**Supporting Information**

**Modular Supramolecular Ureidopyrimidinone Polymer Carriers for Intracellular Delivery**

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## Materials and Methods.

### General

All solvents were purchased from commercial sources and used as received, unless stated otherwise. Water was purified on an EMD Millipore MilliQ Integral Water Purification System. $^1$H-NMR data was obtained using a Varian 7600-AS NMR autosampler with Agilent software Vnmrj Version 3.1 Revision A. LC-MS data was obtained using a C18, Jupiter SuC4300A, 150x2.00 mm column. Fluorescence data were recorded on a Varian Cary Eclipse fluorescence spectrometer using Quartz cuvettes. Fluorescent cell images were acquired with a Leica TCS SP5 AOBS equipped with a 40 x water immersion objective and a temperature-controlled incubation chamber maintained at 37 °C. Multi angle dynamic light scattering experiments were conducted on an ALV/CGS-3 MD-4 compact goniometer system equipped with a ALV-7004 real time correlator (solid state laser: λ = 532 nm; 40 mW). Zeta-potential data was acquired on a Zetasizer Nano ZS. Absorbance of MTT was read out on a Tecan Safire$^2$ plate reader. Agarose gels were imaged using a GE ImageQuant 350 using the Sybrsafe filter. High Pure RNA Isolation Kit was purchased from Roche Life Science. iScript cDNA synthesis kit and IQ SYBR Green Supermix were purchased from Bio-Rad. qPCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System

### Synthesis of monomers

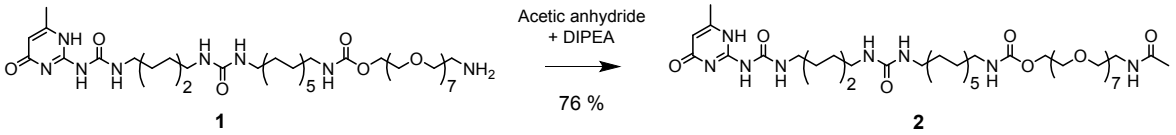
**Synthesis of Boc-protected UPy-PEG$_7$-Amine** is described in Kieltyka *et al. ChemComm.* **2012**.
$^1$HNMR (400 MHz, $CDCl_3$): δ (ppm) 13.16 (s, 1H), 11.84 (s, 1H), 10.08 (s, 1H), 5.85 (s, 1H), 5.11 (s, 1H), 4.93 (s, 1H), 4.72 (s, 1H), 4.48 (s, 1H), 4.22 (s, 2H), 3.4-3.75 (m, 28H), 3.1-3.35 (m, 10H), 2.24 (s, 3H), 1.66 (d, 12H), 1.20-1.55 (m, 37H), 0.97 (t, 2H), 0.61 (q, 1H). LCMS: [M] calcd. 989.26; found 989.68 $[M+H]^+$, 445.50 $[M+2H\text{-}Boc]^{2+}$, 1011.58 $[M+Na]^+$, 506.42 $[M+H+Na]^{2+}$.


**1**

### Synthesis of cationic UPy-PEG$_7$-Amine 1:

Boc deprotection was commenced by addition of approx. 15 mL TFA to 140 mg (141 µmol), stirring for 1 h while on ice. The reaction was removed from ice and the mixture was dried with a nitrogen flow for 0.5 h. The dried mixture was dissolved in 10 mL of $dH_2O$ and lyophilized for 72 h.

Yield: 70%. $^1$HNMR (400 MHz, $CDCl_3$): δ (ppm) 13.13 (s, 1H), 11.80 (s, 1H), 10.04 (s, 1H), 7.96 (s, 3H), 5.83 (s, 1H), 4.20 (t, 2H), 3.83 (t, 2H), 3.5-3-.75 (m, 26H), 3.05-3-3 (m, 10H), 2.23 (s, 3H), 1.1-1.65 (m, 28H). LCMS: [M] calcd. 889.15; found 889.75 $[M+H]^+$, 445.50 $[M+2H]^{2+}$.

