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Efficient Gene Delivery of Tailored Amphiphilic

Polypeptides by Polyplex Surfing

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27

28 **ABSTRACT**

29 Within this study, an amphiphilic and potentially biodegradable polypeptide library based on
30 poly[(4-aminobutyl)-L-glutamine-*stat*-hexyl-L-glutamine] (P(AB-L-Gln-*stat*-Hex-L-Gln)) was
31 investigated for gene delivery. The influence of varying proportions of aliphatic and cationic
32 side chains affecting the physicochemical properties of the polypeptides on transfection
33 efficiency was investigated. A composition of 40 mol% Hex-L-Gln and 60 mol% AB-L-Gln
34 (**P3**) was identified as best performer over polypeptides with higher proportions of protonatable
35 monomers. Detailed studies of the transfection mechanism revealed strongest interaction of **P3**
36 with cell membranes, promoting efficient endocytic cell uptake and high endosomal release.
37 Spectrally, time-, and z-resolved fluorescence microscopy further revealed the crucial role of
38 filopodia surfing in polyplex-cell-interaction and particle internalization in lamellipodia
39 regions, followed by rapid particle transport into cells. This study demonstrates the great
40 potential of polypeptides for gene delivery. Thereby, the amphiphilic character improves
41 performance over cationic homopolypeptides, and the potential biodegradability is
42 advantageous toward other synthetic polymeric delivery systems.

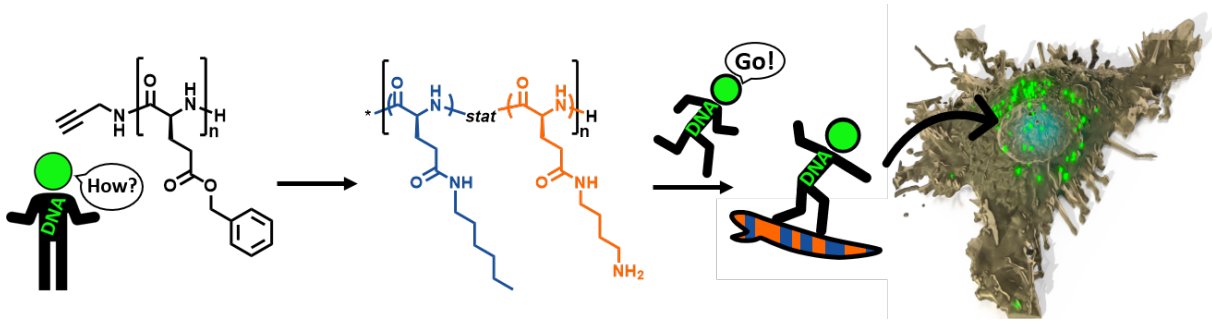
43 **KEYWORDS:** Ring opening polymerization, polypeptide, gene delivery, filopodia surfing,
44 transfection, fluorescence microscopy

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47 TOC

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50

51 INTRODUCTION

52 Synthetic polypeptide-based materials are analogues to functional, *e.g.*, antimicrobial peptides¹
53 and a powerful tool often used in drug,^{2, 3} and gene delivery^{4, 5} and controlled protein release.⁶
54 In particular, the development of suitable vectors for gene delivery, offering versatile
55 possibilities for the treatment of diseases with genetic origin or for vaccination, has attracted
56 increasing attention.^{7, 8} To fulfill its tasks, a gene delivery vector must combine several features,
57 *e.g.*, extracellular protection of nucleic acids from nucleases, uptake into targeted cells and
58 sufficient release of the cargo at its site of action.^{9, 10} Cellular uptake plays a crucial role in
59 transfection, as one of the main barriers that must be overcome and being critical for the
60 intracellular fate of the gene delivery vector and its cargo.^{11, 12} To study the uptake mechanism
61 of polyplexes, high-resolution imaging provides a powerful tool, *e.g.* to track active uptake *via*
62 endocytosis by polyplex surfing on filopodia, which are thin membrane protrusions for
63 scanning the cellular environment.¹³

