Electronic Supplementary Material (ESI) for Nanoscale.

SUPPORTING INFORMATION

Histidine-rich stabilized polyplexes for cMet-directed tumor-targeted gene transfer

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EXPERIMENTAL SECTION

pDNA binding assay by electrophoresis

For pDNA gel-shift assay, a 1 % agarose gel was prepared by dissolving agarose in TBE buffer (trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g, in 1 L of water) and boiling it up to 100 °C. After addition of GelRed for the detection of the nucleic acid, the agarose solution was casted in the electrophoresis unit and left to form a gel. Polyplexes were prepared as described above containing 200 ng of pDNA in 20 µL HBG. If indicated, increasing amounts of heparin (100 I.E. or 500 I.E.) or TCEP (4 mM) were added to the sample after completed polyplex formation. For evaluation of long-term stability in serum, the polyplexes were performed as described in *Methods* "pDNA polyplex stability in 90 % serum via gel shift assay" section but were further incubated for 24 h, 48 h or 72 h. Next, 4 µL of loading buffer (prepared from 6 mL of glycerol, 1.2 mL of 0.5 M EDTA, 2.8 mL of H_2O, 0.02 g of bromphenol blue) were added to each sample before they were placed into the sample pockets. Electrophoresis was performed at 120 V for 80 min.

Determination of HGF receptor/c-Met levels

For each tested cell line 80 000 cells in 100 µL FACS buffer (10% FBS in PBS) were incubated with the monoclonal mouse anti-human HGFR/c-Met antibody (1:200 dilution) or IgG control for mouse primary antibodies (1:100 dilution) for 1 h on ice and subsequently washed twice with FACS buffer. The cells were then stained with Alexa 488-labeled goat anti-mouse (1:400 dilution) secondary antibody for 1 h on ice, washed, counterstained with DAPI (1 µg/mL) and analyzed on a Cyan™ ADP flow Cytometer (Dako, Hamburg, Germany) using Summit™ acquisition software (Summit, Jamesville, NY, USA). DAPI fluorescence was excited at 405 nm and detected with a 450/50 bandpass filter, Alexa-488 fluorescence was excited at 488 nm and detected with a 530/40 nm bandpass filter. The percentage of HGFR positive cells was determined as compared to control IgG stained cells. The mean fluorescence intensity (MFI) corresponds to the arithmetic mean of the living cell population.

Cellular association

Cells were seeded into 24-well plates coated with collagen at a density of 50 000 cells/well. After 24 h, culture medium was replaced with 400 µL fresh growth medium. pDNA polyplexes (N/P 12) in 100 µL HBG, containing 1 µg pDNA (20% of the nucleic acid was Cy5-labeled) were added to each well and incubated on ice for 30 min. Subsequently, cells were washed twice with 500 µL PBS. Cells were detached with trypsin/EDTA and taken up in PBS with 10% FCS. Cellular association was assayed by excitation of Cy5 at 635 nm and detection of emission at 665 nm. Cells were appropriately gated by forward/sideward scatter and pulse width for exclusion of doublets. DAPI (4',6-diamidino-2-phenylindole) was used to discriminate between viable and dead cells. Data were recorded by Cyan™ ADP flow Cytometer (Dako, Hamburg, Germany) using Summit™ acquisition software (Summit, Jamesville, NY, USA).

***In vitro* gene transfer**

DU145 and Huh7 cells were seeded 24 h prior to pDNA delivery using 10 000 or 8 000 cells per well in 96-well plates. Transfection efficiency of oligomers was evaluated using 200 ng pCMVLuc per well. All experiments were performed in quintuplicate. Before transfection, medium was replaced with 80 µL fresh medium containing 10% FCS. Polyplexes formed in 20 µL HBG in sterile Eppendorf caps at 25 °C were added to each well and incubated on cells for 45 min at 37 °C, followed by incubation with fresh medium containing endosomolytic agent chloroquine at concentration of 100 µM (for control experiments without chloroquine only fresh medium was added). After 4 h medium was again replaced by fresh medium and cells were further incubated for 20 h. LinPEI at nontoxic optimum N/P 6 with 4 h longer polyplex incubation on cells was used as positive control, HBG buffer was used as negative control. For all experiments 24 h after transfection, cells were treated with 100 µL cell lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity in the cell lysate was measured using a luciferase assay kit (100 µL

Luciferase Assay buffer, Promega, Germany) and a Centro LB 960 plate reader luminometer (Berthold Technologies, Germany).

Fluorescence microscopy

The cells were seeded into eight-well chamber slides coated with collagen at a density of 30 000 cells/well. After 24 h, culture medium was replaced with 240 µL fresh growth medium. pDNA polyplexes (N/P 12) in 60 µL HBG, containing 600 ng pDNA (20% of the nucleic acid was Cy5-labeled) were added to each well and incubated at 37 °C. After 45 min, DAPI was added and pictures were obtained using Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss AG, Germany).

Proliferation assay

Huh7 and DU145 cells were seeded in 96-well plates at a density of 5 000 cells/well. After 24 h, cells were transfected with cMBP1 (**#1**), cMBP2 (**#2**), alanine control (**#3**) (N/P 12) or linPEI (N/P 6) polyplexes (100 ng pCMVLuc in 20 µL/well). PEG-ylated polyplexes (**#1**, **#2**, **#3**) were incubated with cells for 45 min, linPEI for additional 4 h. Subsequently, the medium was replaced with fresh medium. 0 h, 24 h, 48 h or 72 h after transfections, medium was again removed and replaced by 50 µL of fresh medium and 50 µL CellTiter-Glo® Reagent (Promega, USA). The total cell viability was determined as the ratio of measured luminescent signal proportional to signal of HBG treated cells. Measurement was performed on Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

EGFP expression

DU145 cells were seeded in 24-well plates at the density of 15 000, 25 000 or 50 000 cells/well. On the following day, cells were transfected with cMBP2-targeted single-oligomeric (**#8**), combination (**#8** + **#15**) (N/P 12) or linPEI (N/P 6) polyplexes formed with pEGFP N1. For cMBP2-targeted polyplexes, after 45 min incubation the medium was replaced by fresh medium or medium containing chloroquine (100 µM). After additional 4 h, medium was again

replaced by fresh medium. At the same time medium was first replaced for linPEI with the total incubation time of 4 h and 45 min. 24 h, 48 h or 72 h after transfection, cells were washed with 500 µL PBS, detached with trypsin/EDTA and taken up in PBS with 10% FCS. Samples were analyzed for EGFP expression using flow cytometry (Cyan™ ADP flow Cytometer - Dako, Hamburg, Germany) and Summit™ acquisition software - Summit, Jamesville, NY, USA).

Particle size and zeta potential

Particle size and zeta potential of formulations were measured by laser-light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). A solution of 10 µg pDNA in 50 µL HBG was vigorously mixed with oligomers at N/P 12 in 50 µL HBG and incubated for 30 min. Prior to dynamic light scattering (DLS) measurement, polyplex samples were diluted to 1 mL in 20 mM HEPES pH 7.4. Additionally, experiments at lower polymer concentrations were performed with 8 µg pDNA and polymer at N/P 12 in a total volume of 400 µL HBG and as well incubated for 30 min. Measurements were performed in a folded capillary cell (DTS1060). Zetasizer Nano ZS with backscatter detection (Malvern Instruments, Worcestershire, UK) was utilized, the equilibration time was set to 0 min, the temperature was 25 °C and an automatic attenuator was applied. The refractive index of the solvent, in our case water, was 1.330 and the viscosity 0.8872, the refractive index of polystyrene latex (1.590) was fixed. Each sample was measured 3 times with 10 subruns. Zeta potentials were calculated by the Smoluchowski equation; 10 up to 30 subruns of 10 s at 25 °C (n= 3) were measured.

Particle size measurements in serum

Polyplexes were formed at N/P 12 with 10 µg pDNA in a total volume of 50 µL HBG and incubated for 30 min. After polyplex incubation, 30 µL of HBG and 720 µL of FCS were added to the samples to reach the total FCS concentration of 90 % and further incubated for

0 min, 1 h, 4 h or 24 h at 37 °C prior to DLS measurement as above using Zetasizer Nano ZS with backscatter detection (Malvern Instruments, Worcestershire, UK).

Analytical RP-HPLC

Reversed-phase HPLC (RP-HPLC) was carried out using a Waters HPLC system equipped with a Waters 600E multisolvent delivery system, Waters 996 PDA detector and Waters 717plus autosampler. All structures were analyzed using a Waters Sunfire C18 column (5 µm, 4.6 x 150 mm) and a water/acetonitrile gradient (95:5 – 0:100 in 20 min) containing 0.1 % trifluoroacetic acid. Detection wavelength was 214 nm.

Proton NMR spectroscopy

^{1}H NMR spectroscopy was carried out using a Jeol JNMR-GX 400 (400 MHz) or JNMR-GX 500 (500 MHz). Chemical shifts were calibrated to residual proton signal of the solvent and are reported in ppm. Data is indicated with s = singlet, d = doublet, t = triplet, m = multiplet, comp = complex (group of overlaid protons). Spectra were analyzed using MestReNova (Ver. 9.0, Mestrelab Research). Integrals were normalized to the succinic acid peaks.

Mass Spectrometry

Electrospray ionization (ESI) mass spectrometry was carried out with a Thermoscientific LTQ FT Ultra Fourier transform ion cyclotron and an IonMax source. Water containing 1 % formic acid was used as solvent.

SUPPLEMENT FIGURES AND TABLE

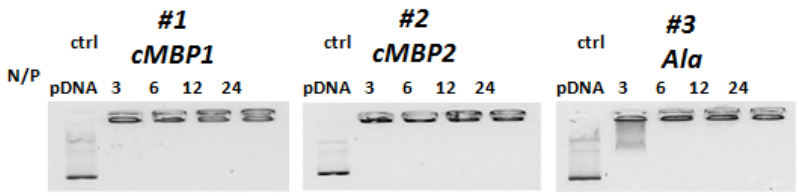


Fig. S1 pDNA binding assay by electrophoresis. Gel retardations of cMBP1-, cMBP2-containing and alanine control polyplexes at increasing N/P ratios were compared to the mobility of the free pDNA.

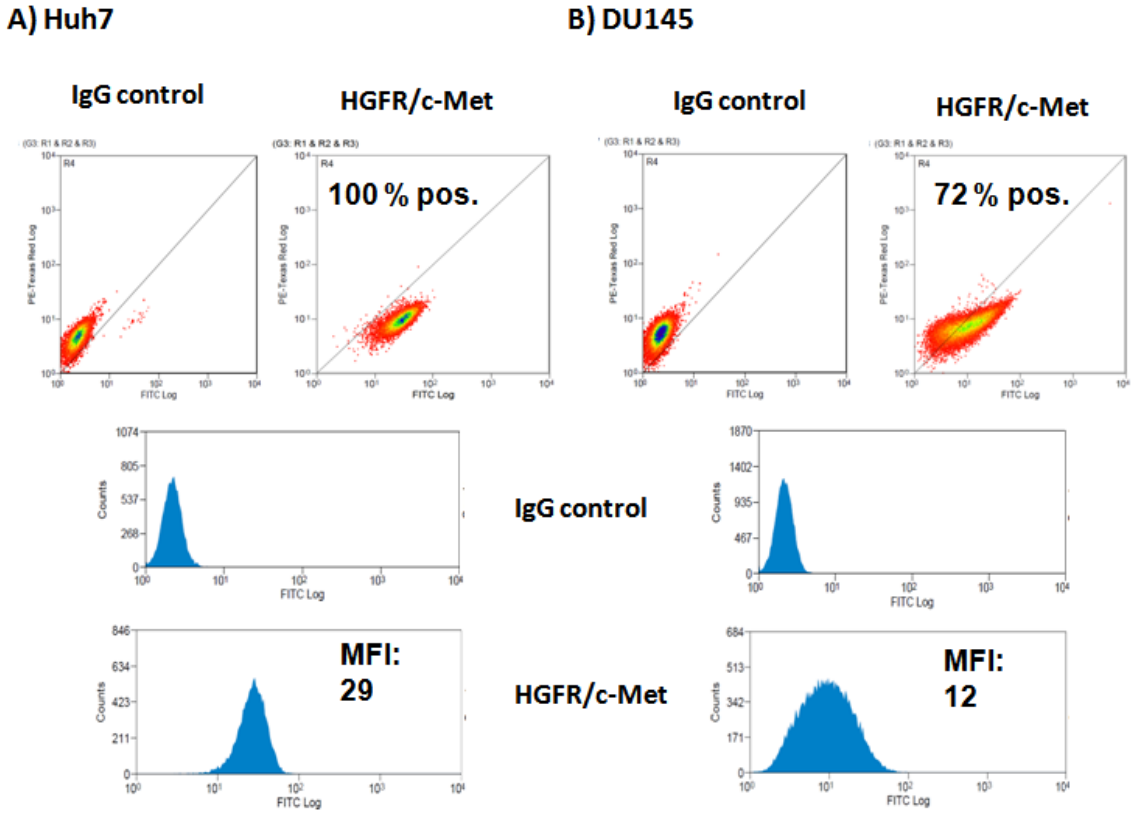


Fig. S2 HGFR/c-Met receptor levels on A) Huh7 and B) DU145 cell line showing the results obtained with the monoclonal mouse anti-human HGFR/c-Met antibody and IgG control. Alexa 488-labeled goat anti-mouse secondary antibody was used for the detection of receptor expression by flow cytometry. MFI stands for mean fluorescence intensity.

