Supporting Information

Inhibitor-GCPII interaction: selective and robust system for targeting cancer cells with structurally diverse nanoparticles

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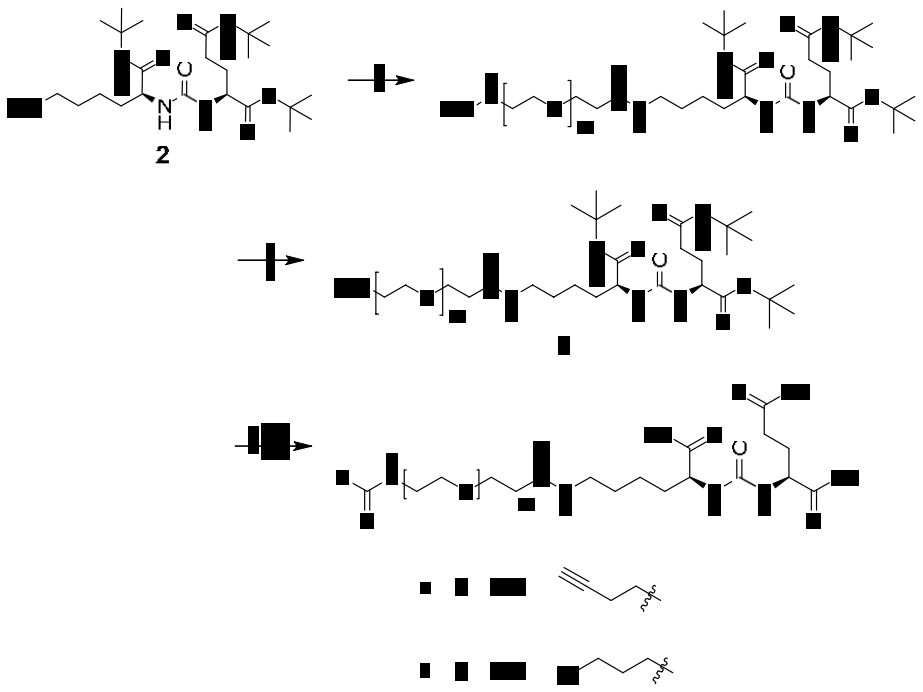
Synthesis of GCPII inhibitors

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. All final compounds were purified using a preparative scale Jasco PU-986 HPLC (flow rate 10 ml/min), equipped with a YMC C18 Prep Column, 5 μm, 250 x 20 mm. Purity was tested on an analytical Jasco PU-1580 HPLC (flow rate 1 ml/min), invariable gradient 2-100% in 30 min unless otherwise stated, equipped with a Watrex C18 Analytical Column, 5 μm, 250 x 5 mm. Final compounds were of at least 99% purity. Structures were confirmed by HRMS at LTQ Orbitrap XL (Thermo Fisher Scientific).

Compound **1** (1-amino-40-(4-bromobenzyl)-39,47-dioxo-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxa-40,46,48-triazahenpentacontane-45,49,51-tricarboxylic acid TFA salt) was synthesized according to the procedure described by Sacha et al,[1] but replacing Boc-NH-PEG_5-COOH with Boc-NH-PEG_{12}-COOH (PurePEG, LLC). The overall yield was 10%. HRMS (ESI-pos.) $C_{46}H_{80}BrN_4O_{20}$ $[M+H]^+$ calc. 1087.4549; found 1087.4525.
Compound **2** was prepared as previously described.[2] Compounds **4** and **5** (GCPII inhibitors with alkyne or azide) were synthesized as shown in Scheme S1.



Scheme S1: Synthesis of **3**, **4**, and **5**. a) Z-NH-PEG_{12}-COOH , TBTU, DIEA, DMF; b) H_2, $Pd(OH)_2$, MeOH; c) 1) 4-pentynoic acid, TBTU, DIEA, DMF 2) TFA; d) azidobutyrate-NHS, DIEA, DMF 2) TFA.

Compound **3** (1-amino-39,47-dioxo-3,6,9,12,15,18,21,24,27,30,33,36-dodecaoxa-40,46,48-triazahen-pentacontane-45,49,51-tricarboxylic acid tri-*t*-butyl ester). A 511 mg (0.68 mmol, 1.1 eq) portion of Z-NH-PEG_{12}-COOH (IRIS Biotech) was dissolved in 1.5 mL DMF, and 297 mg TBTU (0.775 mmol, 1.25 eq) and 150 µl DIEA (0.87 mmol, 1.4 eq) were added in one portion. The reaction mixture was left stirring for 30 min, and 307 mg (0.62 mmol, 1.0 eq) **2** and 118 µl DIEA (0.68 mmol, 1.1 eq) dissolved in 1.5 ml DMF, were added to the reaction mixture in one portion. The reaction was left overnight, and the mixture was rotary evaporated to dryness. The crude product was dissolved in MeOH and a catalytic amount of $Pd(OH)_2$ was added (roughly 15 mg). The reaction flask was purged with hydrogen, and the deprotection was allowed to continue for 2 h with slightly elevated hydrogen pressure (1.05 Atm). Reaction was monitored by analytical HPLC (gradient 30-80% ACN in 30 min, Z-protected compound t_R = 19.5 min; deprotected product t_R = 12.5 min). The product was used in the next step without further purification.