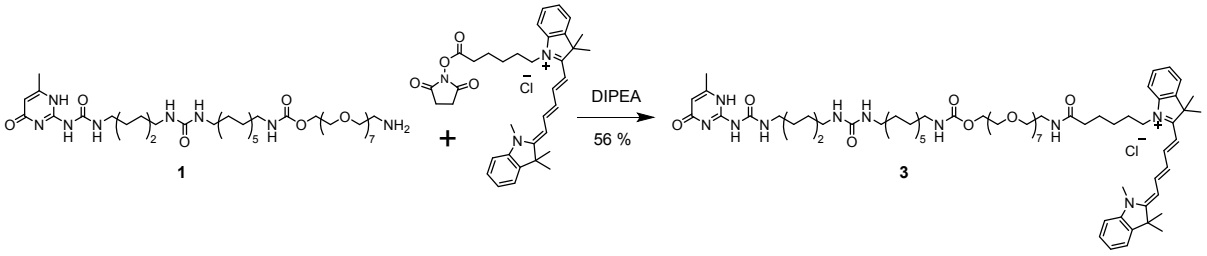



1 2

**Synthesis of acetylated UPy-PEG$_7$-Amine 2:**

45 mg (45 µmole) of Boc-protected **1** was deprotected with TFA, similar as described previously. After drying with a nitrogen flow the yellowish gel-like material was dissolved in 10 mL $CHCl_3$, after which an excess of DIPEA (approx. 3 mL) was added. Next, 14 µL (135 µmole = 3 eq.) of acetic anhydride was added and the reaction was stirred at RT overnight. After overnight reaction, the mixture was dried and dissolved in approx. 3 mL $dH_2O$ and 0.5 mL DMSO and dialysis was performed with a cellulose ester dialysis membrane with a MWCO of 500-1000 Da. Dialysis was started in 1.5 L $dH_2O$ while stirring gently. Over the course of 72 h the $dH_2O$ was refreshed three times. The compound was then lyophilized for 48 h and dried under vacuum at 50 °C for 24 h.

Yield: 76%. $^1$HNMR (400 MHz, $CDCl_3$): δ (ppm) 13.16 (s, 1H), 11.83 (s, 1H), 10.07 (s, 1H), 6.43 (s, 1H), 5.84 (s, 1H), 4.97 (s, 1H), 4.73 (s, 1H), 4.51 (s, 1H), 4.19 (s, 2H), 3.6-3.75 (m, 26H), 3.55 (t, 2H), 3.43 (q, 2H), 3.10-3.30 (m, 8H), 2.24 (s, 3H), 1.99 (s, 3H), 1.20-1.70 (m, 28H). LCMS: [M] calcd 930.18; found 466.50 $[M+2H]^{2+}$, 931.75 $[M+H]^+$, 953.67 $[M+Na]^+$, 477.42 $[M+H+Na]^{2+}$.




1 3

**Synthesis of UPy-PEG$_7$-Amine-Cy5 3**:

7.4 mg (7.5 µmole) of Boc-protected **1** was deprotected with TFA, similar as described above. The resulting gel-like mixture was dissolved in 5 mL $CHCl_3$, after which an excess of DIPEA (approx. 2 mL) was added. To this mixture 5.47 mg (8.9 µmole, = 1.2 eq.) of Cy5 Dye NHS ester (Lumiprobe, mw: 616.19 Da) was added. The reaction was protected from light using aluminum foil and stirred overnight. After overnight reaction the mixture was dried and dissolved in 3 mL DMSO. Dialysis was performed using a regenerated cellulose membrane with a MWCO of 1 kDa. Dialysis was started in 1.5 L $dH_2O$ with 20% DMSO, after 18 h the buffer was replaced with pure $dH_2O$ and after 42 h the water was refreshed once more. The compound was then lyophilized for 48 h.

Yield: 56%. $^1$HNMR (400 MHz, $CDCl_3$): δ (ppm) 13.17 (s), 11.84 (s), 10.07 (s), 7.8-8.0 (m), 7.32-7.5 (m), 7.0-7.2 (d), 6.2-6.4 (m), 5.7-5.9 (d), 5.14 (s), 4.20 (t), 4.04 (t), 3.53-3.73 (m), 3.49 (s), 3.45 (t), 3.1-3.3 (m), 2.15-2.35 (m), 2.08 (s), 1.1-1.9 (m), 0.95 (d), 0.86 (d). LCMS: $[M]^+$ calcd. 1354.81; found 677.75 $[M+H]^{2+}$, 452.25 $[M+2H]^{3+}$, 459.50 $[M+H+Na]^{3+}$, 688.50 $[M+Na]^{2+}$, 1353.92 $[M]^+$.

**Formulation of supramolecular UPy aggregates.** Monomers were dissolved in MeOH at 2.5 mM (**1**), 1.25 mM (**2**) and 0.44 mM (**3**). For preparation of the various aggregates the desired amounts of monomer solutions were injected in MiliQ water and equilibrated by means of shaking for a minimum of two h. For complex formation with siRNA the siRNA was injected in the mixture together with the UPy monomers (in case of 1 step preparation), or after the minimum of 2 h equilibration (in case of 2 step preparation).

**Nile Red (NR) encapsulation.** UPy polymers were prepared at 50 µM concentrations. NR was added to the solution to a final concentration of 5 µM and samples were equilibrated by means of shaking for 5 min. NR was excited at 550 nm and the emission intensity was recorded from 565 nm to 800 nm. 5 scans were performed and averaged.

**FRET Measurements.** UPy polymers were prepared at 50 µM concentrations. In addition, UPy-Cy5 reporter was added to a final concentration of 0.5 µM (1 %) prior to equilibration. Samples were excited at 520 nm and emission intensity was recorded from 540 nm to 800 nm. 5 Scans were performed and averaged. Samples were measured without and in the presence of Nile Red (final concentration 3 µM).