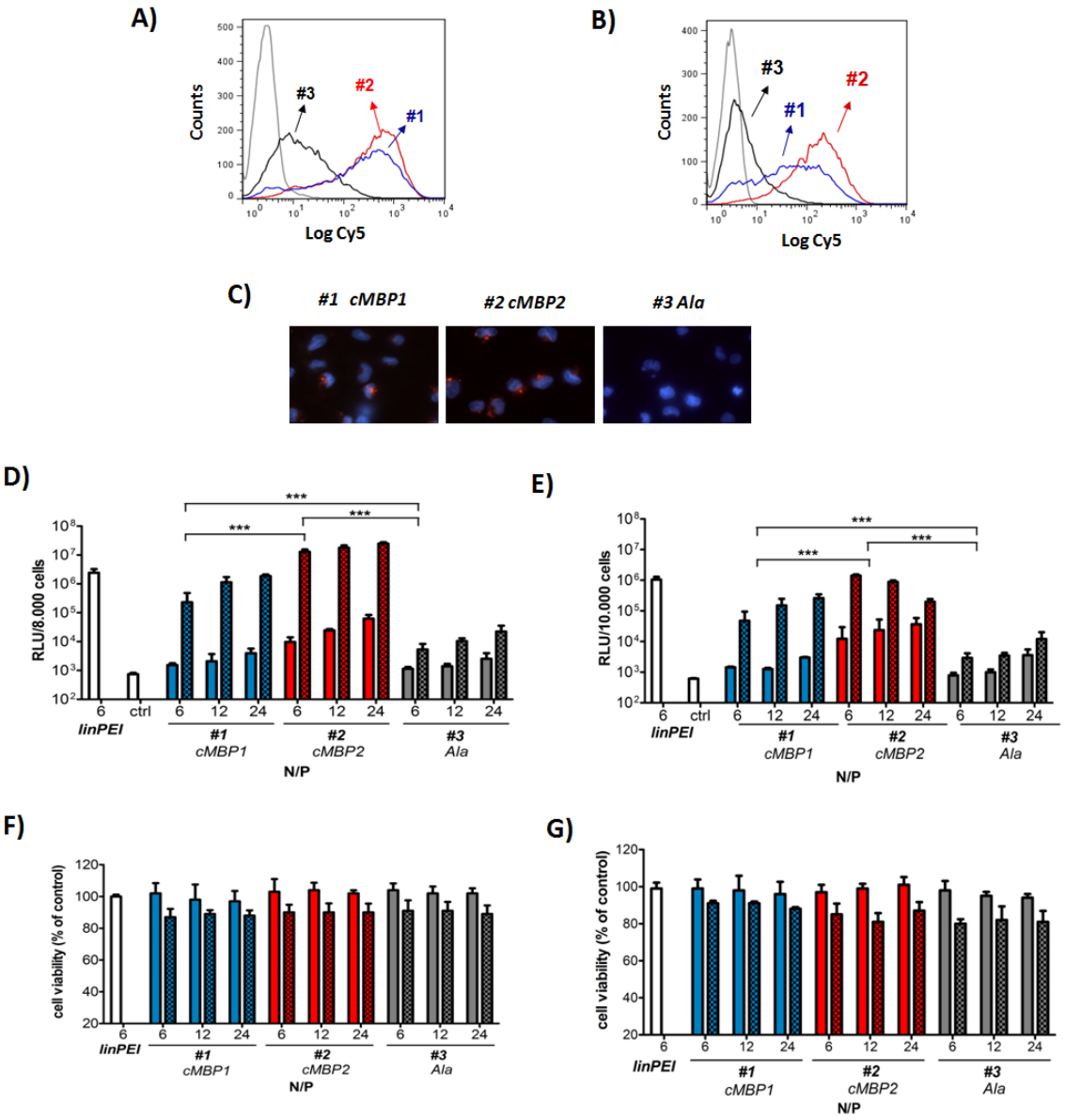


Fig. S3 Cell association of oligomer/Cy5-pDNA polyplexes at N/P 12 to Huh7 cells after 30 min incubation at 4 °C as determined by flow cytometry. Logarithmic X-scale represents Cy5 fluorescence of polyplexes bound to Huh7 (A) or DU145 cells (B). "Count" represents the cumulative cell counts with indicated Cy5 fluorescence after appropriate gating by forward/sideward scatter and pulse width. Dead cells (DAPI positive, < 2%) were excluded from analysis. C) Fluorescence microscopic images of cellular uptake of the cMBP1-, cMBP2-containing and alanine control polyplexes formed with Cy5-labeled pDNA at N/P 12 in Huh7 cells (blue: DAPI staining, red: Cy5). Luciferase gene transfer in Huh7 (D) and DU145 (E) cells without (no pattern) or with (pattern) the addition of endosomolytic chloroquine, as obtained with the cMBP1- and cMBP2-containing polyplexes in comparison to the alanine control polyplexes (45 min incubation). Linear PEI 22 kDa (linPEI) at optimal non-toxic ratio (N/P 6, w/w 0.8; 4 h incubation) was used as positive control, HBG treated cells served as negative control. Data are presented as mean value (±SD) out of quintuplicate. Corresponding cell viability (MTT) assays of Huh7 (F) and DU145 (G) cells after transfections. Cell viability was calculated as percentage to cells treated with HBG.

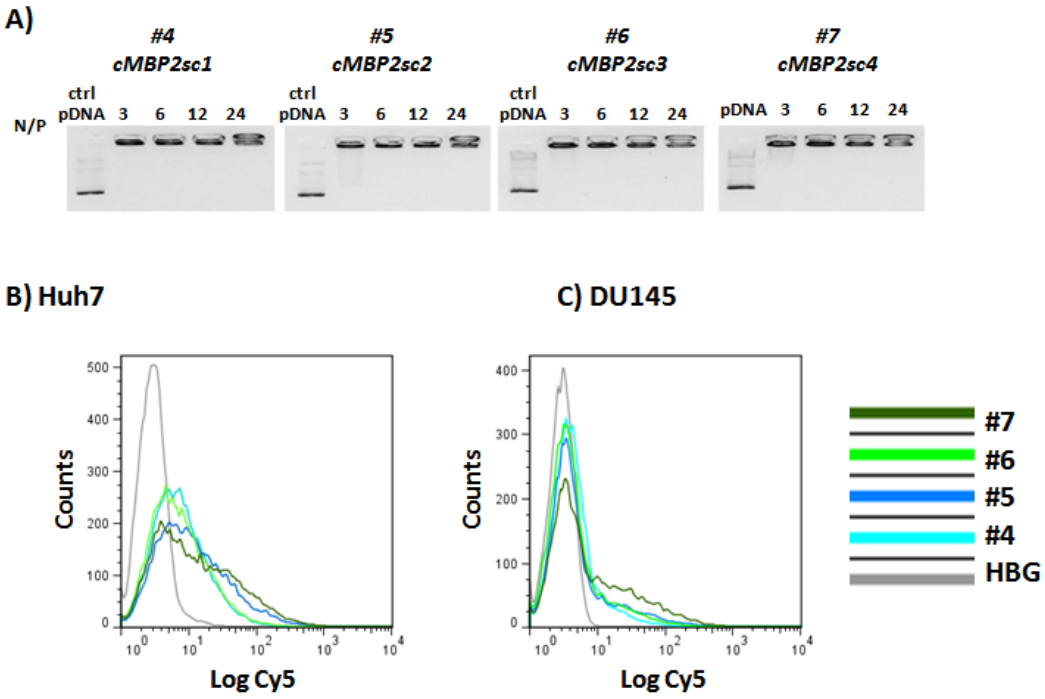


Fig. S4 A) pDNA binding assay by electrophoresis. Gel retardation of the scrambled peptide-equipped polyplexes at increasing N/P ratios was compared to the shift of free pDNA. B, C) Cellular association of polyplexes formed with Cy5-labeled pDNA and conjugates with the conjugated scrambled sequences on B) DU145 and C) Huh7 cells after 30 min incubation at 4 °C as determined by flow cytometry.

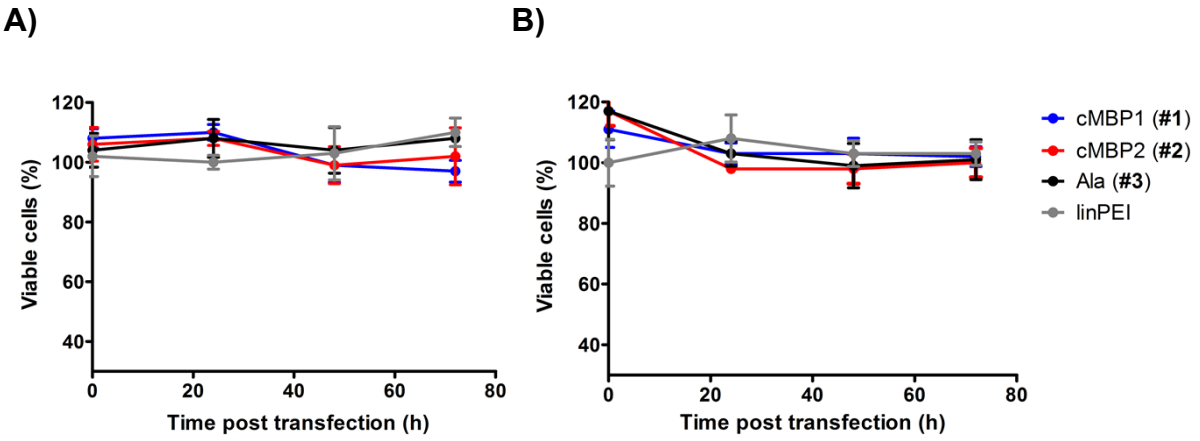


Fig. S5 Influence of transfection with cMBP1 (**#1**), cMBP2 (**#2**), alanine control (**#3**) (N/P 12) or linPEI (N/P 6) polyplexes on proliferation of A) Huh7 and B) DU145 cells. The measurement was performed 0 h, 24 h, 48 h and 72 hours after transfection using CellTiter-Glo® reagent. The values are presented as the relative luminiscent signal over the signal of HBG treated cells (n=5).

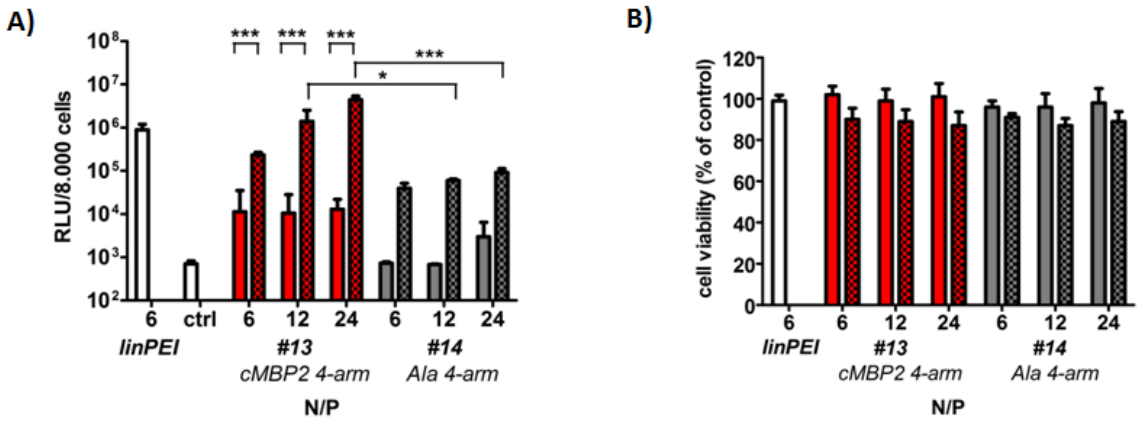


Fig. S6 Influence of additional polycationic arms. A) pDNA transfection efficiencies of 4-arm structures with (pattern) or without chloroquine (no pattern) and B) corresponding cell viabilities. Data are presented as mean value (±SD) out of a quintuplicate.

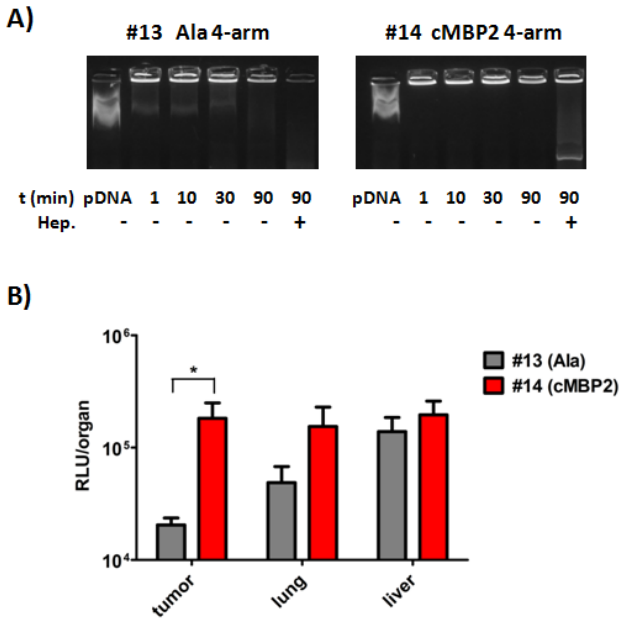
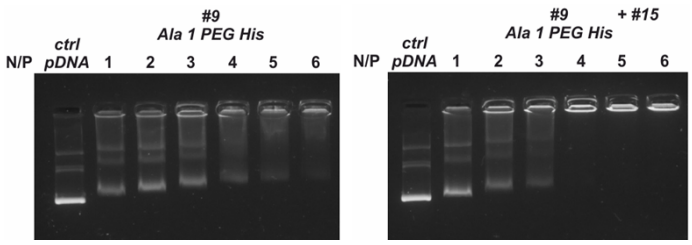
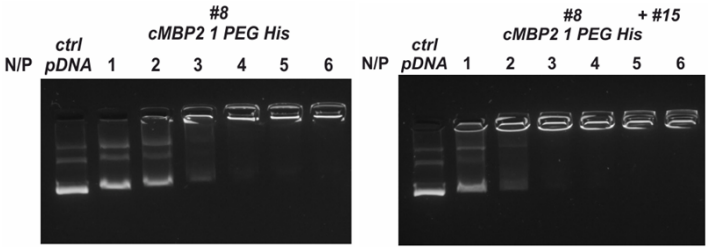


Fig. S7 A) Serum stability of the polyplexes (N/P 12) formed with 4-arm PEGylated cMBP2-targeted (**#14**) or non-targeted control (**#13**) oligomers analyzed at different serum incubation times by an agarose gel shift assay. Where indicated, heparin was added to the polyplexes after incubation in serum for 90 min. B)*In vivo* gene expression at 48 h after i.v. administration of pCMVLuc polyplexes formed with the cMBP2-targeted (**#13**) and alanine control (**#14**) 4-arm PEG_{24} oligomers at N/P 12 into Huh7 tumor bearing mice (N=5, mean±SEM) in tumor, lung and liver. Luciferase gene expression is presented as relative light units per organ or tumor (RLU/organ). Lysis buffer RLU values were subtracted. Liver weight was around 1.5 g, lung weight around 210 mg and Huh7 tumor weight 282±197 mg.

A)



B)



C)

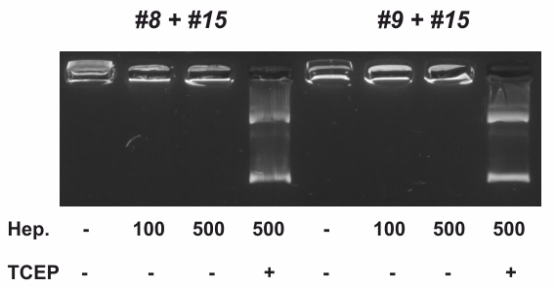
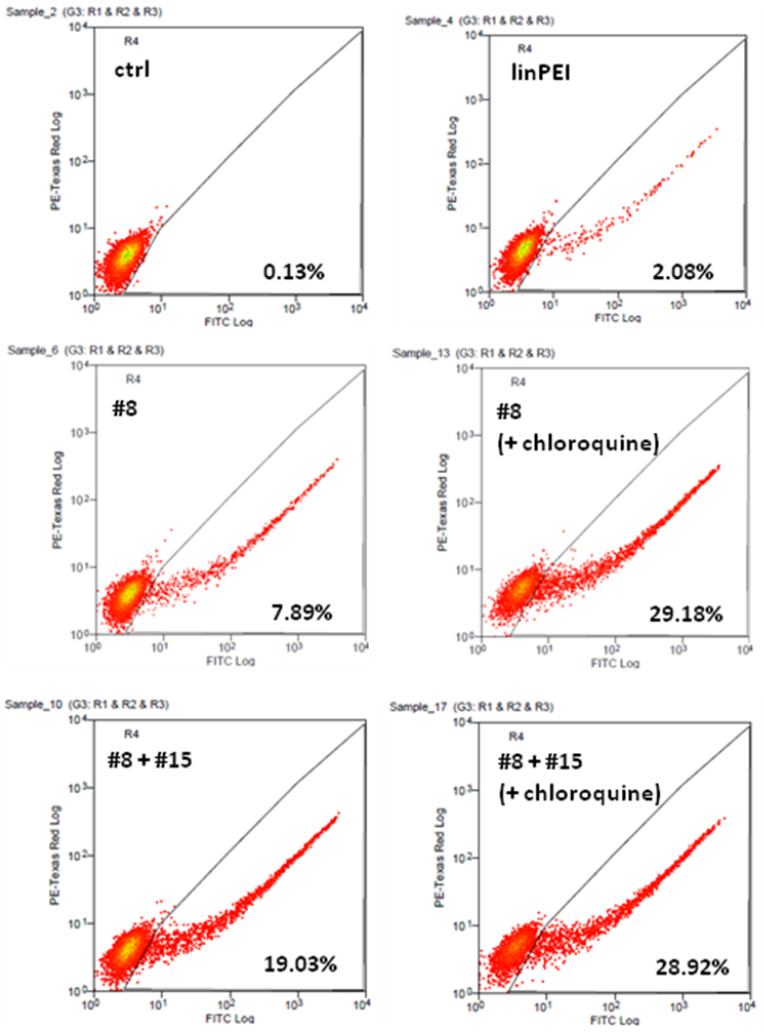