64 Among non-viral gene delivery vectors, especially cationic polymers, including polypeptides,
65 have been evaluated. One prominent example is poly(L-lysine) (PLL) as one of the first non-
66 viral vectors, successfully complexing nucleic acids into polyplexes but revealing low
67 transfection efficiency due to lacking endosomal escape capability.^{14, 15} Besides protonation,
68 also the secondary structure is a decisive factor for the delivery efficiency of polypeptides. As
69 firstly observed for cell-penetrating peptides (CPP), an ordered α -helical or β -sheet¹⁶ secondary
70 structure promotes cell penetration and membrane interaction, while a random coil formation
71 as observed for PLL lacks this ability.^{17, 18} The interaction of CPPs with cellular membranes is
72 further enhanced by incorporation of hydrophobic moieties along the amino acid sequence
73 creating secondary amphipathic cationic peptides with changed charge distribution, resulting in
74 improved cellular uptake.^{19, 20} However, these short peptides often fail to sufficiently condense
75 and bind genetic material.^{21, 22} Ring opening polymerization (ROP) of amino acid *N*-
76 carboxyanhydrides (NCA) allows to generate high molar mass compounds with tailor-made

77 properties and functionalities by the choice of different monomers and initiators.^{5, 23, 24}
78 Furthermore, additional post modification of polypeptides is a valuable method for further fine
79 tuning of the materials. Click chemistry is widely used in this regard as both alkenyl and azide
80 functions are completely inert in ROP. Therefore, various strategies for introducing clickable
81 moieties were demonstrated, such as functional monomers^{25, 26} or the use of a suitable
82 initiator.²⁷ Alternatively, simple starting materials like poly(γ -benzyl-L-glutamate) (PBLG)^{28, 29}
83 or poly(L-glutamic acid) (PGA)^{30, 31} can be explored for the creation of more complex
84 structures. In this regard, post-modifications such as esterification, aminolysis and amidation
85 are applied and allow applications of polypeptides in gene^{32, 33} and drug delivery.³⁴ The
86 potential of PGA has already led to a polymer-drug-conjugate, PGA-paclitaxel (CT-2103)
87 [XYOTAX], which is currently in clinical trials for cancer therapy.^{35, 36} So far, for gene
88 delivery, mainly cationic homopolypeptides with ordered secondary structures have been
89 investigated, showing improved efficiency compared to their random coil counterparts,^{37, 38} and
90 only few studies explored the potential of amphiphilic polypeptides in transfection.³⁹⁻⁴¹
91 In this study, we now combine the beneficial properties, *i.e.*, an ordered secondary structure
92 and a tailored cationic/hydrophobic balance, for the design of synthetic polypeptides for gene
93 delivery. We present a small library of synthetic amphiphilic polypeptides **P1** to **P5** based on
94 poly[(4-aminobutyl)-L-glutamine-*stat*-hexyl-L-glutamine] (P(AB-L-Gln-*stat*-Hex-L-Gln)). The
95 amphiphilic polypeptides were prepared *via* a four-step synthetic route, characterized in terms
96 of their physicochemical properties depending on the ratio of AB-L-Gln- and Hex-L-Gln,
97 formulated into DNA polyplexes and screened for their gene delivery potential. Here, the
98 amphiphilic polypeptide **P3**, which has a high proportion of hydrophobic side chains and
99 ordered secondary structures but still remains soluble in aqueous buffers, showed the highest
100 efficiency. To gain insight into the transfection mechanism and the influence of the
101 cationic/hydrophobic balance on membrane interactions, endosomal release ability and cellular
102 uptake were studied. For an even deeper understanding of the polyplex acquisition, the transport

103 and the internalization process by the cells, spectrally, time- and z-resolved fluorescence
104 microscopy of live and fixed cells and complementary transmission electron microscopy were
105 employed. Thereby, an filopodia and lamellipodia-based initial acquisition mechanism of the
106 amphiphilic polypeptides was identified, which is followed by cellular internalization. Overall,
107 this study highlights the importance of the cationic/hydrophobic balance of amphiphilic
108 polypeptides for transfection efficiency and further reveals the mechanism of the so far
109 underestimated and unstudied step of initial acquisition of amphiphilic polypeptides by
110 lamellipodia and filopodia. These findings are thus giving essential insights for the further
111 design of tailor-made polypeptides for efficient gene delivery.