Compound **4** (5,13,20-trioxo-16-oxa-4,6,12,19-tetraazatetracos-23-yne-1,3,7-tricarboxylic acid). A 17 mg (0.174 mmol, 1.2 eq) portion of 4-pentynoic acid was dissolved in 1 ml DMF along with 79 mg TBTU (0.209 mmol, 1.4 eq) and 90 µl DIEA (0.522 mmol, 3.6 eq). After 20 min, the mixture was added to 158 mg (0.145 mmol, 1.0 eq) of **3** dissolved in 1.5 ml DMF. The reaction was left overnight and the mixture was rotary evaporated to dryness. Crude product was purified using preparative scale HPLC (gradient 30-80% ACN in 60 min, t_R = 22.0 min) and the pure intermediate was rid of all solvents by dry freezing. The final deprotection was performed by adding 1 ml TFA and the reaction mixture was alternately sonicated and stirred for 15 min. TFA was removed by flow of nitrogen, and the product was purified using preparative scale HPLC (gradient 15-50% ACN in 60 min, t_R = 27.0 min) to obtain 23 mg of product (isolated yield = 47%). Analytical HPLC (gradient 2-100% ACN in 30 min, t_R = 16.1 min). HRMS (ESI-neg.) $C_{44}H_{78}N_4O_{21}$ $[M-2H]^{-2}$ calc. 498.25065; found 498.25012.

Compound **5** (56-azido-5,13,53-trioxo-16,19,22,25,28,31,34,37,40,43,46,49-dodecaoxa-4,6,12,52-tetraazahexapentacontane-1,3,7-tricarboxylic acid). A 53 mg (0.053 mmol, 1 eq) portion of **3** was

dissolved in 1 ml DMF, and 15 mg (0.064 mmol, 1.2 eq) of azidobutyrate-NHS (BroadPharm) along with 18 µl DIEA (0.106 mmol, 2.0 eq) were added. The reaction was left overnight, and the mixture was rotary evaporated to dryness. To the crude protected product 1 ml TFA was added and the reaction mixture was alternately sonicated and stirred for 15 min. TFA was removed by flow of nitrogen, and the product was purified using preparative scale HPLC (gradient 15-50% ACN in 60 min, t_R = 33.0 min) to obtain 24 mg of pure product (isolated yield = 44%). Analytical HPLC (gradient 2-100% ACN in 30 min, t_R = 15.4 min). HRMS (ESI-neg.) $C_{43}H_{79}N_7O_{21}$ $[M-2H]^{2-}$ calc. 513.75917; found 513.75952.

Synthesis of HPMA polymers

Atto488-amine (Atto488) was obtained from ATTO-TEC GmbH. The azoinitiator 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) was obtained from Wako Pure Chemical Industries Ltd. *Tert*-butanol, dimethyl sulfoxide, and ethyl acetate were purchased from Sigma-Aldrich. All other chemicals and solvents were of analytical grade.

Synthesis of monomer chain transfer agent and copolymer precursor
N-(2-hydroxypropyl)methacrylamide (HPMA) was synthesized by reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane in the presence of sodium carbonate as previously described.[3,4] 2-methyl-*N*-(6-oxo-6-(2-thioxo-1,3-thiazolidin-3-yl)hexyl)prop-2-enamide (Ma-Acap-TT)[3,4] and the chain transfer agent 2-cyanopropan-2-yl ethyl carbonotrithioate were synthesized as previously described.[5]

The copolymer precursor poly(HPMA-*co*-Ma-Acap-TT) was prepared by reversible addition-fragmentation chain transfer (RAFT) copolymerization.[6,7] HPMA (0.5 g, 3.49 mmol) dissolved in 4.6 ml *tert*-butanol was mixed with a solution of Ma-Acap-TT (350 mg, 1.16 mmol) dissolved in 606 µl dimethyl sulfoxide. 2-Cyanopropan-2-yl ethyl carbonotrithioate (2.39 mg, 1.16×10^{-5} mol) and 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (1.79 mg, 5.82×10^{-6} mol) were introduced into the polymerization ampule. The mixture was bubbled with argon for 10 min and the ampule was sealed. Copolymerization was carried out at 40 °C for 16 h. Polymer precursor was isolated by precipitation into ethyl acetate, filtered off, washed with ethyl acetate and diethyl ether, and dried in a vacuum. The terminating trithiocarbonate group was removed as described by Perrier.[4,5] The copolymer precursor poly(HPMA-*co*-Ma-Acap-TT) with molecular weight M_n = 37,900 g/mol, M_w = 39,600 g/mol, dispersity $Đ$ = 1.04, and content of reactive TT groups of 19.4 mol% was obtained.