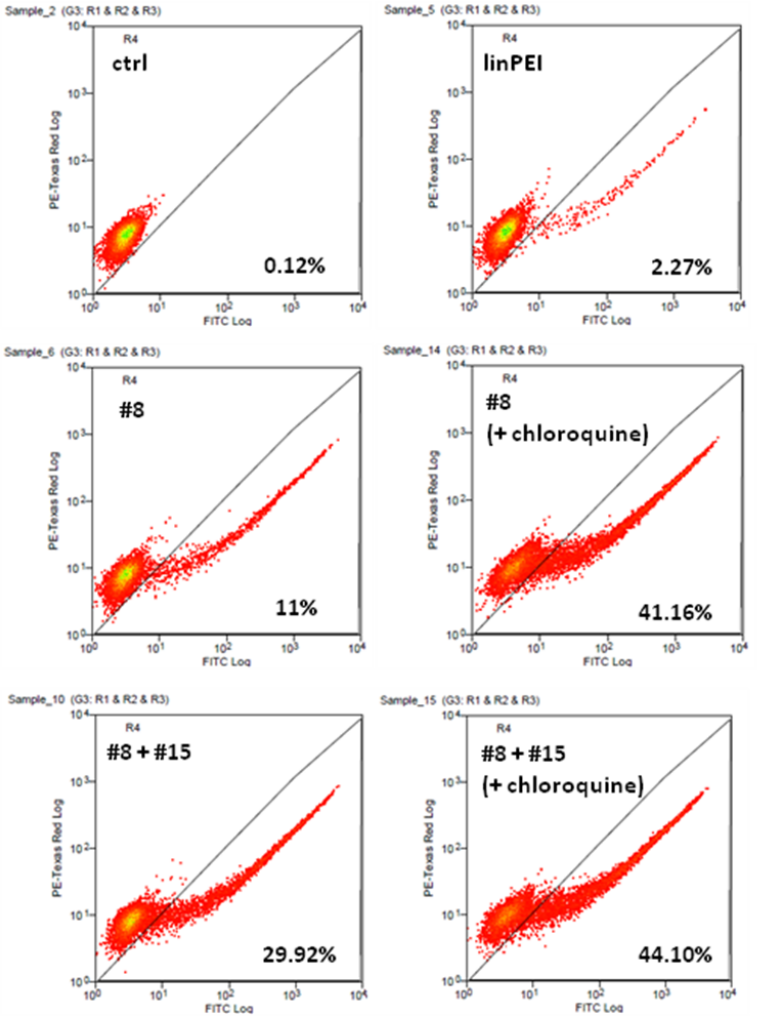
Fig. S8 Gel shift assays of histidines-enriched A) alanine and B) cMBP single and bi-oligomeric polyplexes at lower N/P ratios. C) Gel shift assay of cMBP and alanine histidines-modified polyplexes in the presence of increasing amunts of heparin (100 or 500 I.E.) and reducing agent TCEP (4 mM).

A)


Sample_2 (G3: R1 & R2 & R3)
R4
ctrl
0.13%
Sample_4 (G3: R1 & R2 & R3)
R4
linPEI
2.08%
Sample_6 (G3: R1 & R2 & R3)
R4
#8
7.89%
Sample_13 (G3: R1 & R2 & R3)
R4
#8
(+ chloroquine)
29.18%
Sample_10 (G3: R1 & R2 & R3)
R4
#8 + #15
19.03%
Sample_17 (G3: R1 & R2 & R3)
R4
#8 + #15
(+ chloroquine)
28.92%
PE-Texas Red Log
FITC Log

24 h

B)


Sample_2 (G3: R1 & R2 & R3)
R4
ctrl
0.12%
Sample_5 (G3: R1 & R2 & R3)
R4
linPEI
2.27%
Sample_6 (G3: R1 & R2 & R3)
R4
#8
11%
Sample_14 (G3: R1 & R2 & R3)
R4
#8
(+ chloroquine)
41.16%
Sample_10 (G3: R1 & R2 & R3)
R4
#8 + #15
29.92%
Sample_15 (G3: R1 & R2 & R3)
R4
#8 + #15
(+ chloroquine)
44.10%
PE-Texas Red Log
FITC Log

48 h

C)

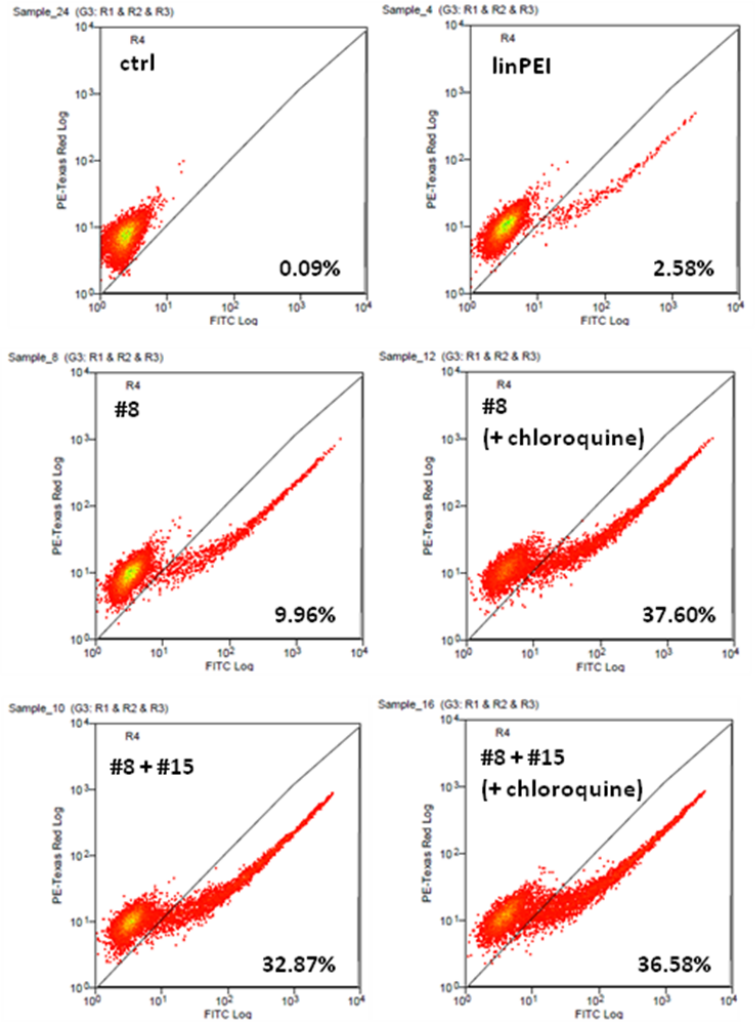


Fig. S9 EGFP expression of DU145 cells transfected with pEGFP N1-containing cMBP2-targeted single-oligomeric (**#8**) or combination (**#8** + **#15**) polyplexes (N/P 12) in the absence or presence of chloroquine at A) 24 h, B) 48 h or C) 72 h after transfection.

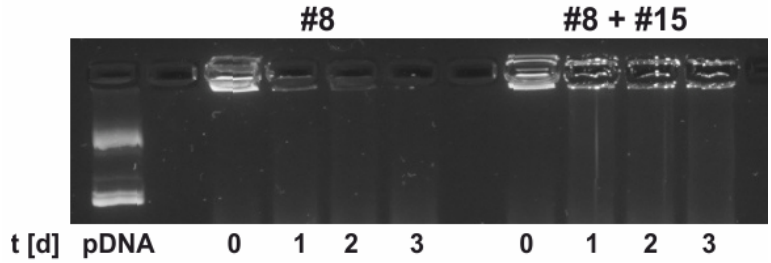


Fig. S10 Serum stability of cMBP2-targeted single-oligomeric (**#8**) and combination (**#8** + **#15**) polyplexes over a period of 3 days.

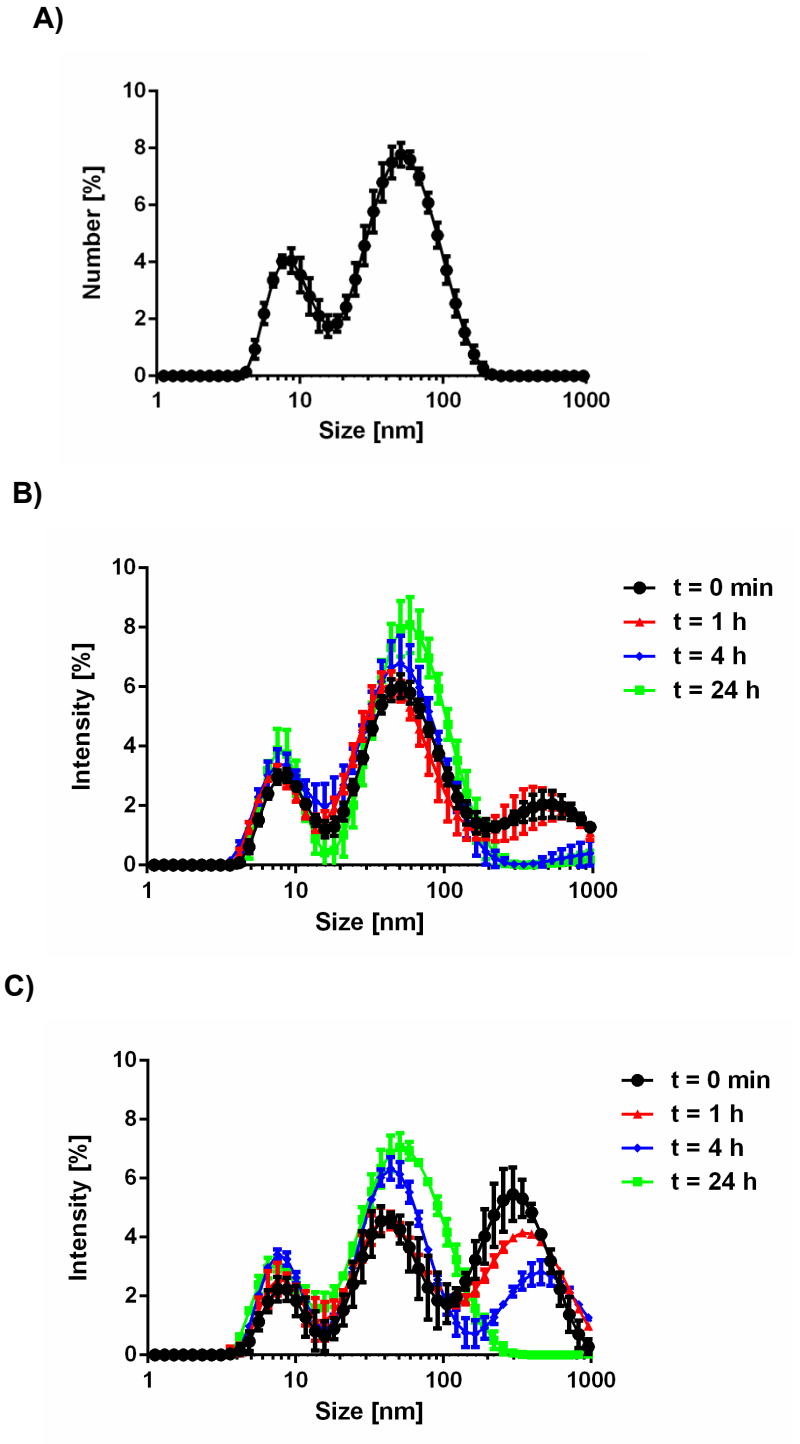


Fig. S11 Size measurements of cMBP2-targeted single-oligomeric (**#8**) (B) and bi-oligomeric (**#8 +#15**) (C) polyplexes in serum with different incubation times (0 min, 1 h, 4 h and 24 h). A) blank measurement of serum only. An increased particles size and decrease in the intensity was observed over time (peak on the right). A longer detection time was observed with the bi-oligomeric polyplexes.

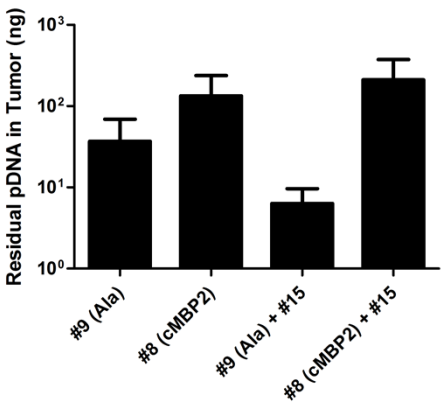


Fig. S12 Residual pDNA in tumor after intravenous injection of histidines-enriched control or cMBP2 single- or bioligomeric polyplexes.

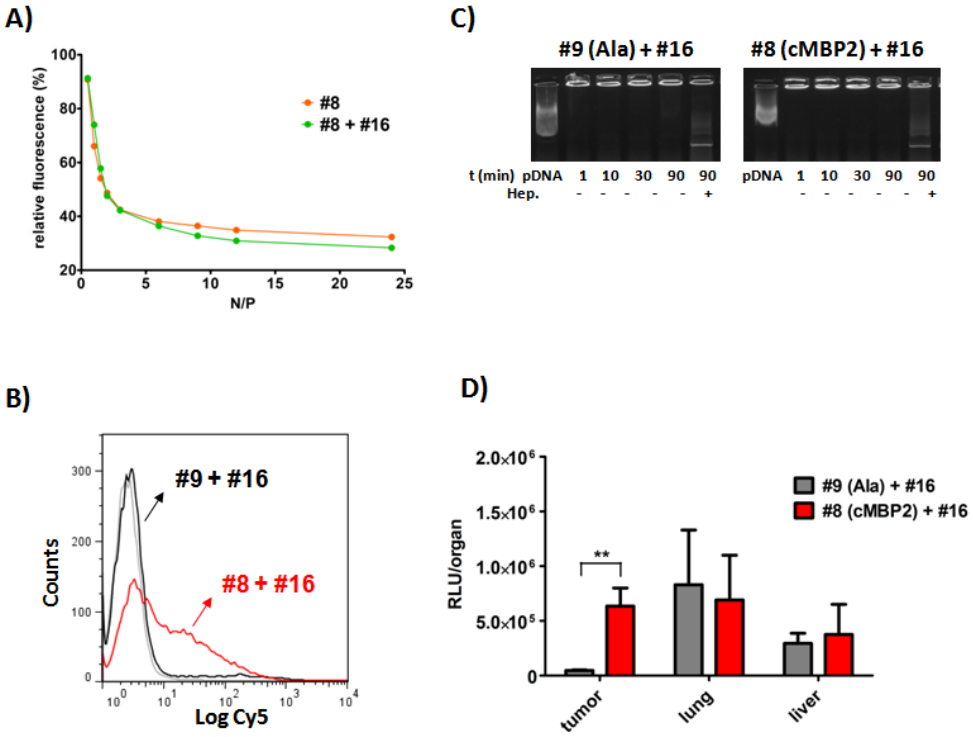
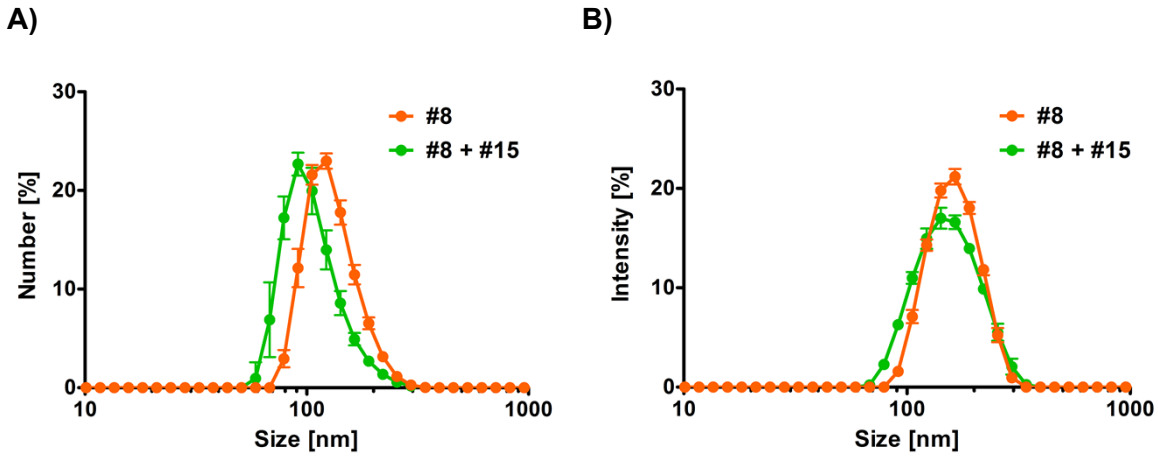


