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Supplemental Information

Antitumoral Cascade-Targeting Ligand for IL-6 Receptor-Mediated Gene Delivery to Glioma

Shanshan Wang, Sören Reinhard, Chengyi Li, Min Qian, Huiling Jiang, Yilin Du, Ulrich Lächelt, Weiyue Lu, Ernst Wagner, and Rongqin Huang

Supplemental Information

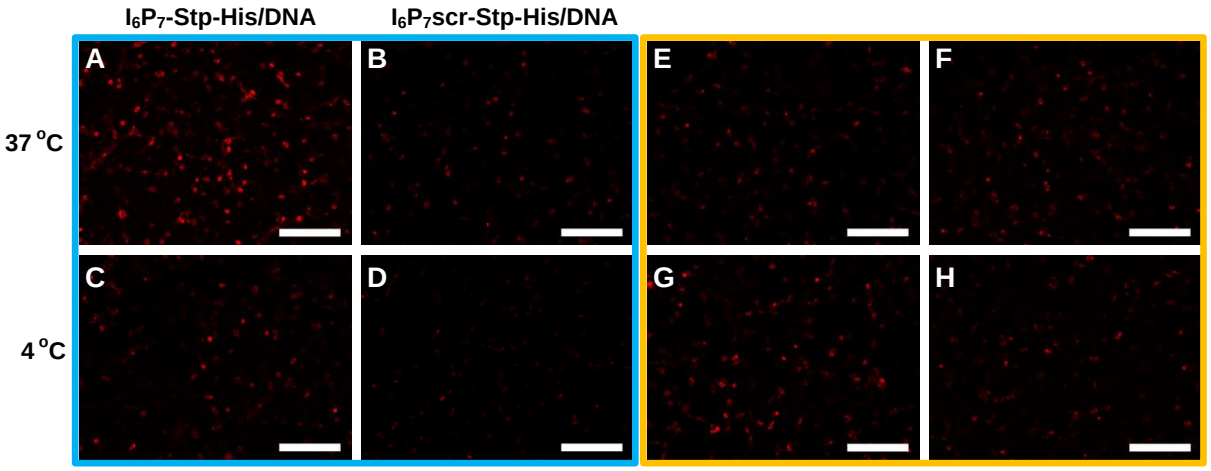


Figure S1 Exploration of cellular uptake mechanism. Cellular uptake of I_6P_7-Stp-His/DNA (A, C) and I_6P_7scr-Stp-His/DNA (B, D) into U87 cells after 1 h at 37 °C (A-B) or 4 °C (C-D). Cellular uptake of I_6P_7-Stp-His/DNA into U87 cells pretreated with 100× excess I_6P_7 (E), PhAsO (F), filipin (G) or colchicine (H) after 1 h at 37 °C. DNA was pre-labeled with YOYO-3. Red: YOYO-3. Scale bar=200 μm.

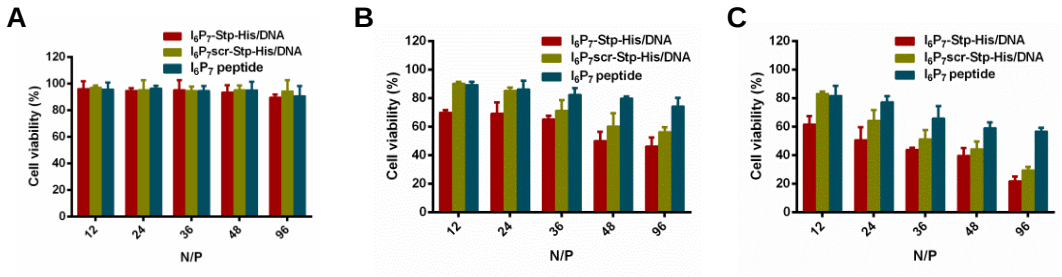


Figure S2 Cell inhibiting ability of the NPs and I_6P_7 peptide on U87 cells. Cell viability of U87 cells after 24 h (A), 48 h (B) and 72 h (C) incubation with I_6P_7-Stp-His/DNA, I_6P_7scr-Stp-His/DNA NPs (at indicated N/P ratios) and I_6P_7 peptide. Data are expressed as mean ± S.D. (n = 4).