112 **EXPERIMENTAL**

113 **Ring opening polymerization of BLG-NCA.** In a glovebox filled with argon, BLG-NCA
114 (12 g, 45.68 mmol) was added to a fire-dried Schlenk flask and dissolved in 48 mL
115 dimethylformamide (DMF, $c_M = 0.95$ M). For the initiator stock solution, 200 μ L propargyl
116 amine (3.12 mmol) were diluted in 1 mL anhydrous DMF ($c = 2.60$ M). The polymerization
117 was initiated by adding 175 μ L of the initiator stock solution (monomer to initiator ratio
118 (M/I) = 100) to the highly agitated reaction solution. The solution was stirred overnight at room
119 temperature (RT) under inert atmosphere. The conversion of the monomer was followed by
120 infrared (IR) measurements (Figure S1). The resulting solution was precipitated in a large
121 excess of diethyl ether. The solid was separated by centrifugation (2132 g, 4 °C, 4 min) and
122 washed three times with 30 mL diethyl ether. All supernatants were discarded. A colorless
123 powder was obtained by freeze-drying. Yield: 8.88 g (88 %). The polymer was characterized
124 by proton nuclear magnetic resonance (1 H-NMR) spectroscopy in deuterated trifluoroacetic
125 acid (d-TFA) including traces of deuterated chloroform ($CDCl_3$, Figure S2B), size exclusion
126 chromatography (SEC) in dimethylacetamide (DMAc) containing 0.21 wt% LiCl (Figure S3A),
127 IR (Figure S4) and Raman spectroscopy (Figure S5).

128

129 **PGA synthesis via ester hydrolysis of PBLG side chains.** PGA was synthesized according to
130 a known procedure.⁴² To a solution of PBLG (3 g, 15.43 mmol BLG units) in 22.5 mL TFA,
131 3.4 mL HBr (48 %, 62.61 mmol, 4 eq. per BLG unit) were added dropwise. The solution was
132 stirred for 18 h at RT. The reaction mixture was precipitated in a large excess of diethyl ether.
133 The precipitant was separated by centrifugation (2132 g, 4 °C, 4 min) and washed three times
134 with diethyl ether. All supernatants were discarded. A colorless powder was obtained by freeze-
135 drying. Yield: 1.66 g (92 %). The product was characterized by ¹H-NMR spectroscopy in d-
136 TFA including traces of CDCl₃ (Figure S2A), by SEC in 0.08 M Na₂HPO₄/0.05 % NaN₃
137 (pH = 9) solution as eluent (Figure S3B), by IR (Figure S4) and Raman spectroscopy (Figure
138 S5), and by elemental analysis (found: Br 1.79 %).

139
140 **General procedure of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling**
141 **of *n*-hexylamine (HexA) and *N*-(*tert*-butoxycarbonyl)-1,4-diaminobutylamine (BocDAB)**
142 **with PGA.** The detailed amounts of the reactants are summarized in Table S2. PGA was
143 dissolved in DMF (c = 0.47 M). Successively, hydroxybenzotriazole monohydrate (HOBT),
144 1.5 eq. per L-Glu unit), HexA (1.10 - 0.31 eq. per L-Glu unit) and BocDAB (0.5 - 0.1 eq. per L-
145 Glu unit) were added and the reaction mixture was stirred for 10 min. EDC (1.50 eq. per L-Glu
146 unit) was added to the resulting suspension and stirred for 18 h at RT. The reaction mixture was
147 precipitated in a large excess of diethyl ether. The solids were separated by centrifugation
148 (2132 g, 4 °C, 4 min) and washed 3 times with diethyl ether. All supernatants were discarded.
149 After drying under reduced pressure, the residue was washed three times with water. A colorless
150 powder was obtained by freeze-drying. The product was characterized by ¹H-NMR
151 spectroscopy in d-TFA including traces of CDCl₃ (Figure 1) and by SEC in DMAc containing
152 0.21 wt% LiCl (Figure S3A) as well as by IR spectroscopy (Figure S7). The respective amount
153 of BocDAB or HexA (mol% and degree of polymerization (DP)) in each compound,
154 summarized in Table 1, was calculated by area correlation of the characteristic signals in the