Fig. S13 A) pDNA compaction ability of combination polyplexes of 2-arm PEGylated oligomer (**#8**) and 4-arm compacting oligomer (**#16**) compared to single oligomer polyplexes formed only with the oligomer (**#8**) as determined by EtBr exclusion assay. Reduction in EtBr fluorescence was determined as percentage of maximal fluorescence of EtBr containing pDNA solution. B) Cellular uptake of combination polyplexes (total N/P 12) comparing the cMBP2-targeted (red line) and alanine control (black line) nanoparticles by Huh7 cells after 45 min at 37 °C and removal of cell surface-bound polyplexes. C) Serum stability analyzed by gel-shift assay. Polyplexes (N/P 12) were incubated for 30 min with subsequent incubation in 90% serum for 1, 10, 30 or 90 min. Where indicated, heparin was added to the polyplexes to evaluate the influence of this highly polyanionic molecule on polyplex stability. D) *In vivo* gene expression 48 h after i.v. administration of cMBP2-targeted (**#8** + **#16**) and alanine control (**#9** + **#16**) combination polyplexes at total N/P 12 (oligomer **#8** or **#9** at 70% and oligomer **#16** at 30% of the total N/P) into Huh7 tumor bearing mice (N=5, mean±SEM) in tumor, lung and liver. Luciferase gene expression is presented as relative light units per organ or tumor (RLU/organ). Lysis buffer RLU values were subtracted. Liver weight was around 1.5 g, lung weight around 210 mg and Huh7 tumor weight 438±152.



	Z-Average		Number		Intensity		PDI	
	Ø	Std. Dev.	Ø	Std. Dev.	Ø	Std. Dev.	Ø	Std. Dev.
442	155,3	2,146	131,5	2,498	165,8	2,084	0,054	0,012
442+689	143,4	1,201	108,2	3,905	158,3	3,110	0,090	0,005

Fig. S14 A) The size of the cMBP2 single- (**#8**) and bi-oligomeric polyplexes (**#8** + **#15**) at *in vitro* conditions (8 µg pDNA and polymer at N/P 12 in total 400 µl HBG).

Table S1. Particle Size and Zeta Potential of polyplexes by DLS Analysis.

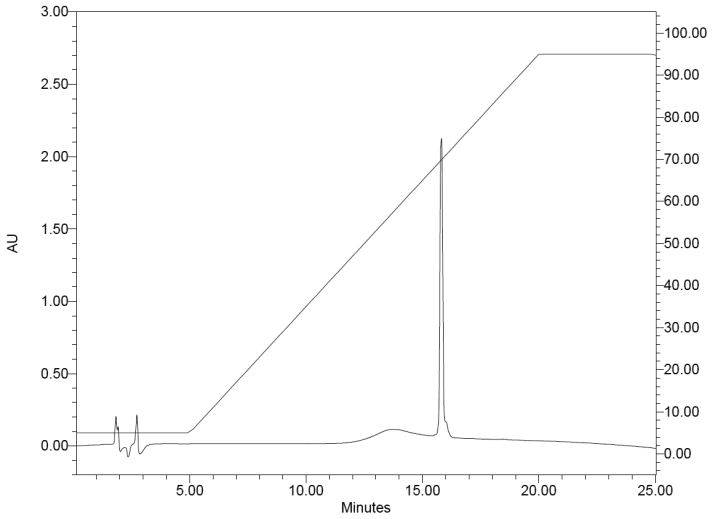
Polymer(N/P 12)	**Average Size (nM)**	**St. Dev.**	**Average Zeta Potential (mV)**	**St. Dev.**
#1 (696)	**205,8**	5,87	**17,3**	0,47
#2 (443)	**195,9**	3,41	**18,2**	0,40
#3 (188)	**197,4**	14,79	**4,7**	0,24
#4 (697)	**186,4**	4,89	**16,0**	0,10
#5 (698)	**191,0**	2,39	**15,8**	0,68
#6 (699)	**253,1**	7,46	**14,6**	0,21
#7 (700)	**216,7**	9,83	**13,2**	0,26
#8 (442)	**162,9**	1,99	**14,3**	0,26
#9 (440)	**401,4**	64,27	**5,1**	0,09
#10 (694)	**430,2**	59,69	**13,2**	0,49
#11 (695)	**478,4**	32,37	**6,0**	1,31
#12 (616)	**177,7**	5,55	**1,3**	0,27
#13 (677)	**179,5**	2,76	**22,4**	0,26
#14 (678)	**390,5**	55,76	**13,2**	0,35
#8 + #15 (442 + 689)	**314,5**	7,16	**14,0**	0,32
#9 + #15 (440 + 689)	**408,9**	69,70	**6,8**	0,11
#8 + #16 (442 + 606)	**239,9**	44,24	**13,8**	0,81
#9 + #16 (440 + 606)	**330,3**	43,12	**8,6**	0,12

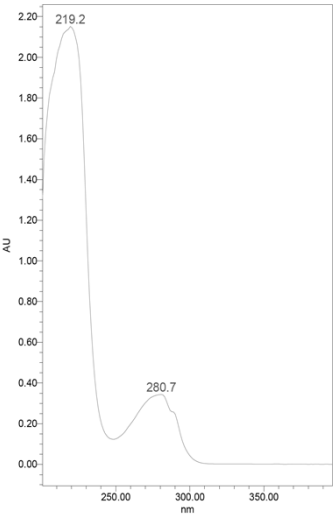
pCMVLuc polyplexes were formed at N/P 12 in HBG buffer at the higher *in vivo* concentrations.

ANALYTICAL DATA

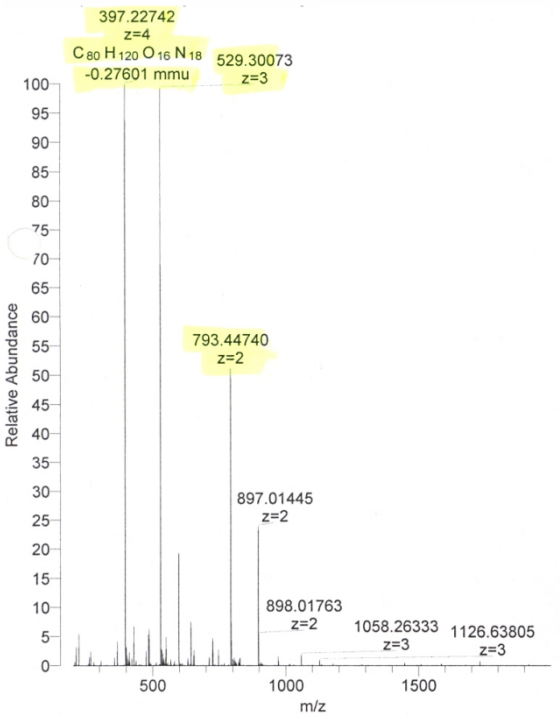
Targeting peptides

cMBP1
Sequence (C->N): K-α[AKLPPWHVSFLY]





RP-HPLC chromatogram UV Spectrum



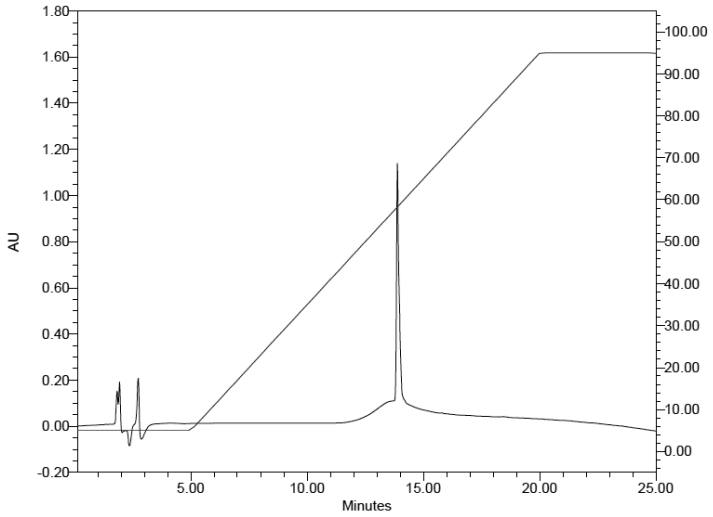
ESI-MS

m/z calcd for $C_{80}H_{116}N_{18}O_{16}$:$[M+2H]^{2+}$ 793.44811, found 793.44740;

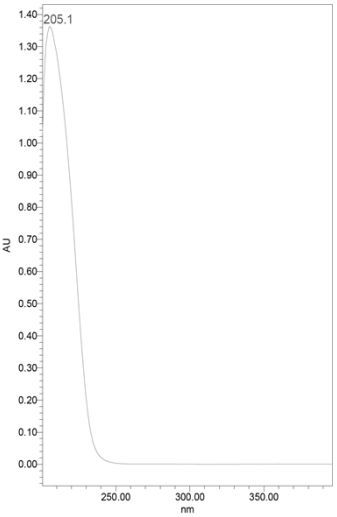
$[M+3H]^{3+}$ 529.30117, found 529.30073; $[M+4H]^{4+}$ 397.22769, found 397.22742.

cMBP2

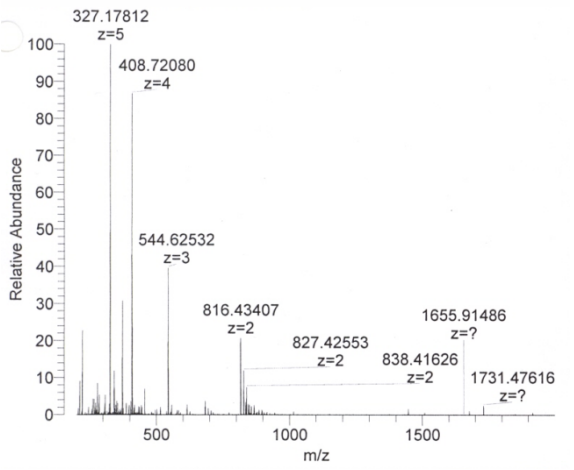
Sequence (C->N): K-$_{\alpha}$[HHHIHDHRSLSK]



RP-HPLC chromatogram



UV Spectrum



ESI-MS

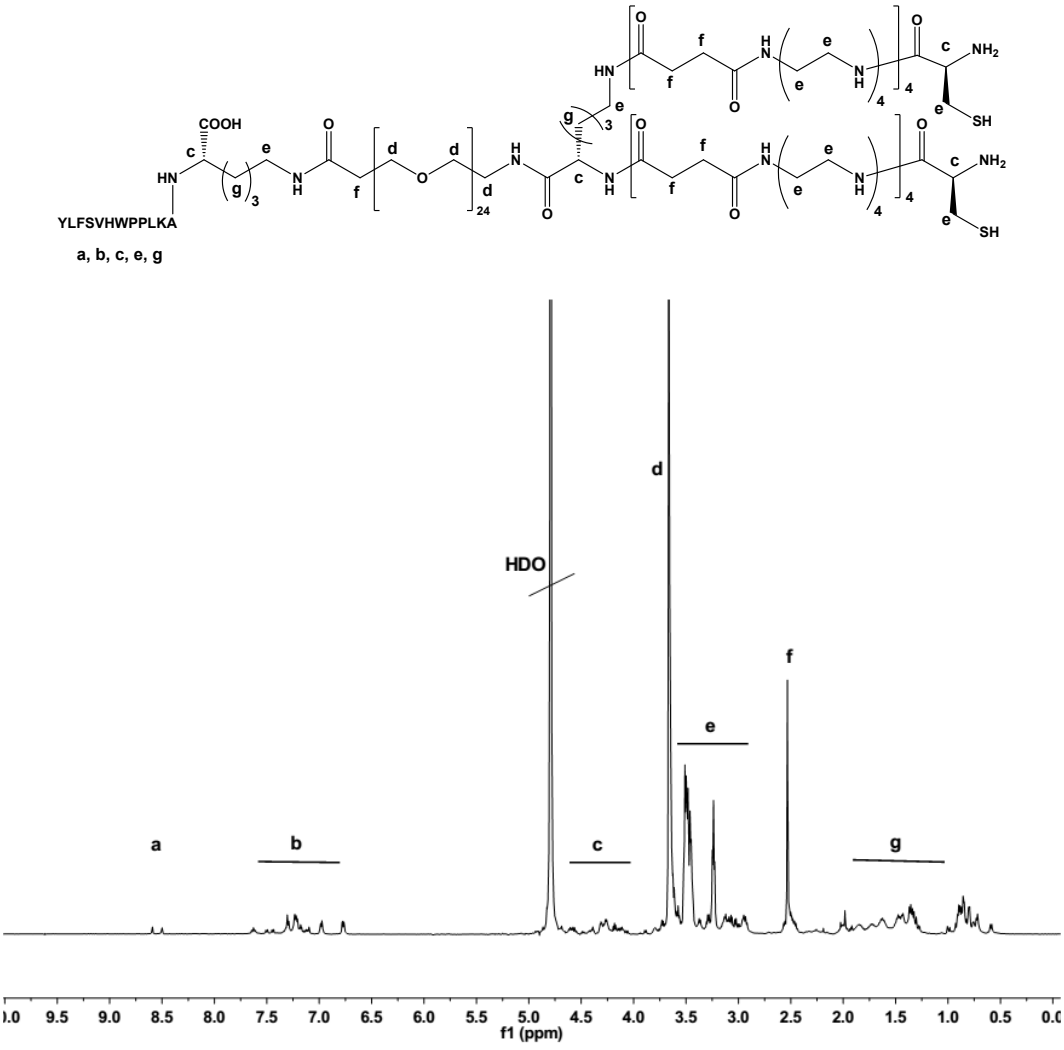
m/z calcd for $C_{70}H_{110}N_{28}O_{18}$: $[M+2H]^{2+}$ 816.43492, found 816.43407; $[M+3H]^{3+}$ 544.62571, found 544.62532; $[M+4H]^{4+}$ 408.72110, found 408.72080; $[M+5H]^{5+}$ 327.17833, found 327.17812.