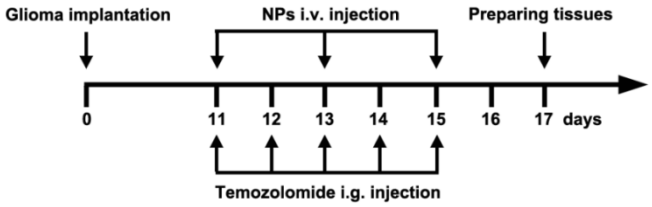
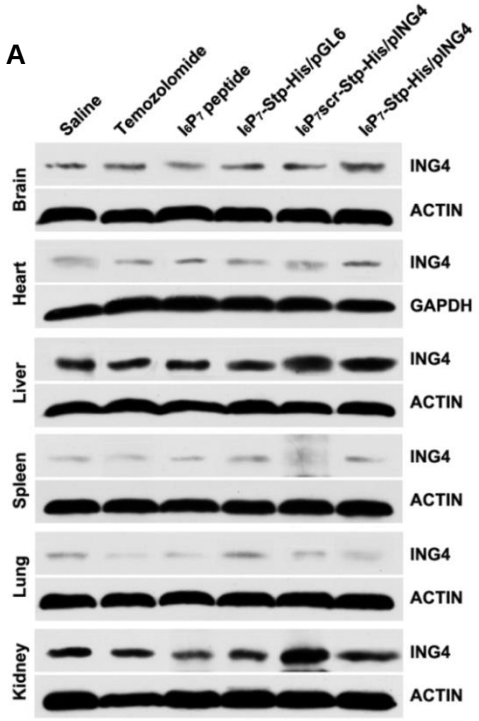


Figure S3 A time line illustrating the time points of NPs i.v. injections and tissue analysis.



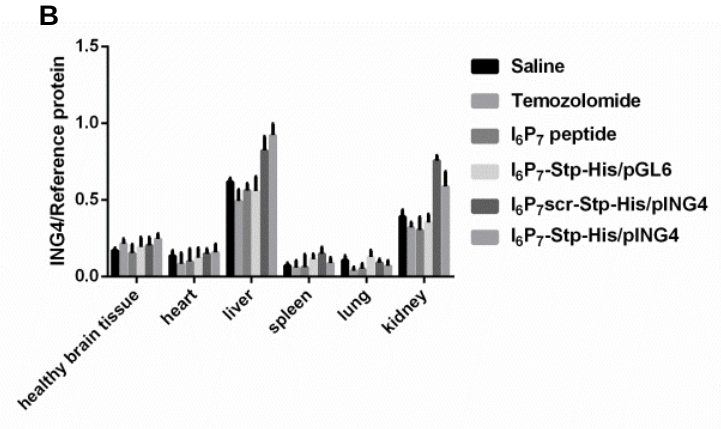


Figure S4 Western blot analysis. (A) Western blot analysis for ING4 expression in tissues for different experimental animal groups and (B) quantitative analysis of the protein levels. Data are expressed as mean ± S.D. (n = 3).

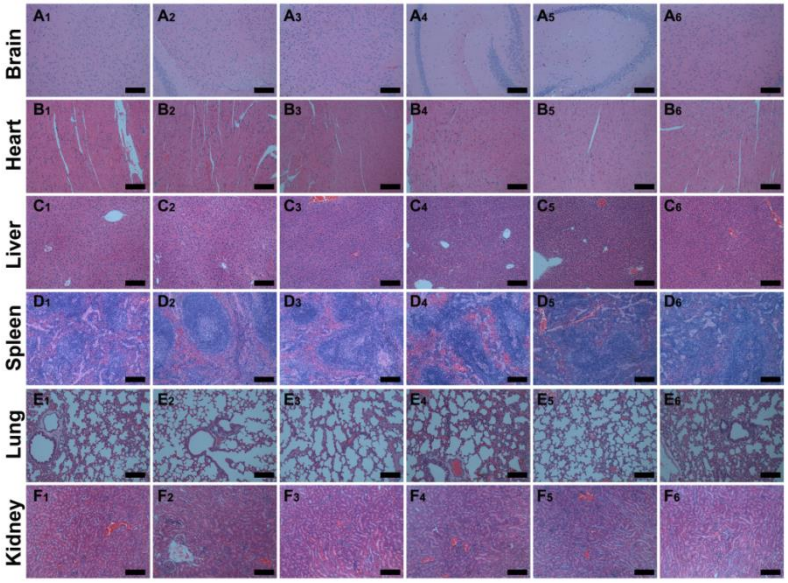


Figure S5 Safety evaluation of the NPs. Histopathological analysis of the brain, heart, liver, spleen, lung and kidney sections stained with hematoxylin and eosin of glioma-bearing mice post i.v. administration of saline (A_1-F_1), temozolomide (A_2-F_2), I_6P_7 peptide (A_3-F_3), I_6P_7-Stp-His/pGL6 (A_4-F_4), I_6P_7scr-Stp-His/pING4 (A_5-F_6) and I_6P_7-Stp-His/pING4 (A_6-F_6). Scale bar = 200 μm.