Determination of molecular weights and polymer composition
The weight-average molecular weights (M_w), number average molecular weights (M_n), and dispersities ($Đ$) of the polymer precursor and conjugates were determined using size exclusion chromatography. The system was configured as previously described.[1,7] The GCPII inhibitor content was determined by quantification of bromine content in polymers using inductively coupled plasma atomic emission spectroscopy (ICP-OES) as previously described.[1]

Synthesis of polymer conjugates
Generally, HPMA copolymer conjugates were prepared by reaction of the copolymer precursor poly(HPMA-*co*-Ma-Acap-TT) containing thiazolidine-2-thione reactive groups (TT) along the polymer chain with a combination of fluorophore (Atto488-amine) and targeting ligand (GCPII inhibitor) according to a previously described procedure.[1]

*Synthesis of fluorescent HPMA polymer without GCPII inhibitor (***pol**, pol*)*

Copolymer precursor poly(HPMA-*co*-Ma-Acap-TT) (65 mg; M_n = 37,900 g/mol, M_w = 39,600 g/mol, $Đ$ = 1.04; 19.4 mol% TT) and Atto488 (3.25 mg, 3.78×10^{-6} mol) were dissolved in 0.5 ml dimethyl sulfoxide. Then, DIEA (2.6 µl, 1.5×10^{-5} mol) was added. The reaction was carried out for 4 h at room temperature. Then, 60 µl of reaction mixture was removed and 2 µl 1-aminopropan-2-ol (AP) was added to remove residual TT reactive groups, providing **pol**. Copolymer with TT reactive groups [rest of reaction mixture, poly(HPMA-*co*-Ma-Acap-ATTO488-*co*-Ma-Acap-TT)] and copolymer with AP-removed TT groups [poly(HPMA-*co*-Ma-Acap-ATTO488-*co*-Ma-Acap-AP)] were isolated by precipitation into ethyl acetate, filtered off, washed with diethyl ether, and dried in a vacuum. The polymer conjugate was purified on a Sephadex LH-20 chromatography column in methanol. Methanol was evaporated, and the conjugate was dissolved in water and purified on a PD-10 chromatography column, then lyophilized. Yield of the aminoreactive HPMA copolymer was 50 mg (pol). Yield of the quenched polymer was 5 mg (**pol**). The Atto488 content was 4.7 wt% and 17 mol% of TT. M_w and $Đ$ were determined as follows: M_w = 42,100 g/mol, $Đ$ = 1.3.

*Synthesis of fluorescent HPMA polymer with GCPII inhibitor (***pol-inh**, pol-inh*)*

Synthesis was very similar to that of polymers without GCPII inhibitor. In the first step, GCPII inhibitor (compound **1**, 9 mg, 8.27×10^{-6} mol) was added to the mixture, and the amount of DIEA was increased correspondingly (7.8 µl, 4.47×10^{-5} mol). Yield of the aminoreactive copolymer with GCPII inhibitor was 45 mg (pol-inh). Yield of the polymer quenched with AP was 5 mg (**pol-inh**). The GCPII inhibitor content was 14.1 wt%, Atto488 content was 4.7 wt% and 10 mol% of TT remained on the polymer. The M_w and $Đ$ were determined as M_w = 47,600 g/mol, $Đ$ = 1,3.

Characterization of prepared particles

Fluorescence spectra measurement

Fluorescence of particles (labeled by Atto488 or Alexa Fluor 488) was determined by measuring the difference in fluorescence of 10 nM or 50 nM solutions of particles, analyzed using a TECAN infinite plate reader (M 1000), with excitation at 500 nm and emission at 520 nm.

Electron microscopy

For morphology analysis, MPyV and Qβ particles were visualized by negative staining. Samples (10 µL, 50 µg/mL) were adsorbed on carbon-coated formvar copper grids (Electron Microscopy Sciences). Grids were washed twice in redistilled H_2O and then contrasted on two drops of 2% solution of phosphotungstic acid (pH 7.2). To prepare sample of NDs, carbon-coated copper grids were placed into a UV-ozonizing chamber (UV/Ozone Pro Cleaner Plus, Bioforce Nanosciences) for 15 min. Then, a droplet of poly(ethyleneimine) (2.5 kDa, 0.1 mg/ml) was placed on the grid. After 10 min incubation, it was removed with a piece of tissue. Then, a droplet of aqueous solution of NDs (50 µg/ml) was placed on the grid, and after 3 min incubation, the liquid was removed with a piece of tissue.[8]

The grids were visualized with a JEOL JEM-1011 transmission electron microscope operated at 80 kV.