Oligomers

^{1}H-NMR Data

#1 (ID 696)

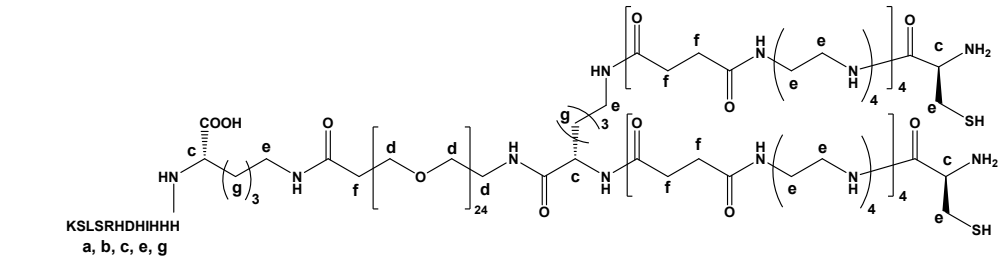
Sequence (C->N): K-$_{\alpha}$[AKLPPWHVSFLY]-$_{\varepsilon}$[PEG_{24}-K-$_{\alpha,\varepsilon}$(Stp_4-C)$_2$]

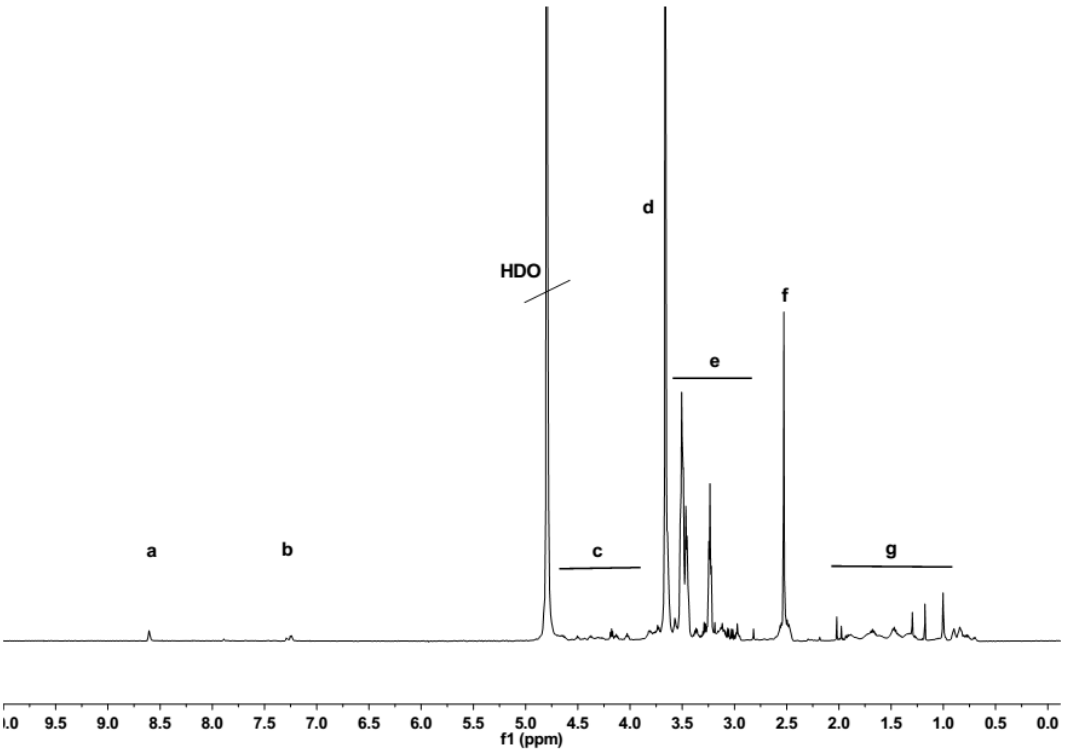


^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.0-1.9 (comp, 53 H, βH alanine, βγδH leucine, βγδH lysine, βγH proline, γH valine), 2.4-2.6 (comp, 34 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- $dPEG_{24}$), 2.9-3.5 (comp, 152 H, -CH_2- tepa, βH cysteine, βH histidine, εH lysine, βH phenylalanine, δH proline, βH serine, βH tryptophane, βH tyrosine), 3.66 (s, 98 H, -CH_2-O- $dPEG_{24}$, -CH_2-N- $dPEG_{24}$), 4.0-4.6 (comp, 16 H, αH amino acids), 4.79 (s, HDO), 7.0-7.4 (comp, 15 H, aromatic H histidine, phenylalanine, tryptophane, tyrosine), 8.61 (s, 1 H, aromatic H histidine).

#2 (ID 443)

Sequence (C->N): K-$_{\alpha}$[HHHIHDHRSLSK]-$_{\varepsilon}$[PEG_{24}-K-$_{\alpha,\varepsilon}$(Stp_4-C)$_2$]

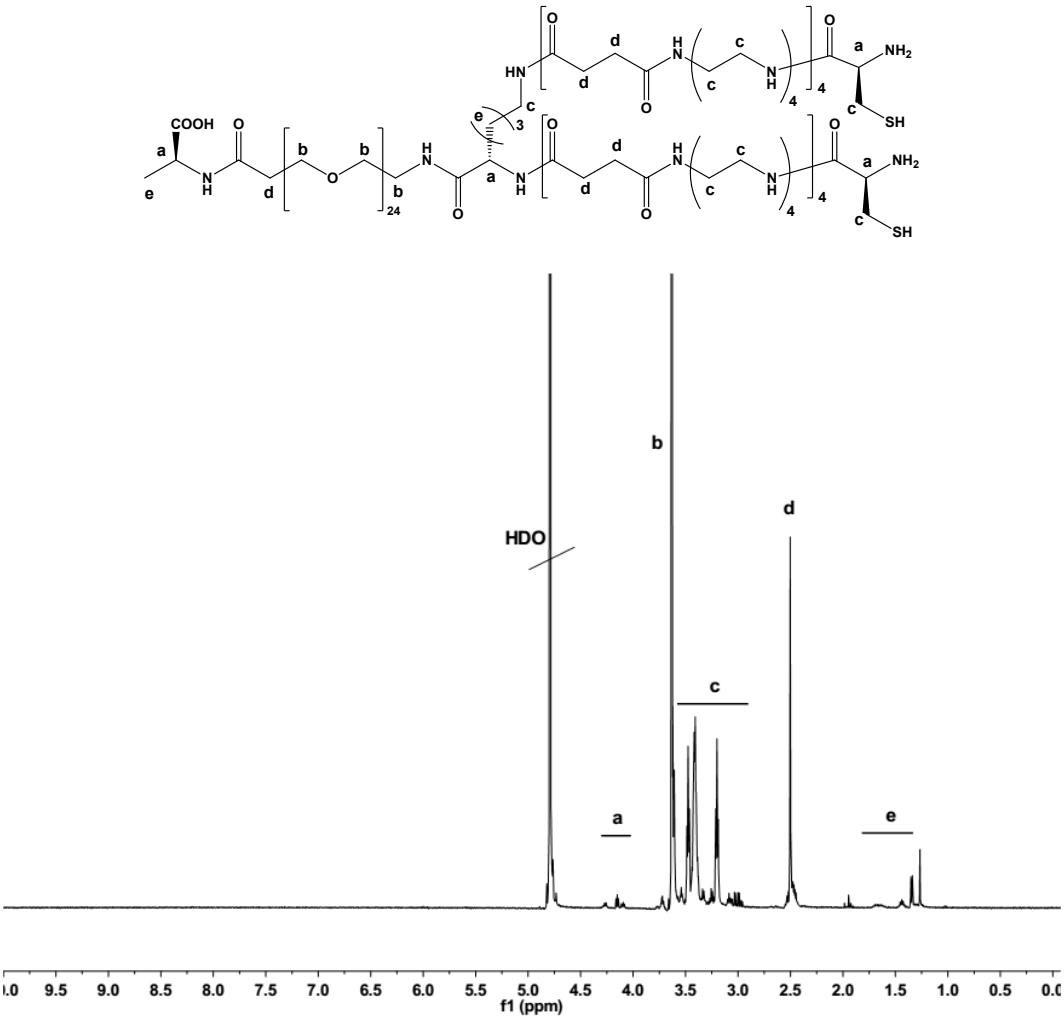




^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.0-2.0 (comp, 40 H, βγH arginine, βγδH isoleucine, βγδH leucine, βγδH lysine), 2.4-2.6 (comp, 34 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- $dPEG_{24}$), 2.8-3.6 (comp, 156 H, -CH_2- tepa, δH arginine, βH aspartate, βH cysteine, βH histidine, εH lysine, βH serine), 3.66 (s, 98 H, -CH_2-O- $dPEG_{24}$, -CH_2-N- $dPEG_{24}$), 3.9-4.6 (comp, 16 H, αH amino acids), 4.80 (s, HDO), 7.24-7.25 (m, 5 H, aromatic H histidine), 8.61 (s, 5 H, aromatic H histidine).

#3 (ID 188)

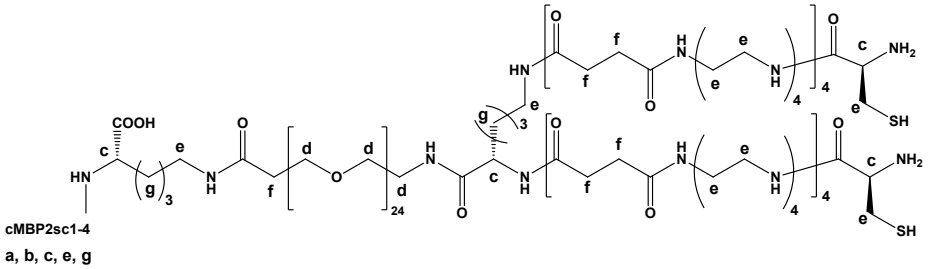
Sequence (C->N): A-PEG_{24}-K-$_{\alpha,\varepsilon}$(Stp_4-C)$_2$

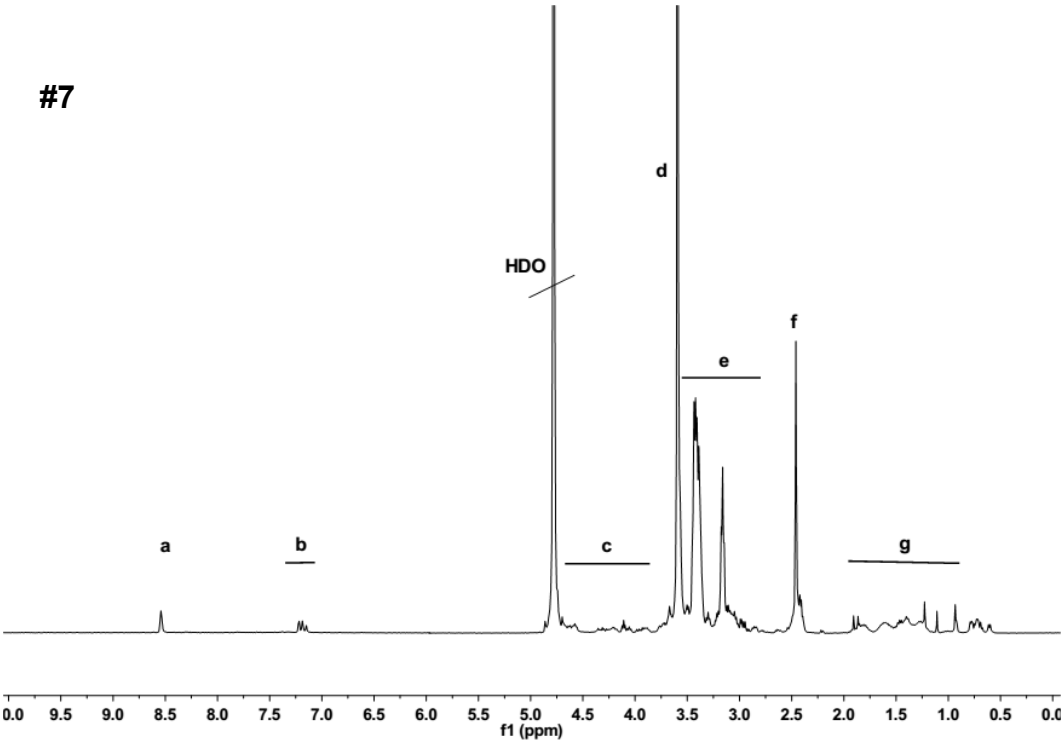


^{1}H-NMR spectrum in D_2O.δ (ppm) = 1.3-1.8 (comp, 9 H, βH alanine, βγδH lysine), 2.4-2.6 (comp, 34 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- $dPEG_{24}$), 2.9-3.5 (comp, 134 H, -CH_2- tepa, βH cysteine, εH lysine), 3.62 (s, 98 H, -CH_2-O- $dPEG_{24}$, -CH_2-N- $dPEG_{24}$), 3.7-4.2 (comp, 4 H, αH alanine, αH cysteine, αH lysine), 4.79 (s, HDO).

#4-7 (ID 697-700)

Sequence (C->N): K-$_{\alpha}$[cMBP2sc1-4]-$_{\varepsilon}$[PEG$_{24}$-K-$_{\alpha,\varepsilon}$(Stp$_4$-C)$_2$]

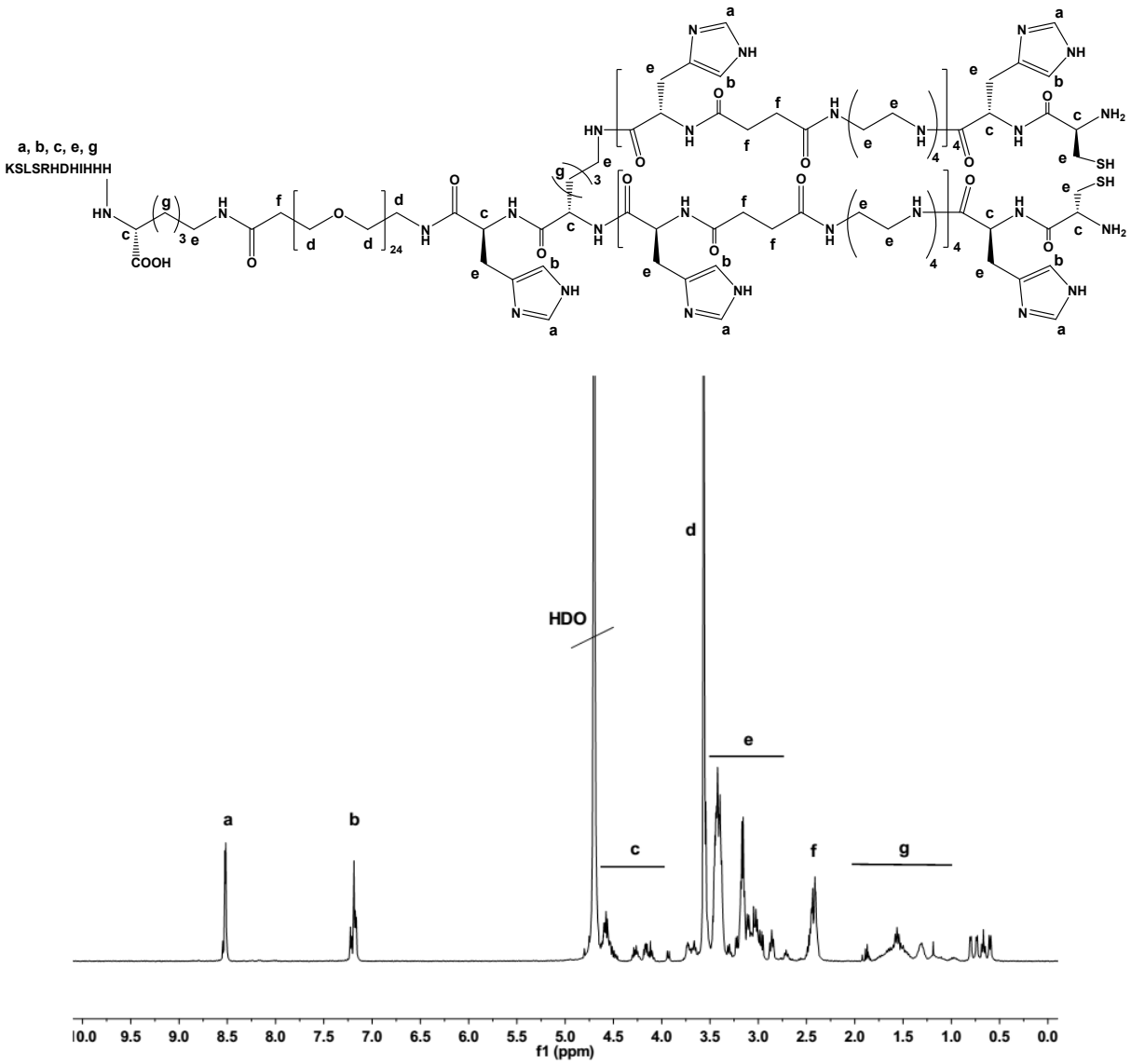




^{1}H-NMR spectrum in D_2O.δ (ppm) = 1.0-2.0 (comp, 40 H, βγH arginine, βγδH isoleucine, βγδH leucine, βγδH lysine), 2.4-2.6 (comp, 34 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- dPEG$_{24}$), 2.8-3.6 (comp, 156 H, -CH_2- tepa, δH arginine, βH aspartate, βH cysteine, βH histidine, εH lysine, βH serine), 3.66 (s, 98 H, -CH_2-O- dPEG$_{24}$, -CH_2-N- dPEG$_{24}$), 3.9-4.6 (comp, 16 H, αH amino acids), 4.80 (s, HDO), 7.24-7.25 (m, 5 H, aromatic H histidine), 8.61 (s, 5 H, aromatic H histidine).