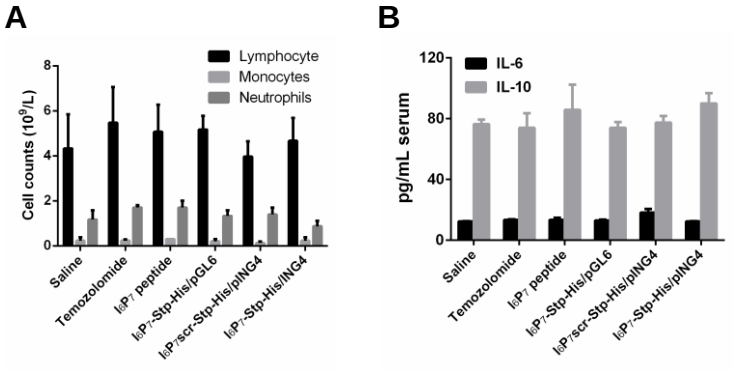


Figure S6 Analysis of haematopoietic cell counts and serum inflammatory cytokine levels of treated mice. After intravenous administration with all preparations, the haematopoietic cell (lymphocyte, monocyte and neutrophils) counts were recorded (A) and IL-6 and IL-10 levels in serum were measured (B). Data are expressed as mean ± S.D. (n = 3).

Supplemental Oligomer Synthesis and Physicochemical Characterizations

Materials

Resins were purchased from Novabiochem GmbH (Hohenbrunn, Germany). Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), syringe reactors (PP reactor with PE frit) were purchased from Multisyntech GmbH (Witten, Germany). All protected (L) amino acids, peptide grade dimethylformamide (DMF), N,N-diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were purchased from Iris Biotech (Marktredwitz, Germany). Fmoc-N-amido-dPEG24-acid was purchased from Quanta Biodesign (Powell, USA).

Loading of a 2-Chlorotrityl Chloride Resin with Fmoc-*L*-Leu-OH

2-Chlorotrityl chloride resin (600 mg, 0.94 mmol chloride) was swollen in water-free DCM for 10 min. Fmoc-*L*-Leu-OH (0.25 eq) and DIPEA (1.5 eq) were added to the resin for 1 h. The reaction solvents were removed and DCM/MeOH/DIPEA (80/15/5) was added for 30 min. After drainage of the reaction mixture, the resin was washed three times with DMF and DCM.

To determine the loading of the resin, a defined amount of resin was treated with 1 mL deprotection solution (20% piperidine in DMF) for 1 h. Afterwards, the solution was diluted and absorption A was measured at 301 nm. The loading was then calculated according to the equation: resin load [mmol/g] = (A*1000)/(m [mg]*7800*d_f) with d_f as dilution factor.

For Fmoc deprotection the resin was treated twice with 20% piperidine in DMF for 10 min each and twice with 20% piperidine in DMF with 2% DBU for 5 min. Reaction progress was monitored by Kaiser tests. Afterwards the resin was washed with DMF and DCM and dried *in vacuo*.

Syntheses of Oligomers I_6P_7-Stp-His and I_6P_7scr-Stp-His

For the random creation of a scrambled sequence of I_6P_7 a computer generated permutation was obtained from an online sequence generator (RANDOM.ORG). After swelling of 0.030 mmol of Fmoc-Leu-chlorotrityl resin in DMF for 30 min, the structures I_6P_7-Stp-His (HO-LRTILSL-PEG_{24}-H-K-α,ε[H-(Stp-H)$_4$-C-NH_2]$_2$) and I_6P_7scr

(HO-LSLRLTI-PEG_{24}-H-K-α,ε[H-(Stp-H)$_4$-C]$_2$) were synthesized with a Syro Wave synthesizer (Biotage, Uppsala, Sweden).

Double couplings were performed with properly protected Fmoc-*L*-amino acids (4 eq) (or Fmoc-PEG_{24}-COOH respectively), DIPEA (8 eq) and HBTU/HOBt (4 eq) in DMF for 60 min. For Fmoc deprotection the resins were treated five times with 20% piperidine in DMF after each double coupling. The resins were washed with DMF (6x) after each double coupling and deprotection. After completion of the synthesis, the resins were washed three times with DCM and dried *in vacuo*.

Cleavage Conditions

The polymers were cleaved from the resin by treatment with 10 mL per g resin cleavage solution TFA/water/TIS (95:2.5:2.5) for 1-2 h. The resins were filtered off and washed twice with TFA. The combined filtrates were concentrated in N2 flow and precipitated by dropwise addition into ice-cold MTBE/n-hexane (1:1). The precipitates were collected by centrifugation. The precipitates were dissolved in water/acetonitrile (7:3) with 10 mM HCl and purified with size exclusion chromatography (Sephadex G10 column). The collected fractions were frozen in $N2_{liq}$ and lyophilized.