TEM image analysis

Analysis of particle size distributions was performed with ImageJ software.[9] Because the ND particles are of irregular shape, we used equivalent circular diameter to express their size.[8] To facilitate comparison of the data, we used the same approach for all NPs.

Equivalent circular diameter (d_{eq}) of a particle is defined as the diameter of a circular particle with the same area as the particle of interest (S), as described by the equation

$$d_{eq} = \sqrt{4S/\pi} \qquad \text{(S1)}$$

The data were plotted as the volume-weighted histogram, in which the contribution of each particle in the distribution relates to the volume of that particle (equivalent to mass for samples of uniform density); the relative contribution of a particle is proportional to the cube of its size. Particle volumes (PV) used for the construction of volume-weighted histograms were calculated as if the particles were spheres of diameter equal to the circular equivalent diameter as described by the equation

$$PV = \frac{4}{3}\pi\left(\frac{d_{eq}}{2}\right)^3 \qquad \text{(S2)}$$

The particle diameters and the respective standard deviations indicated in the manuscript were obtained by fitting of the volume-weighted histograms with Gaussian function.

Stability test – dynamic light scattering (DLS) measurement
DLS was recorded with a Zetasizer Nano ZS system (Malvern Instruments) at different temperatures with 10-min equilibration. Sample concentrations were 0.25 mg/ml.

SDS-PAGE analysis
For electrophoretic analysis, 2 µg of particles was mixed with 2.5 µl of 100 mM dithiothreitol (DTT), 2.5 µl of 10 M urea, and 2 µl of sample buffer (50 mM Tris, pH 6.8, 25% 2-mercaptoethanol, 5% sodium dodecyl sulfate, 0.005% bromophenol blue) in the total sample volume of 10 µl and incubated for 10 min at 70 °C to disassemble the particles. All samples were separated on SDS-PAGE in MOPS buffer (NuPAGE Novex 4-12% Bis-Tris precast polyacrylamide gels, ThermoFisher Scientific). The running time in SDS-PAGE was 50 min with constant voltage 200 V. The gel was scanned for Alexa Fluor 488 (excitation at 488 nm) fluorescence on a PharosFX Molecular Imager (Bio-Rad). Subsequently, proteins were stained with colloidal Coomassie dye G-250 according to the manufacturer's protocol (GelCode Blue Stain Reagent, ThermoFisher Scientific). The Coomassie-stained SDS-PAGE gel was scanned on a Molecular Imager GS 800 densitometer (Bio-Rad).

Preparation of cell lines with switchable GCPII expression
U251 MG cell lines with switchable GCPII expression were prepared analogously as described by Tykvart *et al*. for HEK cell lines.[10] Briefly, U251 MG cells (supplied by ATCC as U373 MG cells) were stably transfected with pTet-Off® Advanced vector (Clontech) using FuGENE® HD transfection reagent (Roche). Geneticin selection was applied and monoclonal populations were prepared using cloning rings. First, individual clones were tested for the ability to alter expression by addition of doxycycline using transient transfection with GFP. The clone with highest expression difference in ON and OFF state (after addition of doxycycline) was selected for further cotransfection with pTRE-Tight-GCPII and pPUR vector (Clonetech) using similar transfection conditions. Similarly, after puromycin selection and clone preparation using cloning rings, the resulting U251 +/- MG clone with highest/lowest GCPII expression was chosen.