#8 (ID 442)

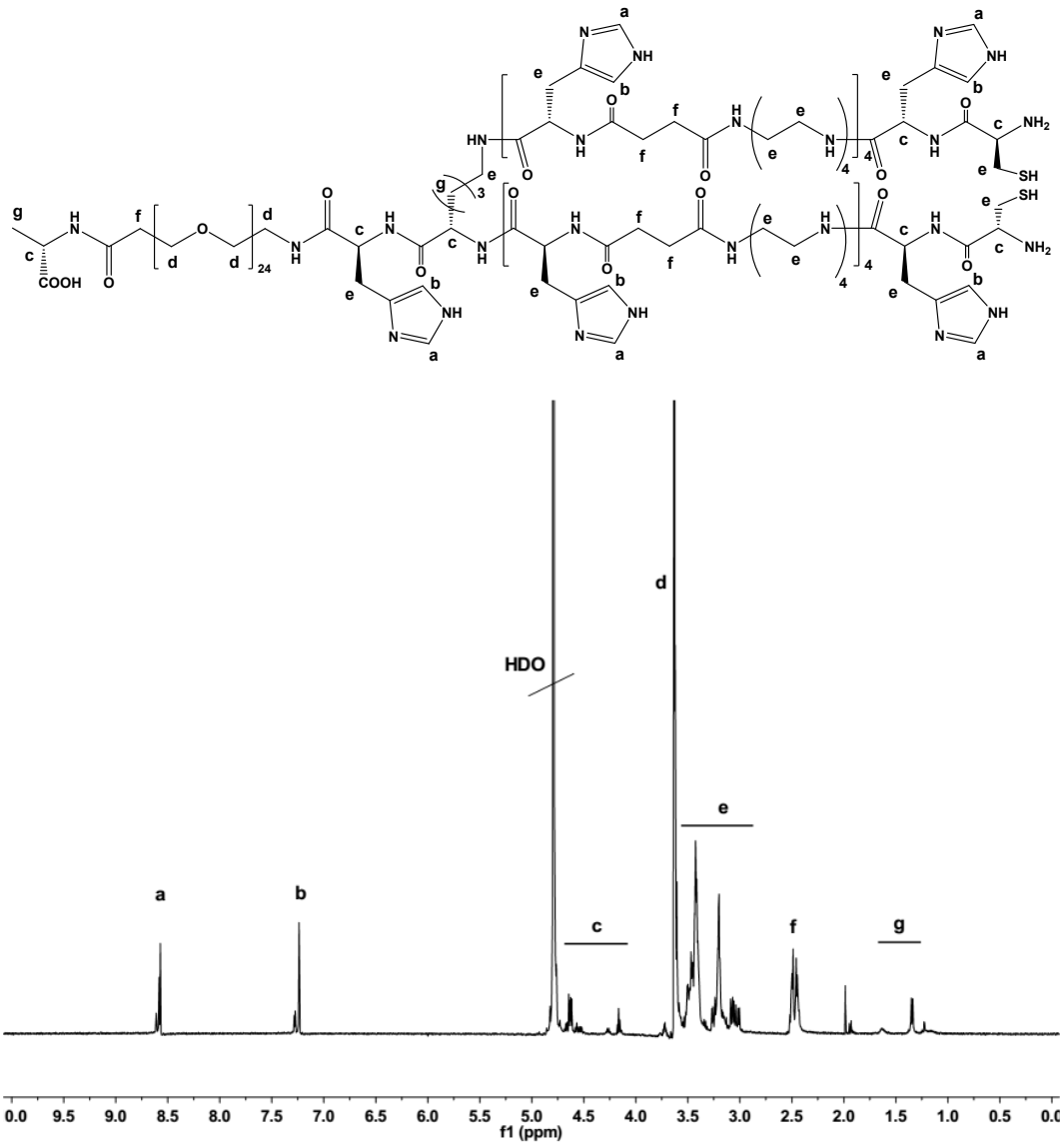
Sequence (C->N): K-$_{\alpha}$[HHHIHDHRSLSK]-$_{\varepsilon}$[PEG_{24}-H-K-$_{\alpha,\varepsilon}$[H-(Stp-H)$_4$-C]$_2$]



^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.0-2.0 (comp, 40 H, βγH arginine, βγδH isoleucine, βγδH leucine, βγδH lysine), 2.4-2.6 (comp, 34 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- dPEG_{24}), 2.8-3.5 (comp, 176 H, -CH_2- tepa, δH arginine, βH aspartate, βH cysteine, βH histidine, εH lysine, βH serine), 3.67 (s, 98 H, -CH_2-O- dPEG_{24}, -CH_2-N- dPEG_{24}), 4.0-4.6 (comp, 27 H, αH amino acids), 4.79 (s, HDO), 7.1-7.3 (m, 16 H, aromatic H histidine), 8.48-8.58 (m, 16 H, aromatic H histidine).

#9 (ID 440)

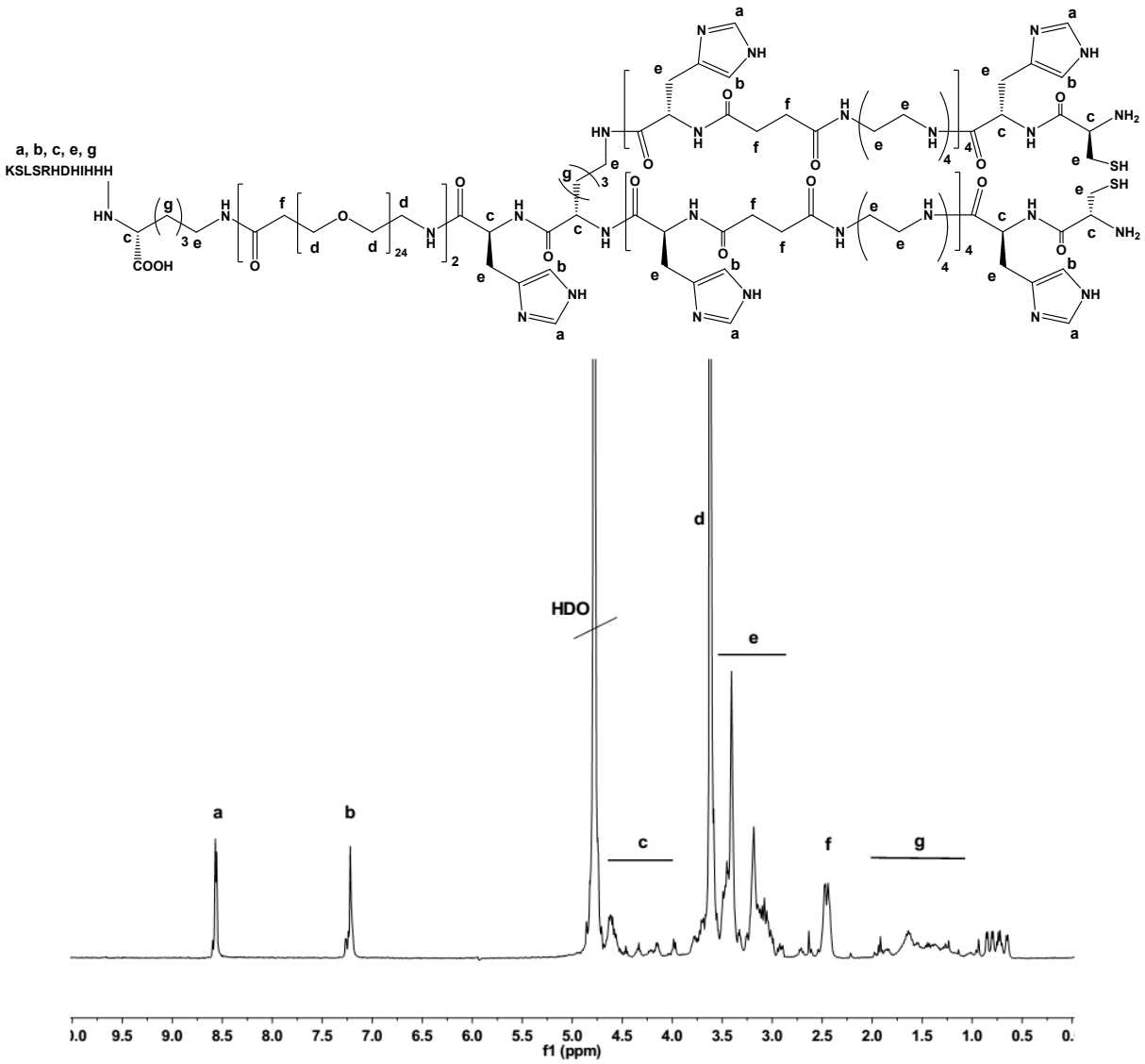
Sequence (C->N): A-PEG_{24}-H-K-$_{\alpha,\varepsilon}$[H-(Stp-H)$_4$-C]$_2$



^{1}H-NMR spectrum in D_2O.δ (ppm) = 1.2-1.6 (comp, 9 H, βH alanine, βγδH lysine), 2.4-2.6 (comp, 34 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- dPEG$_{24}$), 2.9-3.5 (comp, 156 H, -CH_2- tepa, βH cysteine, βH histidine, εH lysine), 3.62 (s, 98 H, -CH_2-O- dPEG$_{24}$, -CH_2-N- dPEG$_{24}$), 4.0-4.7 (comp, 15 H, αH cysteine, αH lysine, αH histidine), 4.79 (s, HDO), 7.24 (m, 11 H, aromatic H histidine), 8.57-8.58 (m, 11 H, aromatic H histidine).

#10 (ID 694)

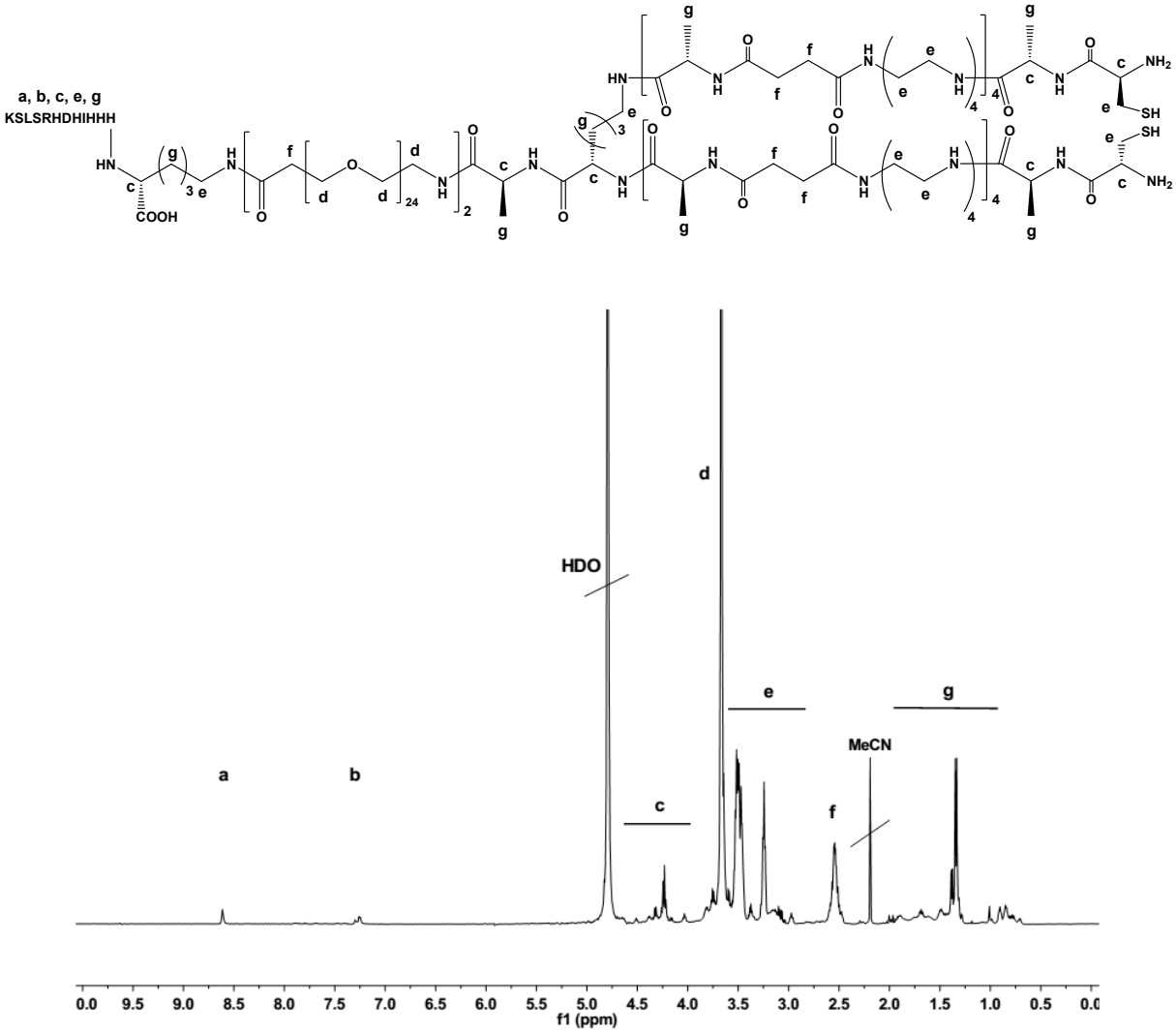
Sequence (C->N): K-$_{\alpha}$[HHHIHDHRSLSK]-$_{\varepsilon}$[(PEG_{24})$_2$-H-K-$_{\alpha,\varepsilon}$[H-(Stp-H)$_4$-C]$_2$]-



^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.0-2.0 (comp, 40 H, βγH arginine, βγδH isoleucine, βγδH leucine, βγδH lysine), 2.4-2.6 (comp, 36 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- $dPEG_{24}$), 2.9-3.5 (comp, 176 H, -CH_2- tepa, δH arginine, βH cysteine, βH histidine, εH lysine, βH serine), 3.62 (s, 196 H, -CH_2-O- $dPEG_{24}$, -CH_2-N- $dPEG_{24}$), 4.0-4.7 (comp, 27 H, αH amino acids), 4.78 (s, HDO), 7.22 (s, 16 H, aromatic H histidine), 8.56-8.57 (m, 16 H, aromatic H histidine).