Proton NMR Spectra

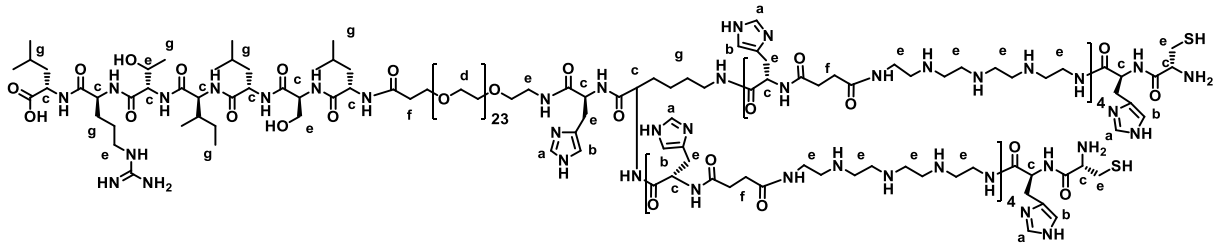
^{1}H NMR spectra were recorded using a Jeol JNMR-GX 400 (400 MHz) by Jeol. All spectra were recorded without TMS as internal standard and therefore all signals were calibrated to the residual proton signal of the solvent. The coupling constant had an accuracy of 0.3 Hz. Chemical shifts are reported in ppm and refer to the solvent as internal standard (D_2O at 4.80). Data are reported as s = singlet, d = doublet, t = triplet, m = multiplet; integration was performed manually. The spectra were analyzed using MestreNova (Ver. 9.0.1-13254 by MestReLab Research).