155 ¹H-NMR spectra using the Peak Analyzer of Origin 9.1.0G (© by OriginLab Corporation),
156 according to a procedure published elsewhere, Figure S8.⁵ In addition, the DP of PBLG was
157 presumed as total DP of **PP1 - PP4** and **P5**. The theoretical molar mass ($M_{n,theo}$) was calculated
158 by adding the molar mass of all 96 units and of the initiator.

159

160 **General procedure of Boc deprotection (P1 to P4).** The detailed amounts of the reactants are
161 summarized in Table S3. In a reaction vessel, poly[(4-(N-tert-butoxycarbonylaminobutyl)-L-
162 glutamine-*stat*-hexyl-L-glutamine] (P(BocAB-L-Gln-*stat*-Hex-L-Gln)) (250 mg) was dissolved
163 in 2.5 mL TFA and stirred overnight at RT. Then, the pH value of the reaction solution was
164 adjusted to 7.4 using a saturated solution of NaHCO₃. The resulting mixture was dialyzed
165 against water with a regenerated cellulose membrane (MWCO 3.5 kDa, SpectraPor RC, Carl
166 Roth) with regular water changes to remove all salt impurities. A colorless powder was obtained
167 by freeze-drying. The product was characterized by ¹H-NMR spectroscopy in d-TFA including
168 traces of CDCl₃ (Figure S9), Raman- (Figure S5) and CD spectroscopy (Figure 1B).

169

170 **Polyplex formulation.** pDNA-loaded polyplexes were formulated either in phosphate buffered
171 saline solution (PBS) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)
172 buffered glucose solution (HBG, 5 % (w/v) glucose, 20 mM HEPES, pH 7.4). Therefore, stock
173 solutions of the polypeptides in PBS or 0.2 M sodium acetate buffer (pH 5.8) were diluted with
174 PBS (PBS polyplex) or HBG buffer (HBG polyplex), respectively. To obtain a nitrogen to
175 phosphate (N/P) ratio of 5 and 10, the polymers were mixed with pDNA dissolved in ultrapure
176 water at a volume ratio of 1:2 followed by vortexing for 10 s. The samples were incubated for
177 15 min at RT prior to usage.

178

179 **Cell culture.** The mouse fibroblast cell line L-929 was cultured in Dulbecco's modified eagle
180 medium (DMEM, 1 g L⁻¹ glucose), supplemented with 10 % (v/v) fetal bovine serum (FBS),

181 100U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (D10) at 37 °C in a humidified 5 % (v/v) CO₂
182 atmosphere.

183

184 **Transfection of L-929 Cells.** For transfection experiments, L-929 cells were seeded in a 48-
185 well format at a density of 0.1×10^6 cells mL⁻¹ in 250 µL D10 per well and incubated for 24 h.
186 The medium was changed to 225 µL fresh D10 1 h prior to treatment. Polyplexes were prepared
187 as described above using DH-pEGFP-N1 pDNA. pKMyC pDNA (not encoding for green
188 fluorescent protein) was used as negative control. The polyplexes were prepared at N/P ratios
189 of 5 and 10 and final pDNA concentrations of 3 and 4.5 µg mL⁻¹ per well. The cells were treated
190 with 25 µL of the samples or respective buffer as control and incubated with the samples for
191 24 h. Subsequently the cells were detached by trypsin-ethylenediaminetetraacetate (trypsin-
192 EDTA), resuspended in Hanks' balanced salt solution (HBSS) buffer (supplemented with 2 %
193 FBS and 20 mM HEPES) and measured *via* flow cytometry ($\lambda_{\text{EX}} = 488$ nm combined with a
194 525/40 nm bandpass filter, FITC channel). Viable single cells expressing green fluorescence
195 protein (GFP) were identified by gating to the negative control (polyplex with pKMyC pDNA).
196 The gating strategy is shown in detail in Figure S10. Cells were further imaged by fluorescence
197 microscopy using brightfield imaging and the FITC channel ($\lambda_{\text{EX}} = 469/35$ nm,
198 $\lambda_{\text{EM}} = 525/39$ nm) ⁴³.

