlue reagent was added (10 µL, 30 min., 37°C), and then transferred to a black 96-well multiplate (80 µL). Cellular viability was determined by quantifying fluorescence (ex. 560 nm and em. 590 nm). Each viability experiment was normalized against a negative control where no reagent/polymer/LPS had been added to the cells. Positive control: 20% DMSO.

Hemolysis. Human RBCs obtained from Skejby Hospital blood bank were isolated from full blood through centrifugation. RBCs were washed with PBS (3x 10 mL, 5 min., 400 rpm) and substrates (5 µL) were added to RBC solutions (45 µL) in clear sterile 96-well multiwall plates and incubated overnight an Eppendorf thermomixer (37°C, 300 rpm). After incubation, PBS (200 µL) was added gently without re-suspension of the samples. After centrifugation (5 min., 400 rpm) 100 µL of the supernatant was collected and placed in a new clear sterile 96-well multiwall plate and absorbance (541 nm) was determined.

Data Analysis. For all data points, at least 3 independent experiments with minimum 3 replicates for each sample were performed and reported as mean ± standard deviation (SD). Data was analyzed in Microsoft Excel and plotted in OriginPro (Origin Lab, v.8.5). Graphs were visually enhanced in Adobe Illustrator CS5. Statistical significance was determined through student's T-test using Excel software. One-tailed unpaired t-test with 95% confidence interval was considered statistical significant if $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

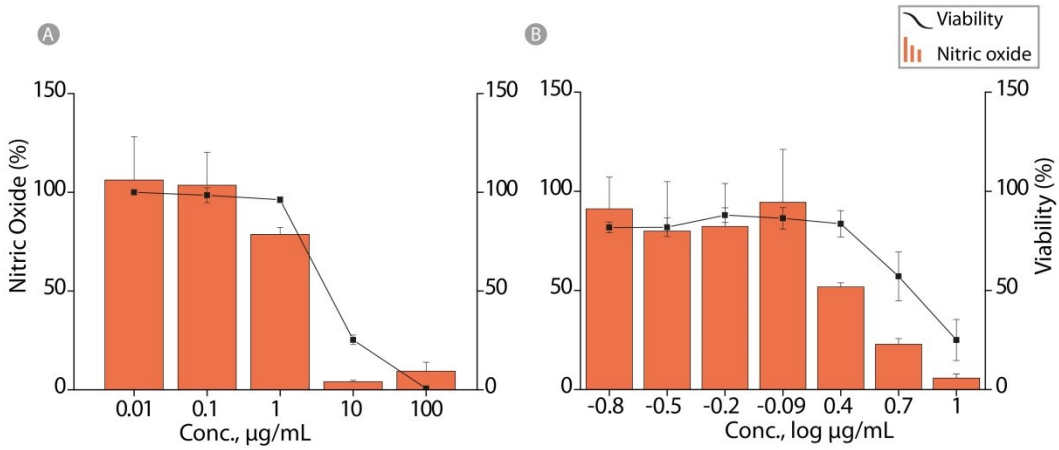

Figure SI 3 Inhibition of NO production and associated cytotoxic effect in LPS stimulated macrophages after 24 h incubation with branched PEI in different concentrations. (A) 0.1 g/L – 10 µg/L 10-fold dilution steps, (B) 10 µg/mL – 1 µg/L using 2-fold dilution steps. Results shown are average of triplicate experiments, reported as mean ±SD.

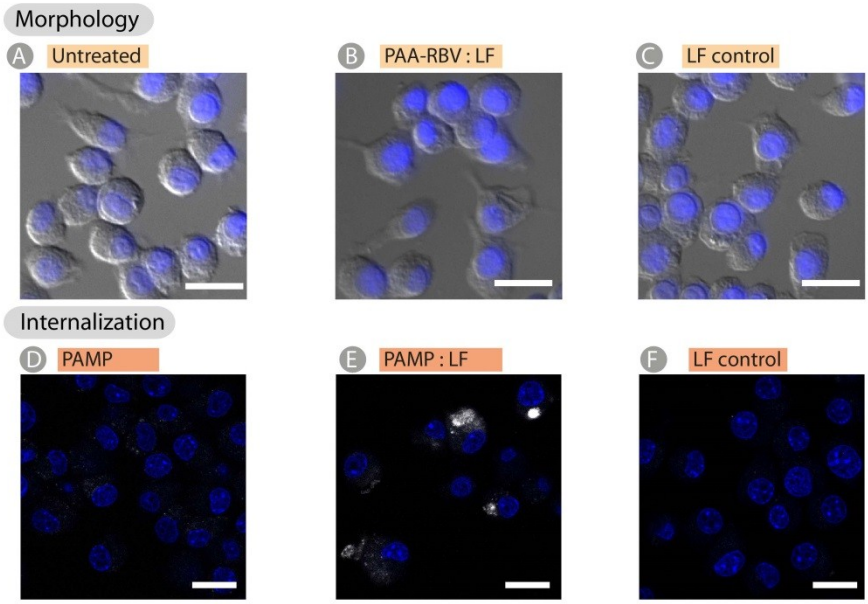

Figure SI 4 Polymer internalization into macrophages as visualized through confocal laser scanning microscopy and DIC microscopy using fluorescently labeled polymers of PAA-RBV MP) at 10 µg/mL upon 24 h incubation. (A)-(C) Visualization of cell morphology of DAPI stained (blue) untreated cells. (D)-(F) Fluorescence (white) upon cellular internalization of DAPI stained (blue) cells. Scale bars correspond to 20 µm.

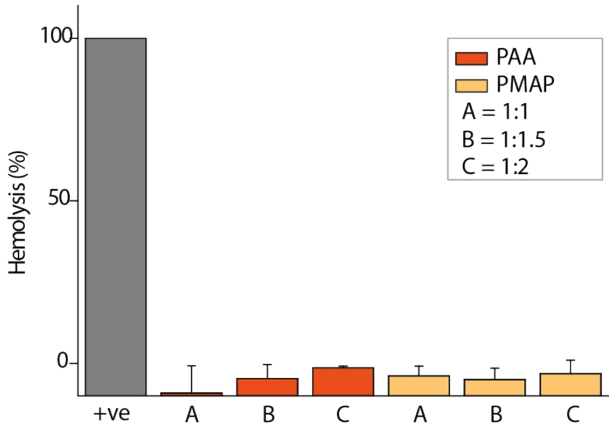

Figure SI 5 Hemocompatibility of polyplexes composed of PEI and anionic MPs in three different ratios (1:1, 1.5:1, and 2:1 PEI-to-MPs) at final MP concentration of 1 μg/mL upon 24 h incubation with RBCs. Results shown are average of triplicate experiments, reported as mean ±SD (n=3).

1. M. B. L. Kryger, B. M. Wohl, A. A. A. Smith and A. N. Zelikin, *Chemical Communications*, 2013, **49**, 2643-2645.