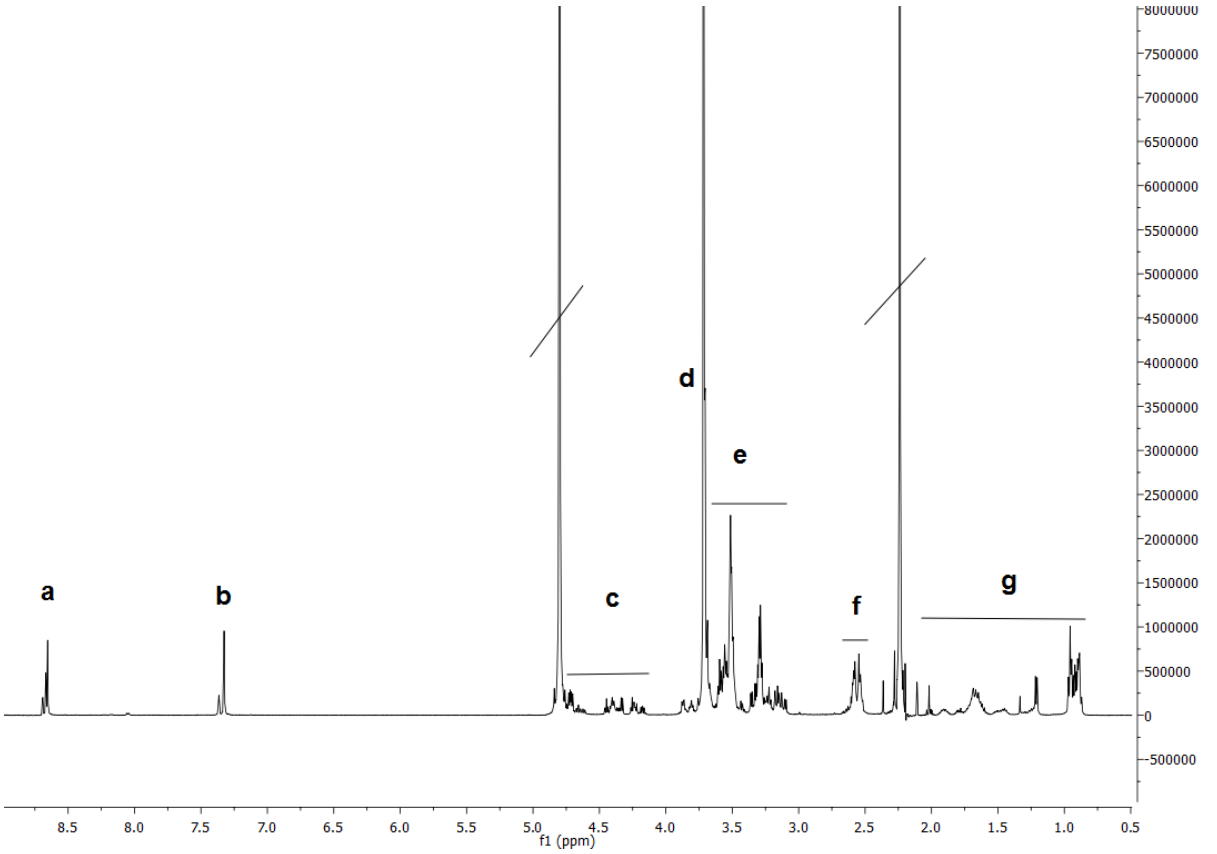
^{1}H Proton NMR Spectra

All ^{1}H NMR spectrum were recorded at 400 MHz in D_2O.

I_6P_7-Stp-His:

Sequence (C→N): LRTILSL-PEG_{24}-H-K-α,ε[H-(Stp-H)$_4$-C]$_2$

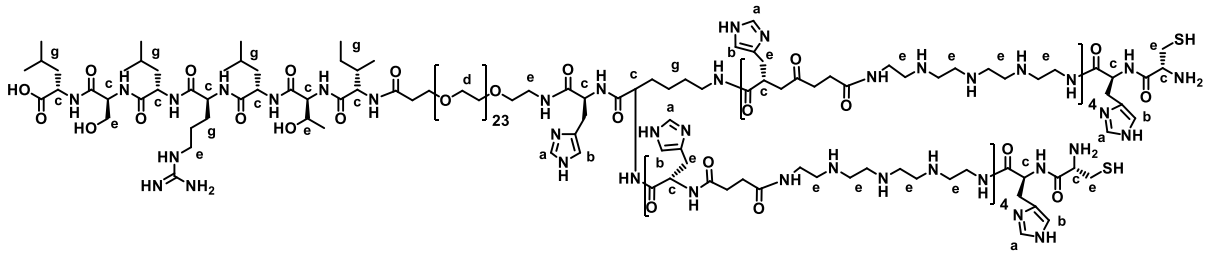


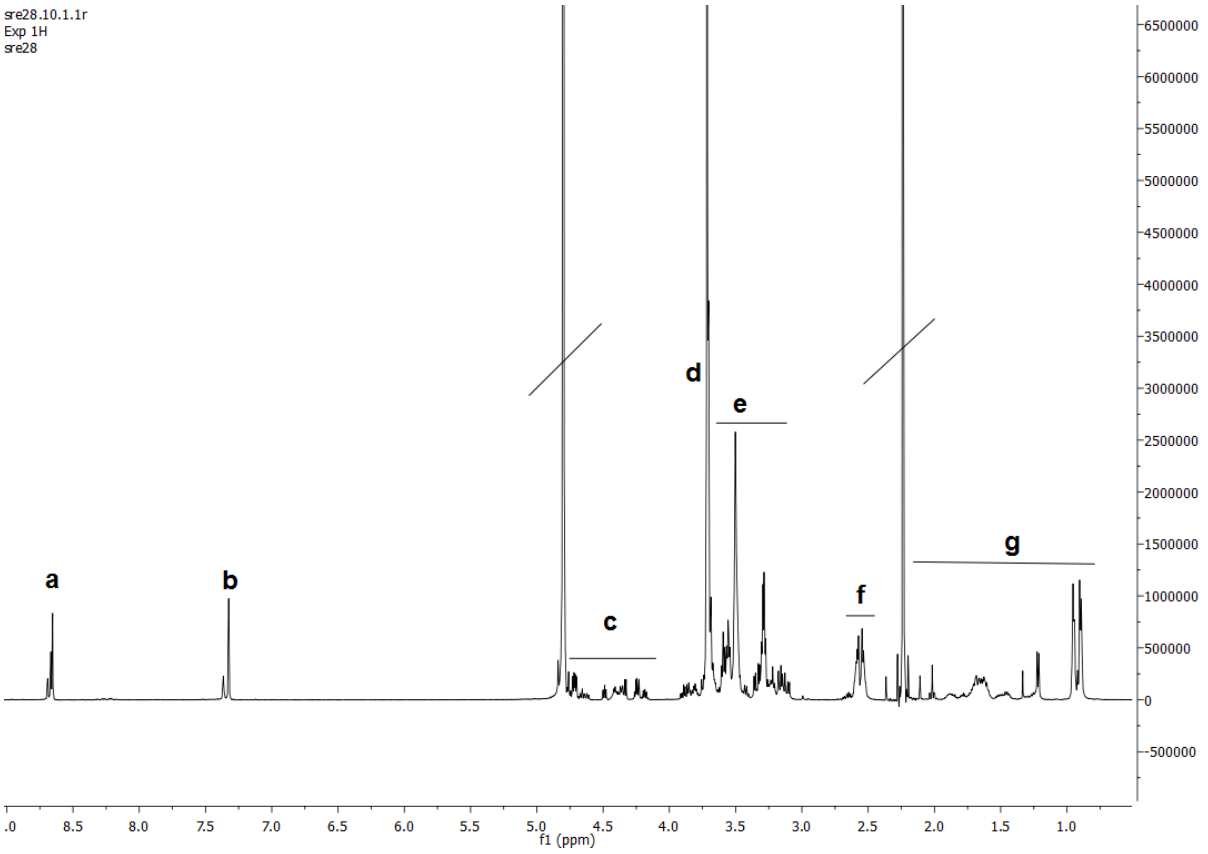


δ (ppm) = 0.85-2.0 (m, 85 H, βγH arginine, βγδH isoleucine, βγδH leucine, γH threonine, βγδH lysine), 2.4-2.6 (m, 34 H, -CO-CH2-CH2-CO- succinic acid, -CO-CH2- dPEG24), 2.8- 3.5 (m, 149 H, -CH2- tepa, δH arginine, βH cysteine, βH histidine, εH lysine, βH serine, -CH2-N- dPEG24), 3.65-3.75 (s, 146 H, -CH2-O- dPEG24), 4.1-4.75 (m, 29 H, αH amino acids), 4.80 (s, HDO), 7.2-7.4 (d, 10 H, aromatic H histidine), 8.6-8.7 (m, 9 H, aromatic H histidine).

I_6P_7scr-Stp-His:

Sequence (C→N): LSLRLTI-PEG_{24}-H-K-α,ε[H-(Stp-H)$_4$-C]$_2$





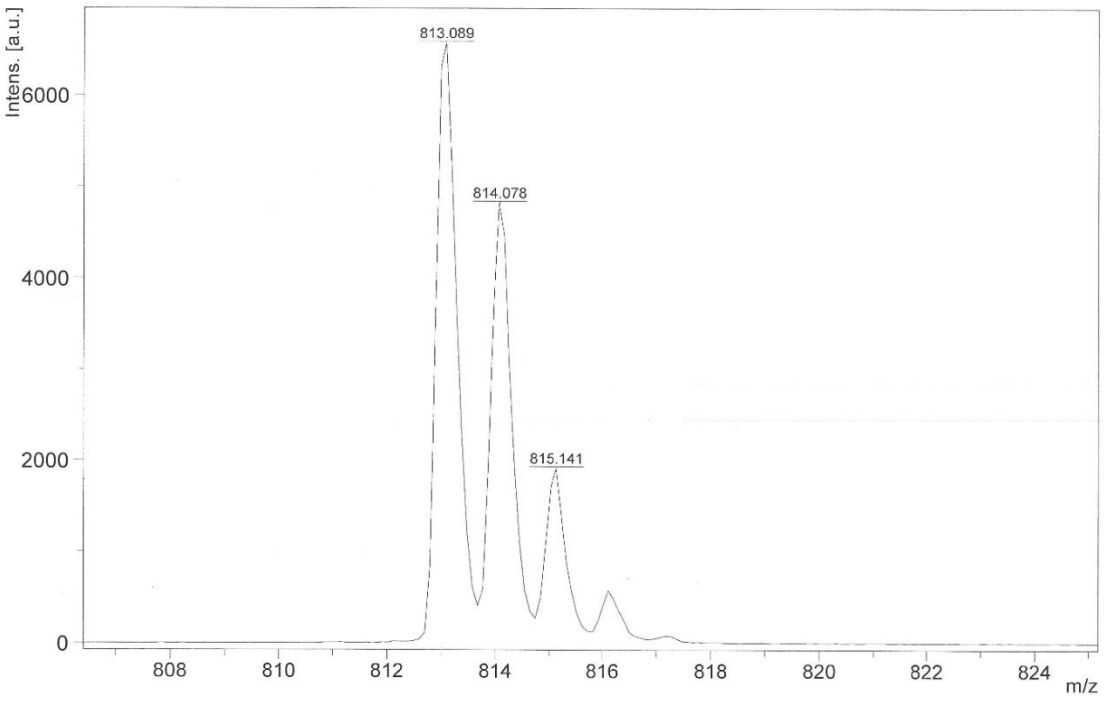
δ (ppm) = 0.85-2.0 (m, 95 H, βγH arginine, βγδH isoleucine, βγδH leucine, γH threonine, βγδH lysine), 2.4-2.6 (m, 34 H, -CO-CH2-CH2-CO- succinic acid, -CO-CH2- dPEG24), 2.8- 3.5 (m, 162 H, -CH2- tepa, δH arginine, βH cysteine, βH histidine, εH lysine, βH serine, -CH2-N- dPEG24), 3.65-3.75 (s, 171 H, -CH2-O- dPEG24), 4.1-4.75 (m, 28 H, αH amino acids), 4.80 (s, HDO), 7.2-7.4 (d, 11 H, aromatic H histidine), 8.6-8.7 (m, 10 H, aromatic H histidine).