#11 (ID 695)

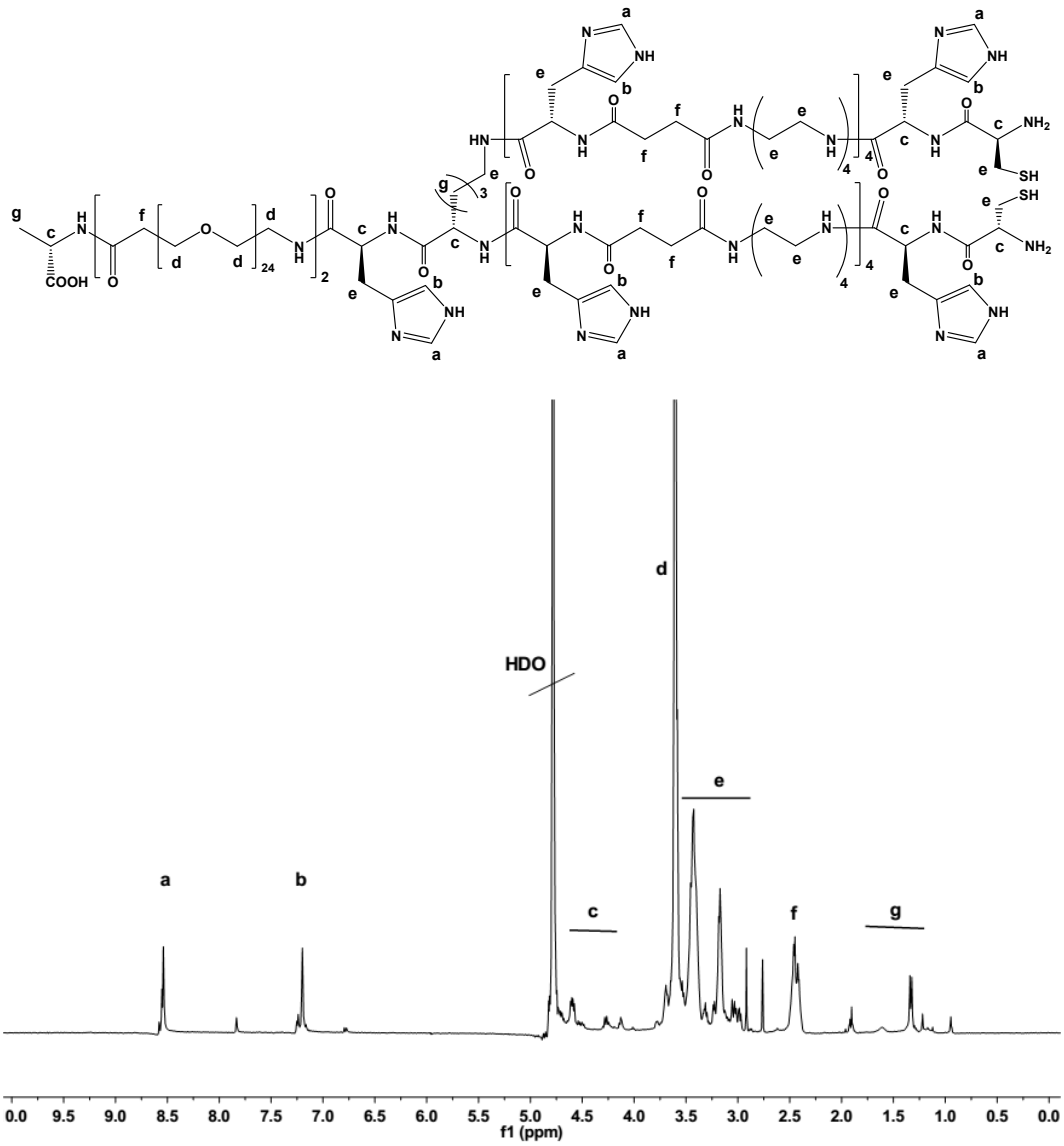
Sequence (C->N): K-$_{\alpha}$[HHHIHDHRSLSK]-$_{\varepsilon}$[(PEG$_{24}$)$_2$-A-K-$_{\alpha,\varepsilon}$[A-(Stp-A)$_4$-C]$_2$]



^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.0-2.0 (comp, 73 H, βH alanine, βγH arginine, βγδH isoleucine, βγδH leucine, βγδH lysine), 2.4-2.6 (comp, 36 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- dPEG$_{24}$), 2.9-3.6 (comp, 154 H, -CH_2- tepa, δH arginine, βH cysteine, βH histidine, εH lysine, βH serine), 3.67 (s, 196 H, -CH_2-O- dPEG$_{24}$, -CH_2-N- dPEG$_{24}$), 4.0-4.7 (m, 27 H, αH amino acids), 4.79 (s, HDO), 7.25-7.30 (m, 5 H, aromatic H histidine), 8.61 (s, 5 H, aromatic H histidine).

#12 (ID 616)

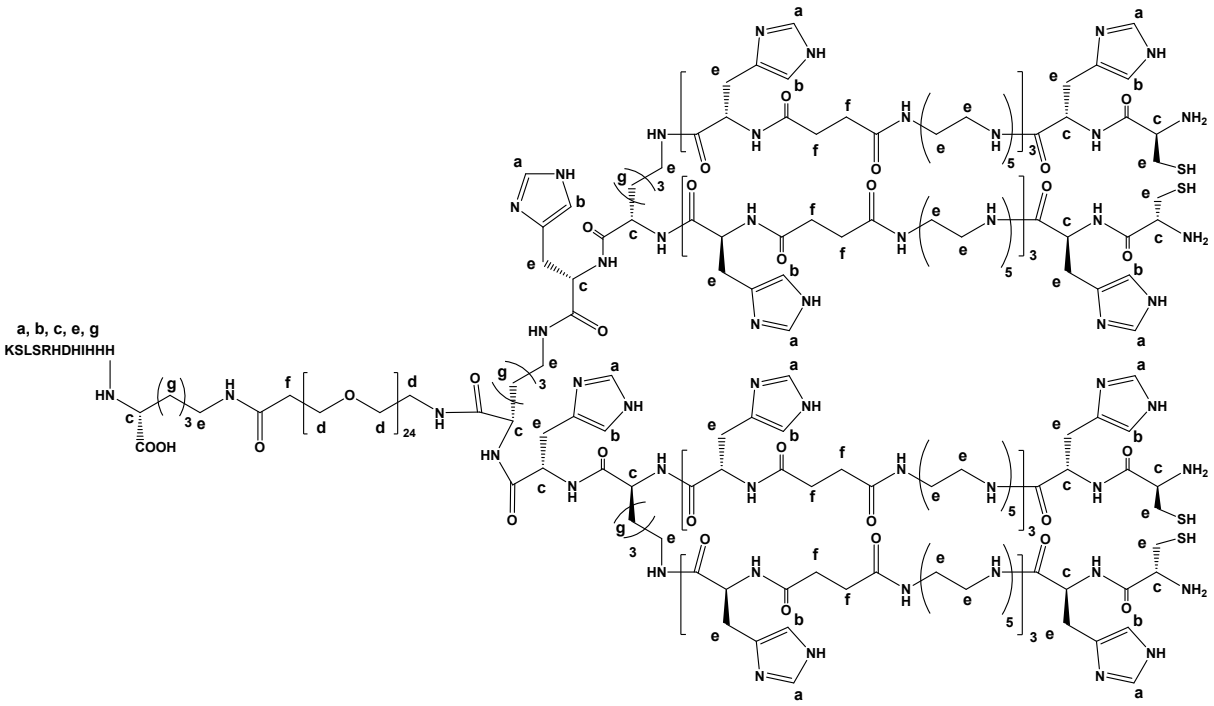
Sequence (C->N): A-$(PEG_{24})_2$-H-K-$_{\alpha,\varepsilon}$[H-(Stp-H)$_4$-C]$_2$

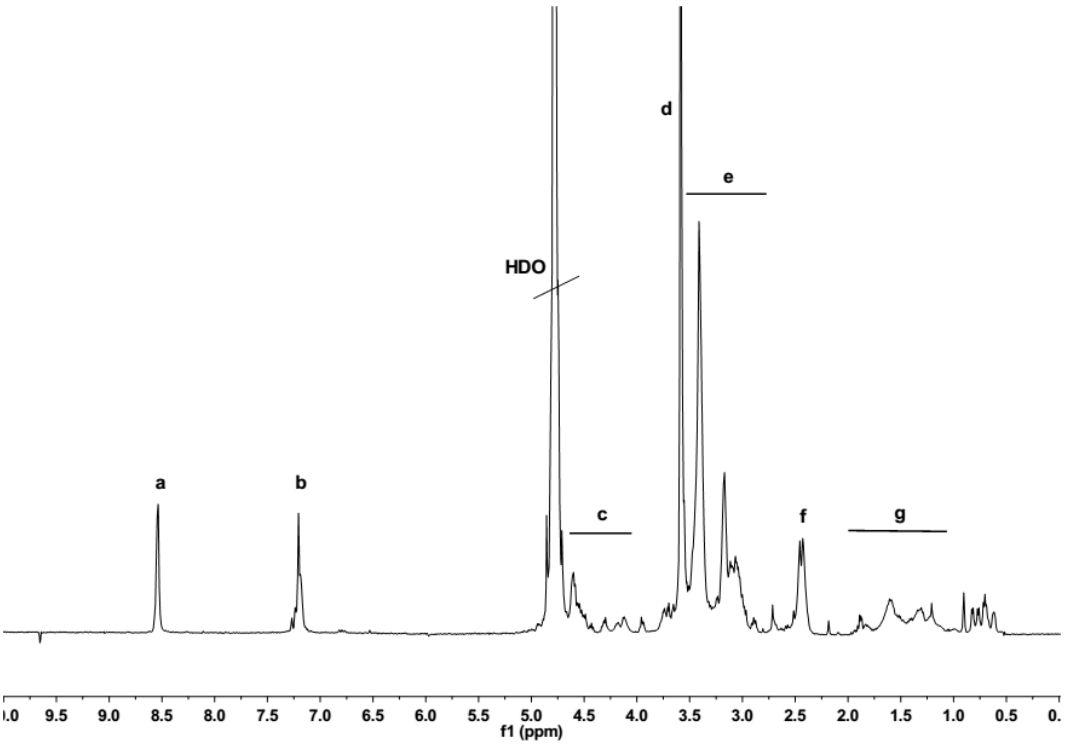


^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.2-1.7 (comp, 9 H, βH alanine, βγδH lysine), 2.4-2.6 (comp, 36 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- dPEG$_{24}$), 2.9-3.5 (comp, 156 H, -CH_2- tepa, βH cysteine, βH histidine, εH lysine), 3.61 (s, 196 H, -CH_2-O- dPEG$_{24}$, -CH_2-N- dPEG$_{24}$), 4.2-4.7 (comp, 15 H, αH cysteine, αH lysine, αH histidine), 4.78 (s, HDO), 7.20 (m, 11 H, aromatic H histidine), 8.54 (m, 11 H, aromatic H histidine).

#13 (ID 677)

Sequence: K-$_{\alpha}$[HHHIHDHRSLSK]-$_{\varepsilon}$[PEG_{24}-K-$_{\alpha,\varepsilon}$(H-K-$_{\alpha,\varepsilon}$(H-(Sph-H)$_3$-C)$_2$)$_2$]

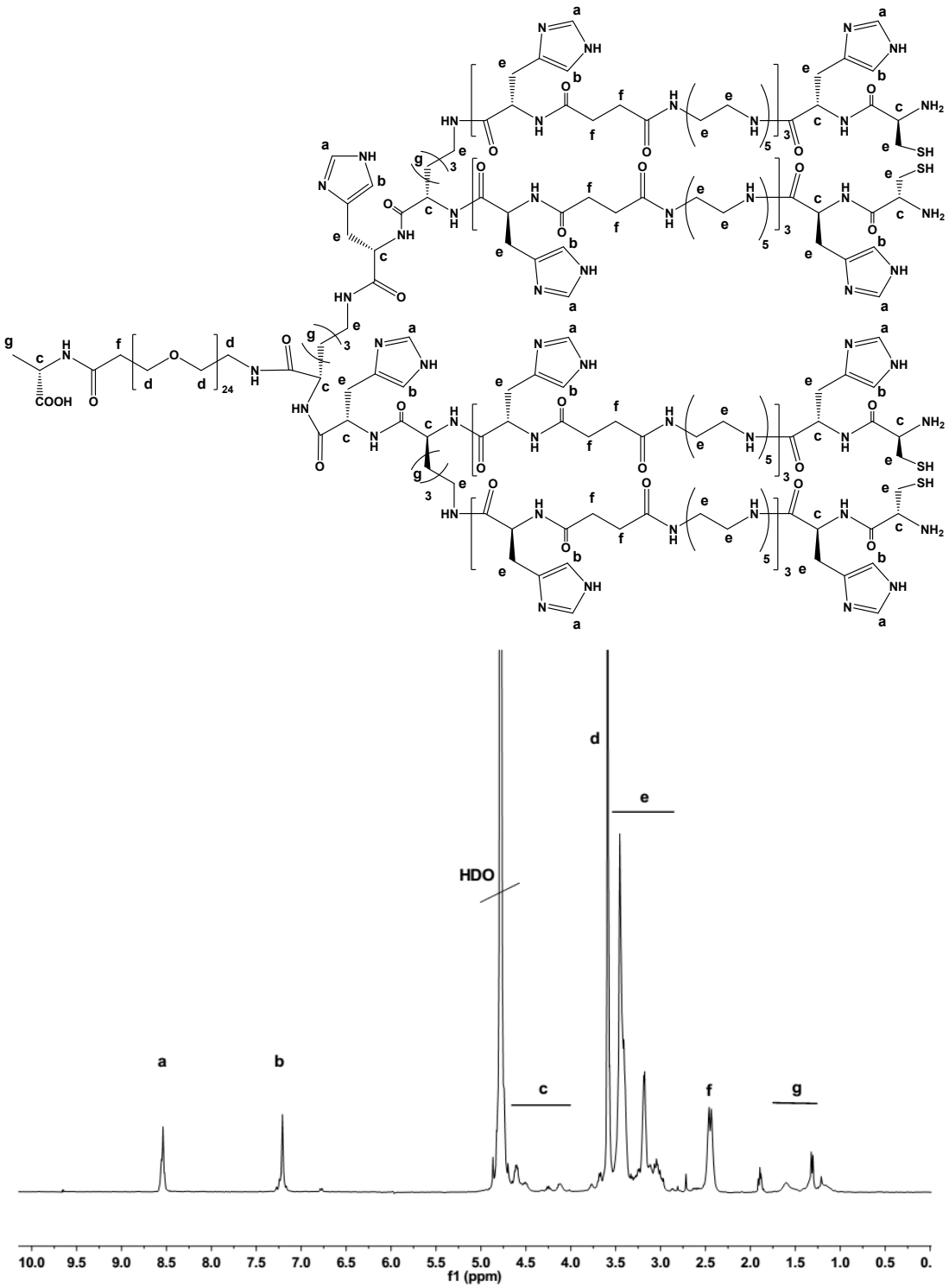




^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.0-2.0 (comp, 52 H, βγH arginine, βγδH isoleucine, βγδH leucine, βγδH lysine), 2.4-2.6 (comp, 50 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- $dPEG_{24}$), 2.8-3.5 (comp, 310 H, -CH_2- peha, δH arginine, βH cysteine, βH histidine, εH lysine, βH serine), 3.63 (s, 98 H, -CH_2-O- $dPEG_{24}$, -CH_2-N- $dPEG_{24}$), 4.0-4.7 (comp, 38 H, αH amino acids), 4.78 (s, HDO), 7.1-7.3 (m, 23 H, aromatic H histidine), 8.55 (s, 23 H, aromatic H histidine).