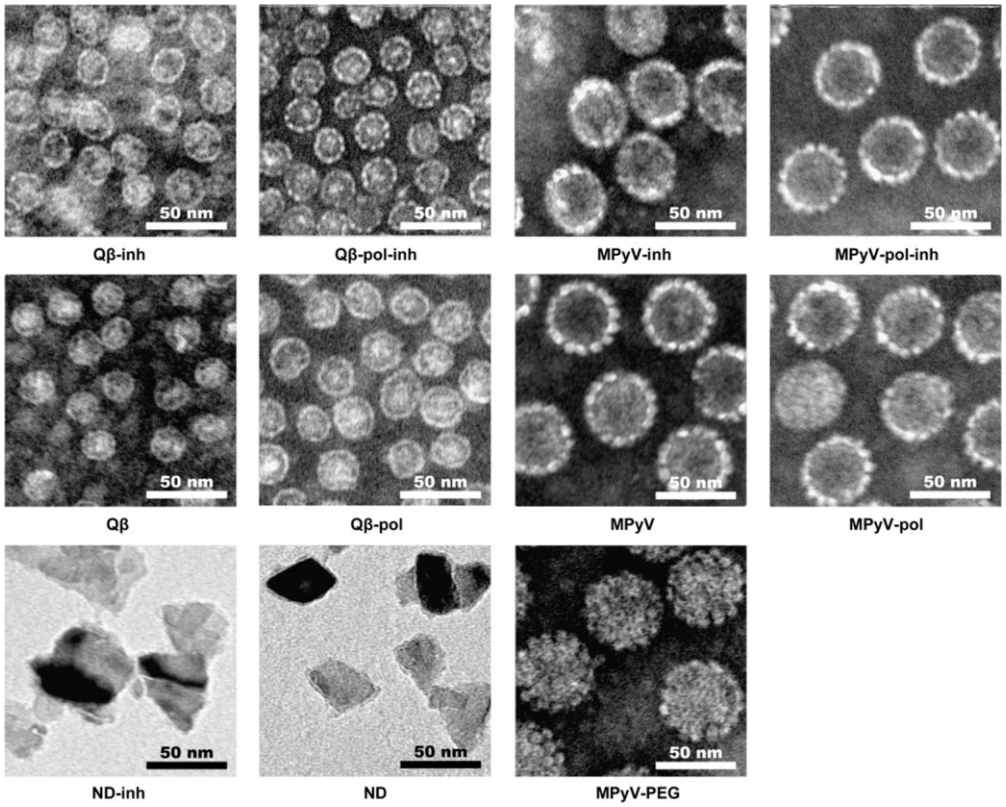


Figure S1. Characterization of all NPs by transmission electron microscopy.

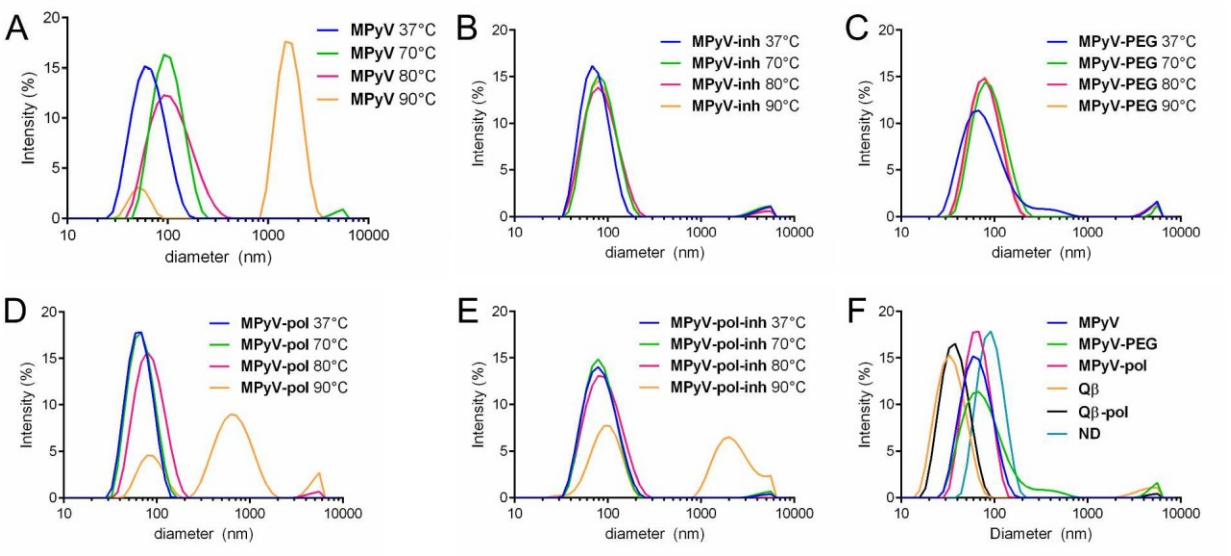


Figure S2. Measurement of the temperature stability of modified NPs in storage buffer (pH 7.4) by dynamic light scattering. Tested temperatures were 37, 70, 80, and 90 °C; the incubation time was 10 min. (A) **MPyV**, (B) **MPyV-inh**, (C) **MPyV-PEG**, (D) **MPyV-pol**, and (E) **MPyV-pol-inh**. (F) Size distribution of all NPs without conjugated inhibitor at 37 °C (negative controls).