Consistent with MS analysis, the oligomer batches (crude products) contained a minor fraction of smaller truncated oligomers in addition to the full-length sequences.

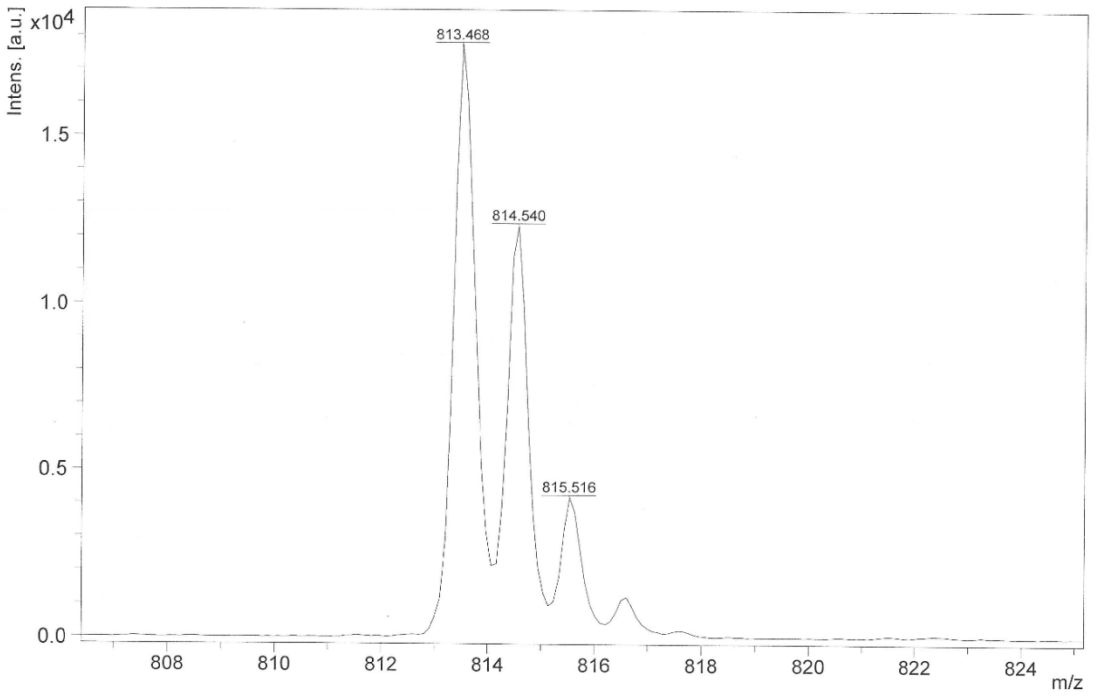
Mass spectrometry

Polymer (10 mg) was dissolved in 1 mL H_2O. Of this solution, 1 µL was spotted on a 1 µL matrix droplet consisting of a dried solution of 2,5-dihydroxybenzoic acid (DHB) in 30:70 [v/v] acetonitrile : 0.1% TFA in water (c = 20 mg/ml). Samples were analyzed using an Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). For one sample spectrum, 150 spectra of respective probes were averaged.