**Dynamic Light Scattering.** For DLS measurements MiliQ water was filtered through a 0.2 µm filter before use. UPy polymers were prepared at 50 µM concentrations. Experiments covered a range of angles between 36° and 148°. Each angle was measured in triplet, for 10 sec, at 20 ° C, using a total of 4 detectors. Raw autocorrelation data of angle 102° was plotted to visualize the trend in decreasing correlation times. To obtain hydrodynamic diameters the data was further analyzed via Cumulant analysis.

**Zeta-Potential.** UPy polymers were prepared at 50 µM concentrations. Approximately 1 mL of sample was injected in a Malvern Disposable capillary cell (DTS1061). Three consecutive measurements with 70 runs of 10 sec were performed and averaged.

**Cell culturing & Imaging.** HK-2 Cells were purchased from ATCC and cultured at 37°C in 95% air/5% $CO_2$ atmosphere in Dulbecco's Modified Eagle Medium (DMEM) 41965-039 supplemented with 10% Foetal bovine serum (FBS) and 1% penicillin streptomycin (P/S). Cells were passed typically twice a week and for experiments cells ranging from passage 5 to 20 were used. Cell imaging was performed on a Leica TCS SP5X confocal microscope. For UPy internalization and siRNA delivery studies cells were seeded in a Lab-Tek Chambered #1.0 Borosilicate Coverglass System 24 h prior to imaging to a confluency of approximately 70%. Right before imaging, the medium on the cells was discarded and cells were washed once with PBS. Upon imaging, the media was removed and replaced by 10 µM UPy polymers dissolved in non-supplemented DMEM. Images were analyzed with ImageJ.

**Viability.** HK-2 cells were seeded in 96 well plates (5000 cells/well) and cultured overnight under normal conditions. The next day medium was removed and UPy polymers in 125 µL DMEM medium containing 2% FBS were added to the cells. Final concentrations of 0, 0.1, 1 and 10 µM were tested, each in sixfold. After 24 h incubation time, 13.75 µL (10%) of a freshly prepared MTT solution (5 mg/mL in PBS, filtered through 0.2 µm filter) was added to the wells (final volume 138.75 µL). Samples were incubated at 37 °C for approximately 2 h and the medium was then removed. 150 µL of acidic isopropanol (isopropanol containing 0.04 M HCL) was added to the wells. The samples were then incubated at 37 °C for 20 min and subsequently 100 µL was transferred to a Costar EIA/RIA 96 wells plate. The absorbance was measured at 570 nm and the absorbance at 650 nm was deducted from this value. Values were normalized to cells receiving no UPy polymer treatment and reported

as average and standard deviation. LIVE/DEAD Viability assay was purchased from thermoFisher. Calcein AM and Ethidium homodimer-1 were diluted in medium to 1 µM before addition to cells.

**Gel retardation assay.** All UPy samples were mixed and equilibrated with 100 ng (6.4 pmol) fluorescently labeled (Alexa488) siRNA (Qiagen #1027284) at N/P ratios 0, 1, 2, 4, 10, 20 in a total volume of 30 µL (containing 5 µL 6x loading dye). For the neutral polymer amounts were used similar to if it was a full cationic polymer, for no N/P ratios can be calculated for a polymer without amines. Samples were run on a 1.5 % agarose gel at 70 volt for 30 min.

**Silencing study.** HK-2 cells were seeded in a 24 wells plate (30,000 cells/well) and cultured in DMEM media supplemented with 10% FBS and 1% Pen/Strep overnight. The next day UPy polymer - siRNA complexes were mixed with media supplemented with 2% FBS to a final volume of 1 ml. Both samples contained 600 ng (45 pmol) TGFBR1 siRNA (Thermo M-003929-02-0005) and were complexed with the two UPy polymers at N/P ratio = 10 prepared via 1-step formulation. As scrambled negative control the fluorescent labeled non targeting siRNA was used with a 50% polymer at N/P ratio = 10. Cells were washed with PBS once and subsequently treated with the medium containing siRNA complexes. After 4 hours incubation, media was discarded and replaced by fresh DMEM supplemented with 10% FBS and 1% Pen/Strep. Approximately 48 hours after transfection, RNA of the cells was extracted using a High Pure RNA Isolation Kit following manufacturers' protocol and immediately afterwards transcribed to cDNA with an iScript cDNA synthesis kit following manufacturers' protocol. Quantitative polymerase chain reaction was performed with 5 ng cDNA and TGBR1 specific primers to quantify TGFBR1 mRNA expression. Normalization of each sample was performed to the expression of the housekeeping gene GAPDH. Reported mRNA expression values are averages and standard deviation from a total of 4 samples per condition, normalized to TGFBR1 expression of untreated cells.