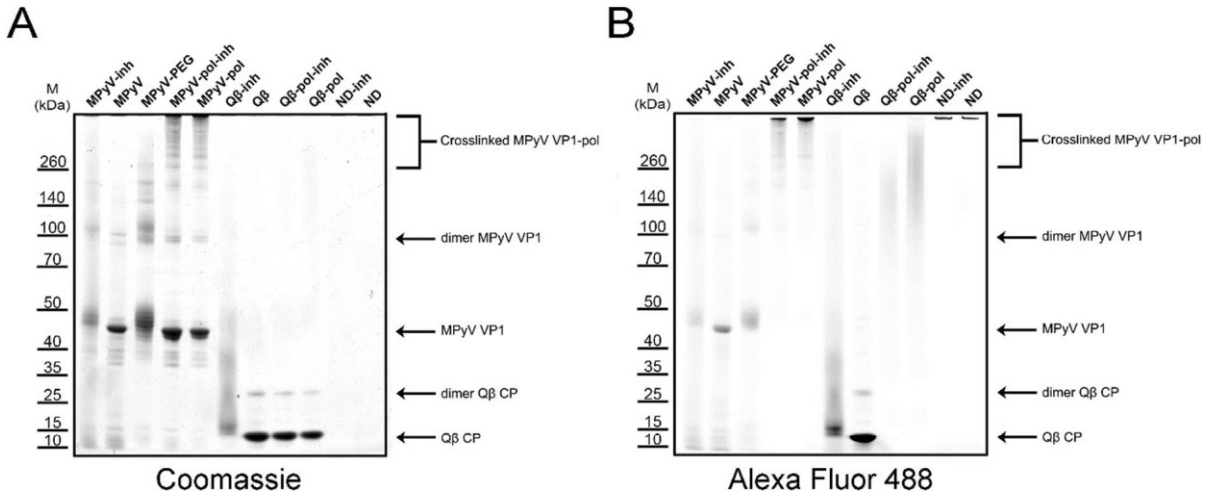


Figure S3. Characterization of all nanoparticles using SDS-PAGE. The gel was (A) stained with Coomassie brilliant blue and (B) scanned for Alexa Fluor 488 fluorescence. The molecular weights are indicated on the left. The particles were disassembled by treatment with dithiothreitol and urea.

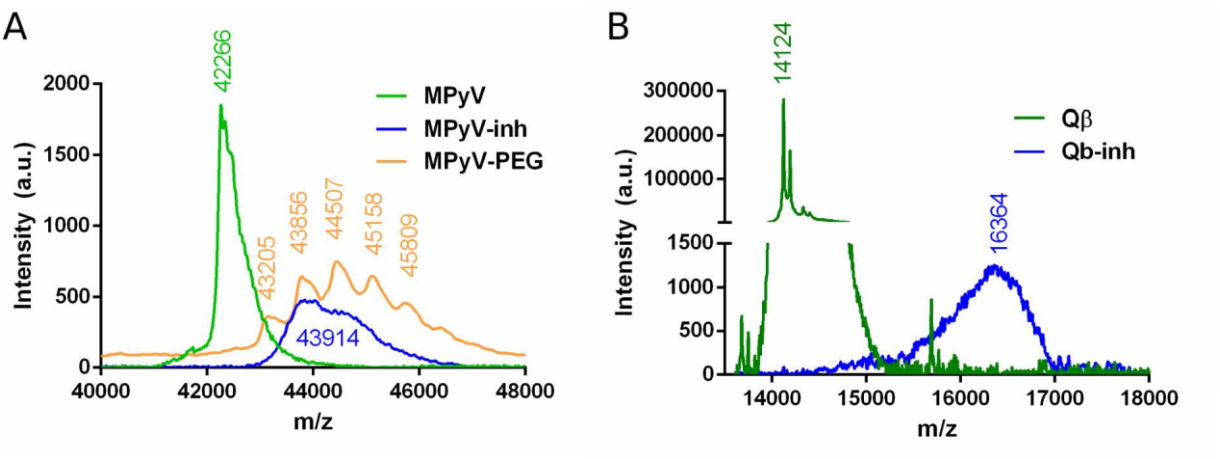


Figure S4. MALDI measurement of disassembled VLPs to subunits. (A) MPyV (MPyV, **MPyV-inh**, **MPyV-PEG**) and (B) Qβ (Qβ, **Qβ-inh**). The marked peaks indicate the corresponding mass.