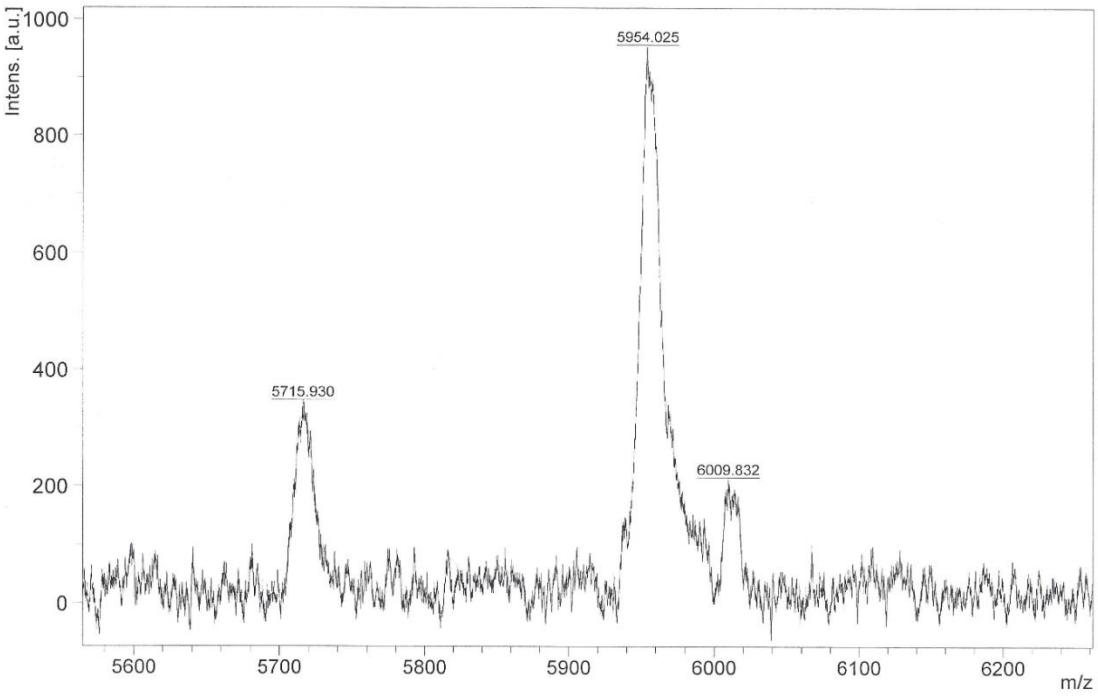
Structure	Calculated (m/z)	Found (m/z)
I_6P_7 ligand	814.53	814.08
I_6P_7scr ligand	814.53	814.54
I_6P_7-Stp-His	5953.56	5954.03
I_6P_7scr-Stp-His	5953.56	5952.77



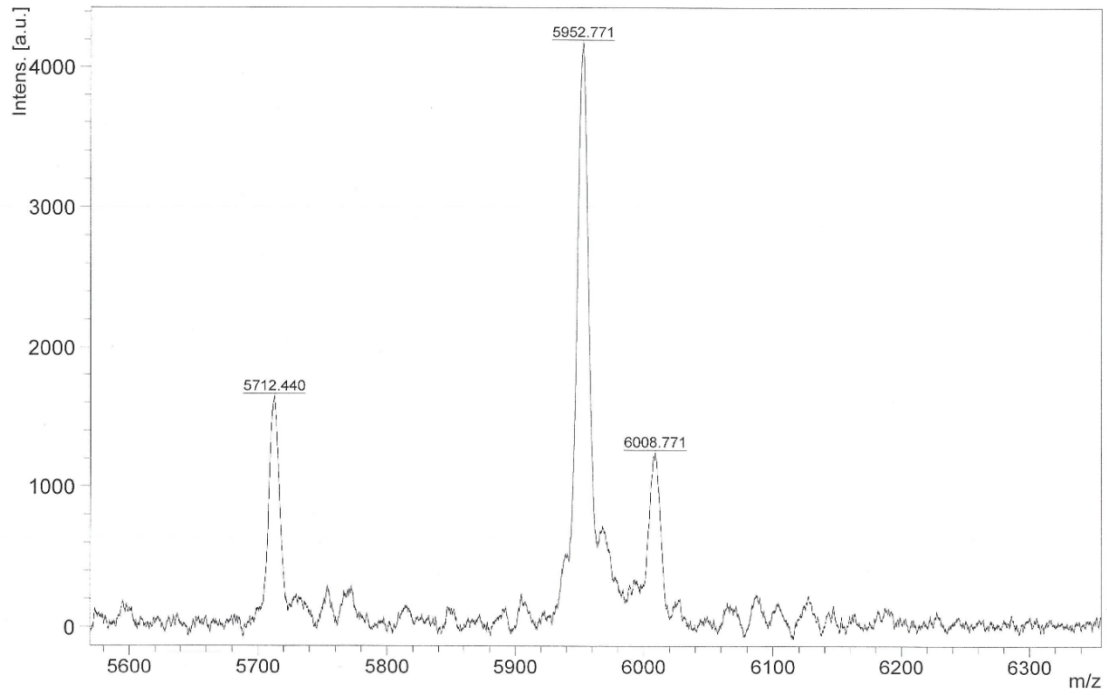
MALDI-spectra of I_6P_7



MALDI-spectra of I_6P_7scr



MALDI-spectra of I_6P_7-Stp-His



MALDI-spectra of I_6P_7scr-Stp-His