#14 (ID 678)

Sequence (C->N): A-PEG_{24}-K-$_{\alpha,\varepsilon}$[H-K-$_{\alpha,\varepsilon}$(H-(Sph-H)$_3$-C)$_2$]$_2$



^{1}H-NMR spectrum in D_2O. δ (ppm) = 1.2-1.7 (comp, 21 H, βH alanine, βγδH lysine), 2.4-2.6 (comp, 50 H, -CO-CH_2-CH_2-CO- succinic acid, -CO-CH_2- $dPEG_{24}$), 2.9-3.6 (comp, 254 H, -CH_2- peha, βH cysteine, βH histidine, εH lysine), 3.63 (s, 98 H, -CH_2-O- $dPEG_{24}$, -CH_2-N- $dPEG_{24}$), 4.0-4.7 (comp, 26 H, αH amino acids), 4.79 (s, HDO), 7.21 (s, 18 H, aromatic H histidine), 8.55 (s, 18 H, aromatic H histidine).

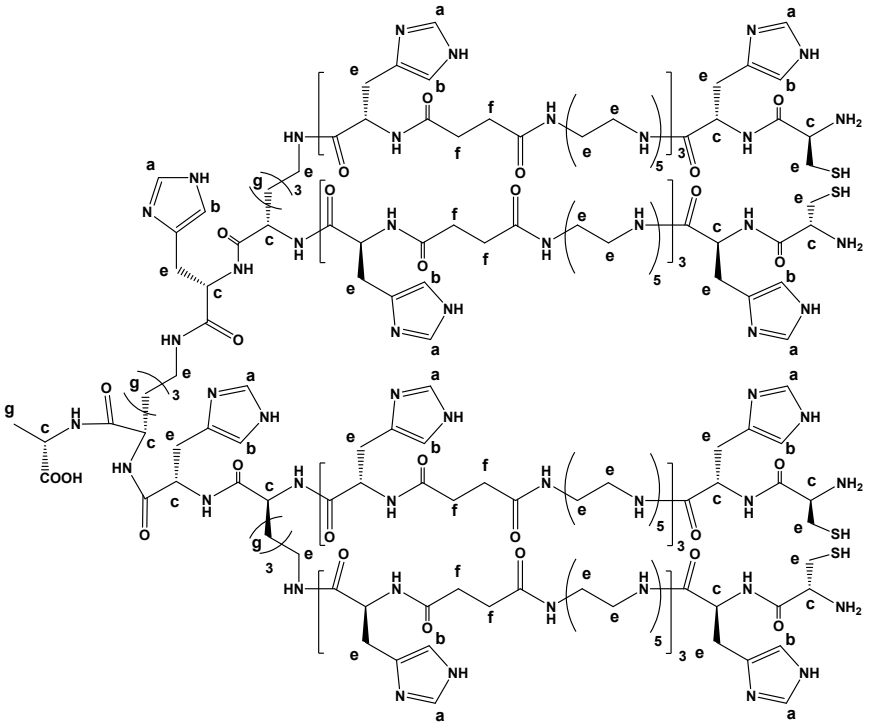
#15 (ID 689)

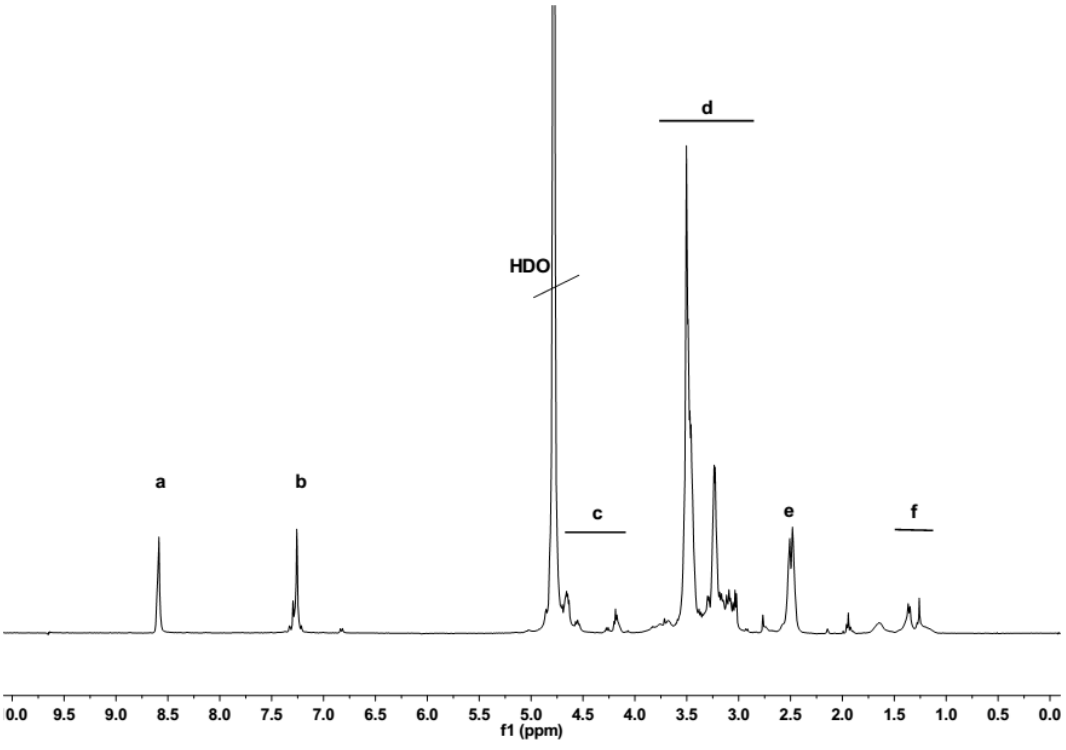
Sequence (C->N): C-H-(Stp-H)$_3$-K-$_{\alpha,\varepsilon}$[(H-Stp)$_3$-H-C]$_2$

^{1}H-NMR spectrum in D_2O.δ (ppm) = 1.1-1.4 (comp, 6H, βγδH lysine), 2.3-2.7 (comp, 36 H, -CO-CH_2-CH_2-CO- succinic acid), 2.9-3.8 (comp, 176 H, -CH_2- tepa, βH cysteine, βH histidine, εH lysine), 4.1-4.7 (comp, 16 H, αH cysteine, lysine, histidine), 4.79 (s, HDO), 7.2-7.4 (m, 12 H, aromatic H histidine), 8.5-8.7 (m, 12 H, aromatic H histidine).

#16 (ID 606)

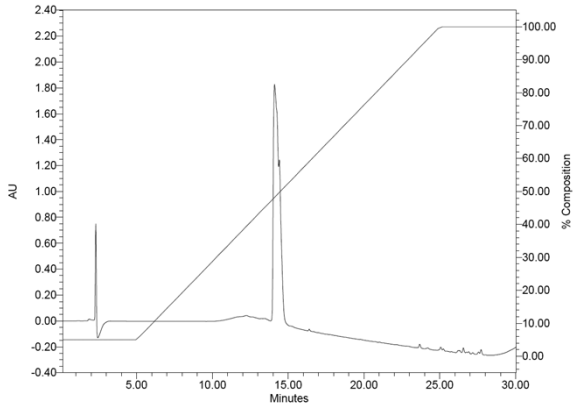
Sequence (C->N): A-K-$_{\alpha,\varepsilon}$[H-K-$_{\alpha,\varepsilon}$(H-(Sph-H)$_3$-C)$_2$]$_2$



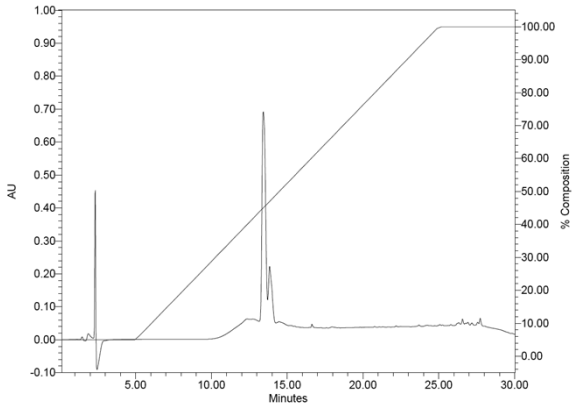


^{1}H-NMR spectrum in D_2O.δ (ppm) = 1.3-1.8 (comp, 19 H, βH alanine, βγδH lysine), 2.4-2.6 (comp, 48 H, -CO-CH_2-CH_2-CO- succinic acid), 2.9-3.7 (comp, 290 H, -CH_2- peha, βH cysteine, βH histidine, εH lysine), 4.1-4.7 (comp, 26 H, αH amino acids), 4.78 (s, HDO), 7.26 (s, 18 H, aromatic H histidine), 8.58-8.59 (m, 18 H, aromatic H histidine).

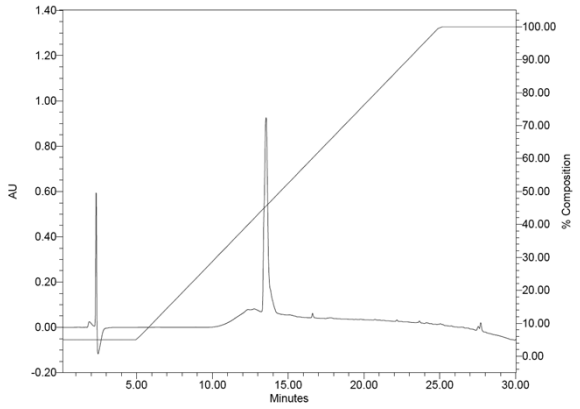
HPLC data



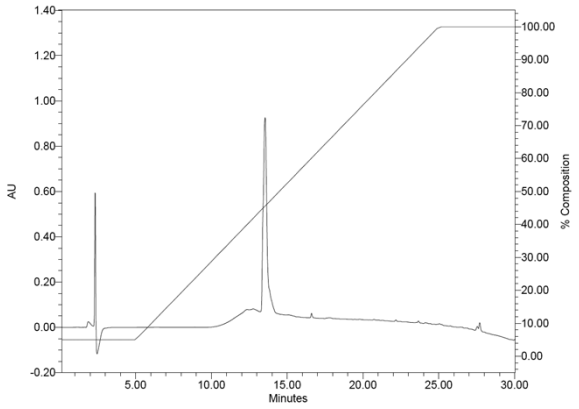
#1 (ID 696)



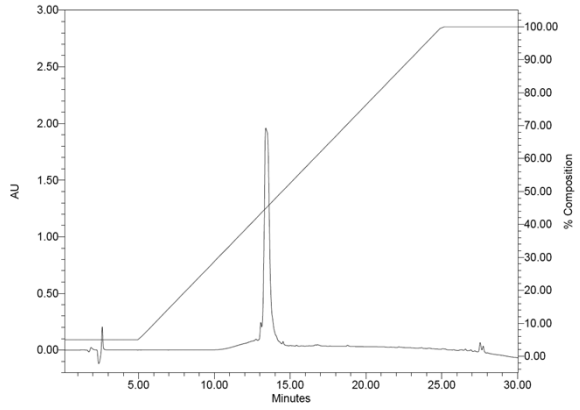
#2 (ID 443)



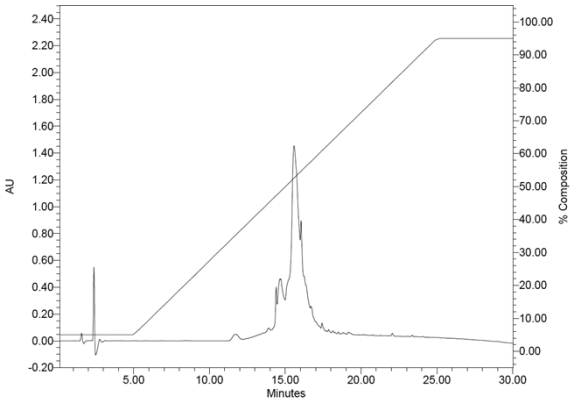
#3 (ID 188)



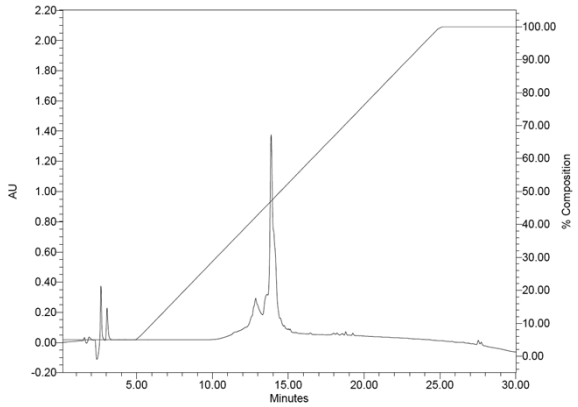
#8 (ID 442)



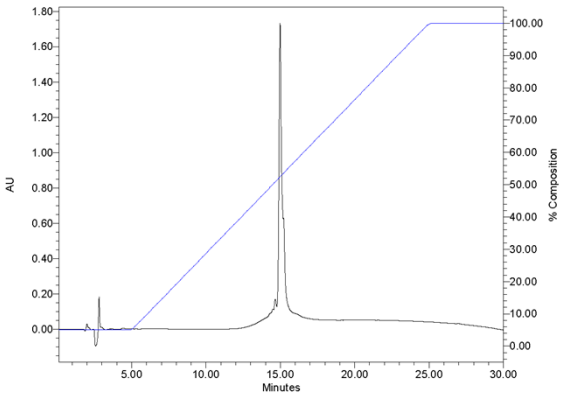
#9 (ID 440)



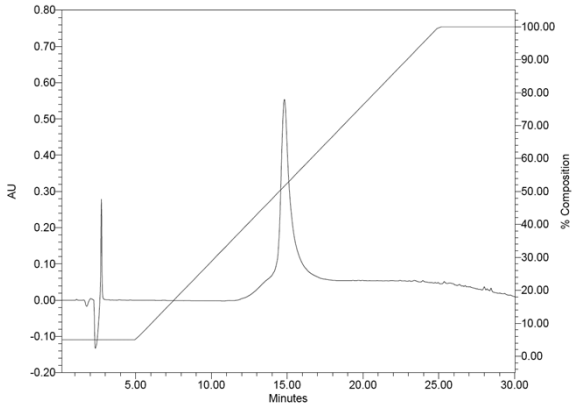
#10 (ID 694)



#12 (ID 616)



#15 (ID 689)



#16 (ID 606)