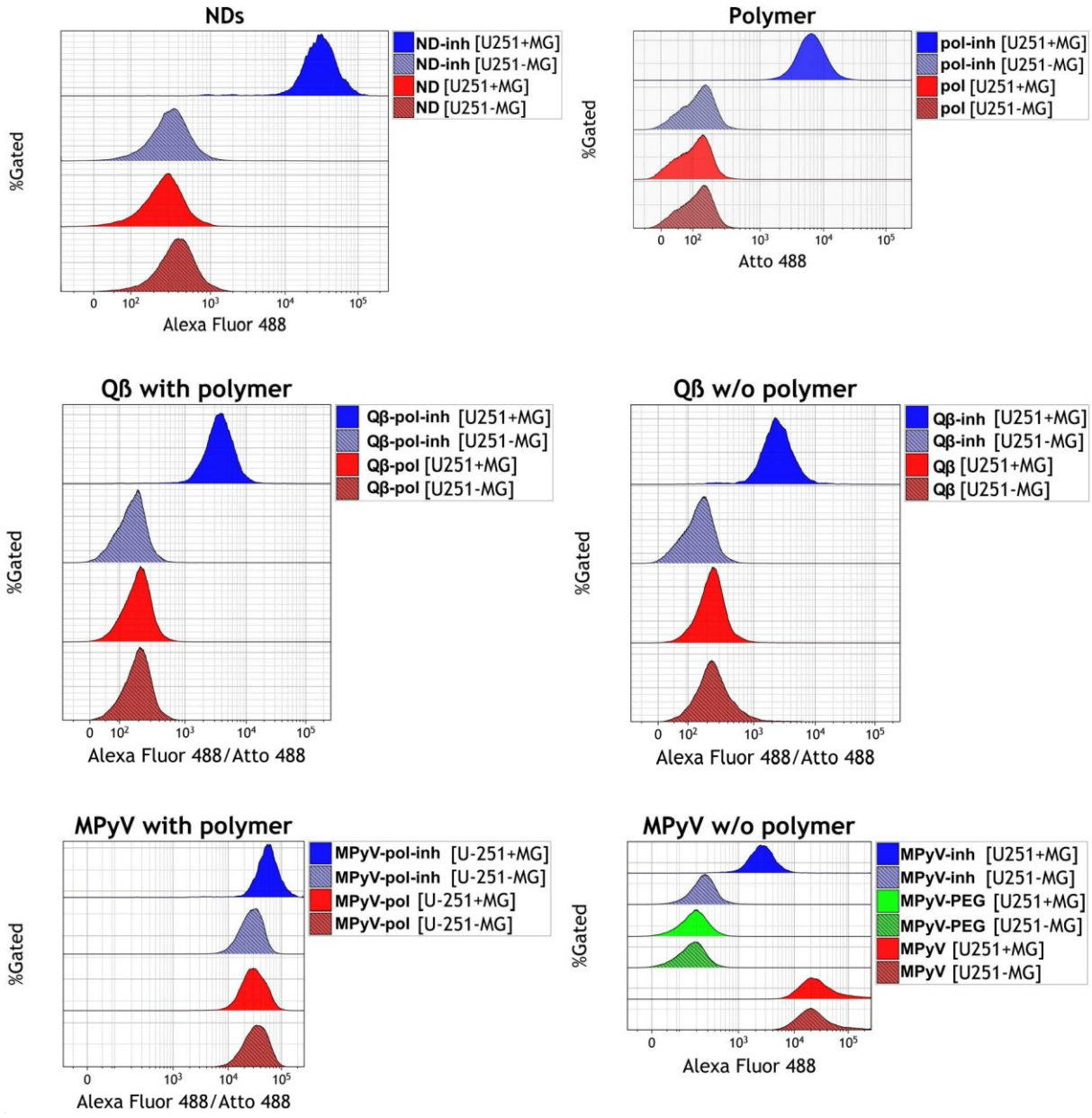
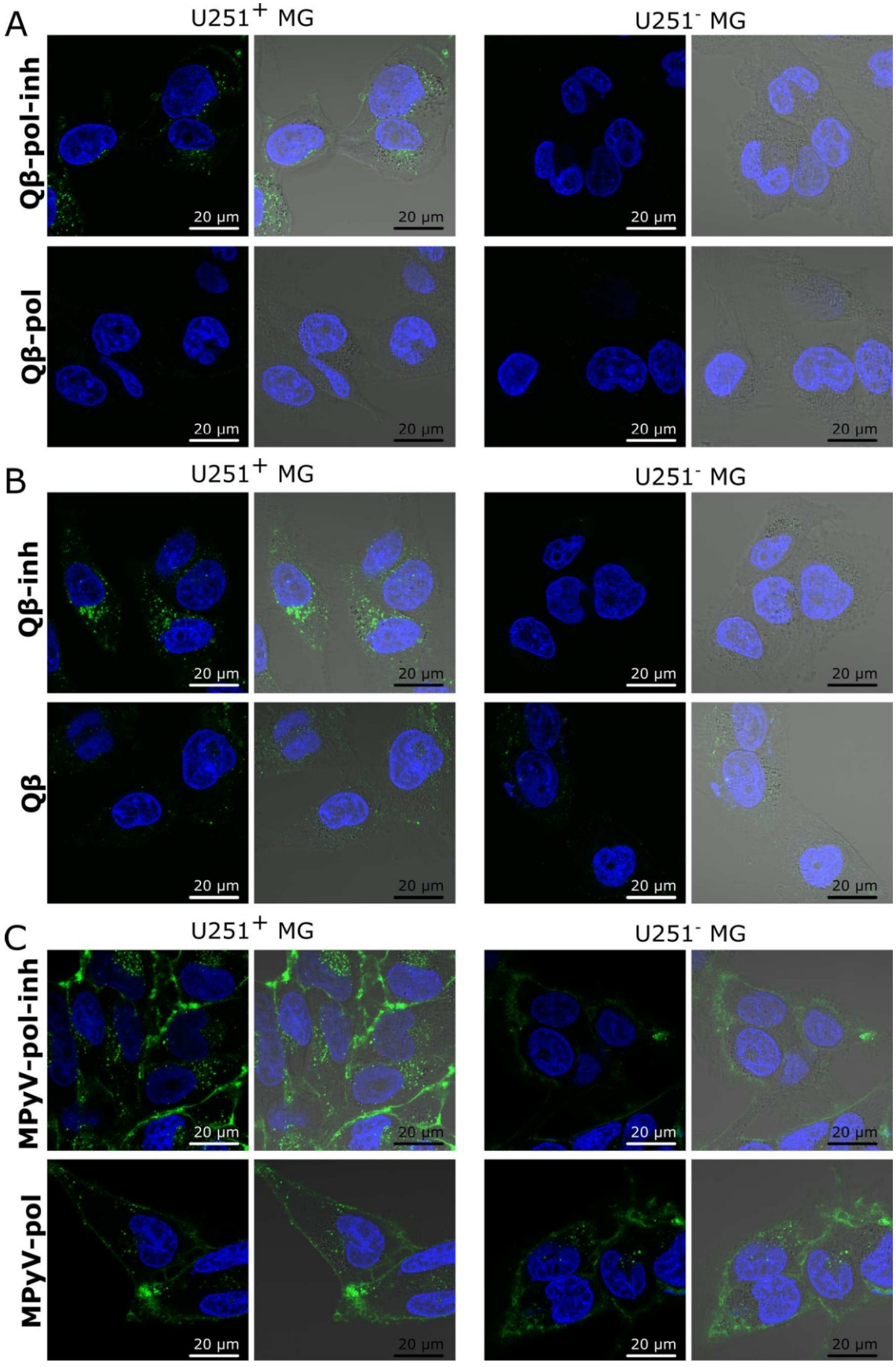


Figure S5. Flow cytometry measurements presented as histogram overlays. Merged data from triplicates are shown for each sample. Cells either with (U-251^{+} MG; solid fills) or without (U-251^{-} MG; hatched fills) GCPII expression were used. The blue histograms denote data for NPs with inhibitor, red for NPs without inhibitor, and green for MPyV VLPs with PEG (additional control). Data were analyzed with Kaluza Analysis Software (v1.5a).


A
U251+ MG
U251- MG
Qβ-pol-inh
Qβ-pol
B
U251+ MG
U251- MG
Qβ-inh
Qβ
C
U251+ MG
U251- MG
MPyV-pol-inh
MPyV-pol
20 µm

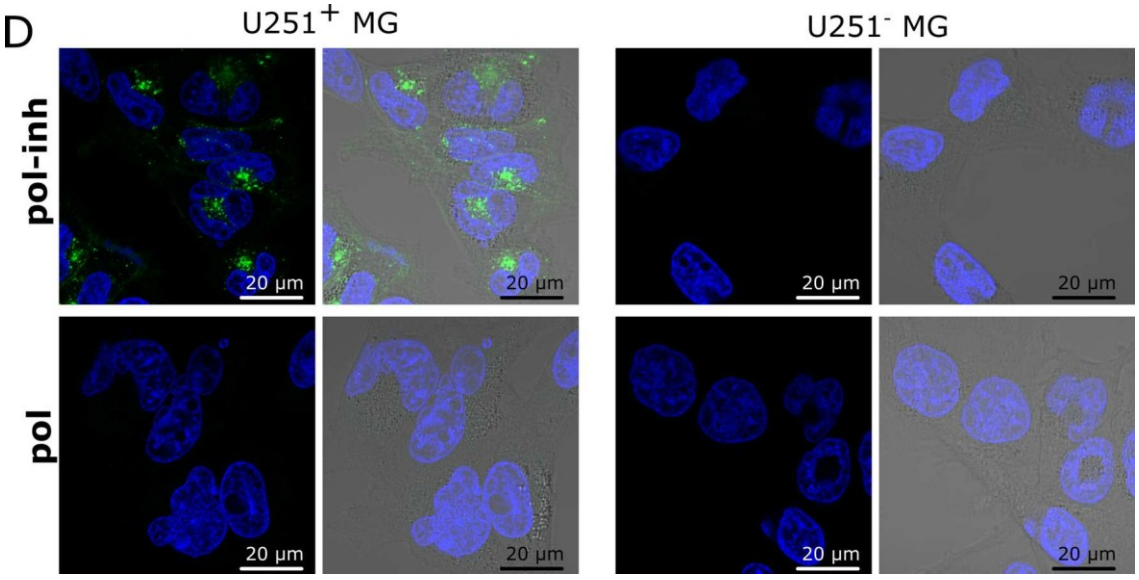


Figure S6. Confocal images of NP uptake by U-251 MG cells. Cells with (U-251^{+} MG) or without (U-251^{-} MG) GCPII expression were incubated with NPs for 1 h. Binding of (A) poly(HPMA)-coated Qβ VLPs, (B) Qβ VLPs, (C) poly(HPMA)-coated MPyV VLPs, and (D) polymer VLPs. Confocal sections of representative cells with corresponding signals in green (NPs conjugated with Alexa Fluor 488 or Atto 488) and blue (nuclei stained with Hoechst) channels are shown. Merge is composed of both channels and bright field image.

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