199

200 **Endosomal escape *via* calcein release assay.** For investigations of endosomal escape, a calcein
201 assay was performed as previously described with slight adaptations of the procedure.^{44, 45} L-929
202 cells were seeded in 24-well ibiTreat plates at a density of 0.1×10^6 cells mL⁻¹ in 500 µL D10
203 and incubated for 24 h. The medium was changed to fresh D10 1 h prior to treatment.
204 Polyplexes were prepared as described above (N/P 10, 4.5 µg mL⁻¹ pDNA per well). For
205 treatment, 10 µL calcein (2.5 mg mL⁻¹) were added per well to give a final concentration of
206 50 µg mL⁻¹, directly followed by 50 µL of the samples. Following 1 h of incubation, the cells

207 were washed twice with warm FC buffer and 500 μ L warm D20 (DMEM supplemented with
208 20 % FBS) was added. The cells were subsequently stained with 8 μ M Hoechst 33342 and
209 imaged *via* confocal laser light scanning microscopy (CLSM). Calcein was excited at 488 nm
210 by applying the argon laser (1 %) using emission filters for 490–544 nm. For excitation of
211 Hoechst, the Argon laser at 405 nm (0.5 %) was used with a detection filter for 410–469 nm.
212 To avoid cross talk, calcein and Hoechst were imaged in two separate tracks using the frame
213 scan modus. Images were acquired using the ZEN software, version 2.3 SP1 (Zeiss) and were
214 processed using ImageJ (Version 1.52a, National Institutes of Health).⁴⁶ Image analysis for
215 quantification of cells with released calcein, was conducted as described before, with slight
216 adjustments of image processing steps and is described in detail in the SI ⁴⁵.

217

218 **Cellular uptake of the polyplexes in L-929 cells measured *via* flow cytometry.** To study
219 cellular uptake *via* flow cytometry, L-929 cells were seeded in a 48-well format as described
220 for transfection experiments. The medium was changed to 225 μ L fresh D10 1 h prior to
221 treatment. For polyplex preparation (N/P 5 and 10, 1.5 μ g mL⁻¹ pDNA), pKMyC pDNA was
222 labeled with YOYO-1 iodide (0.027 nmol per 1 μ g pDNA) and subsequently mixed with the
223 polypeptides as described above. In the following, the assay was performed as described
224 before.⁴⁴ Briefly, 25 μ L of the samples were added per well and the cells were incubated with
225 the samples for 1 h, 4 h and 24 h. Subsequently, the cells were detached and resuspended as
226 described for transfection experiments and Trypan Blue solution was added at a concentration
227 of 0.04 % per well. Additionally, the cells were stained once with propidium iodide (PI) to gate
228 the viable cells. PI was added to a final concentration of 1 μ g mL⁻¹, and the cells were then
229 measured *via* flow cytometry (λ_{EX} = 488 nm, 610/20 nm bandpass filter). PI-negative cells were
230 gated according to the positive control linear poly(ethyleneimine) (LPEI) without pDNA, final
231 concentration of 100 μ g mL⁻¹) and negative control (PBS buffer). YOYO-1 fluorescence was
232 measured using the FITC channel. Viable single cells showing increased YOYO-1 fluorescence

233 intensity (FI) were identified by gating to the negative control (pDNA labeled with YOYO-1)
234 and the relative mean fluorescence intensity (rMFI) of all viable single cells was calculated
235 relative to the negative control. A detailed gating strategy is shown in the SI (Figure S11).

236

237 **Cellular uptake of P2 and P3 polyplexes in L-929 cells imaged by fluorescence microscopy.**

238 To monitor cellular uptake *via* fluorescence microscopy, L-929 cells were seeded into 4-well
239 glass bottom dishes (Greiner) at a density of 0.2×10^6 cells mL⁻¹ in 500 μL D10 and incubated
240 for 24 h. Polyplexes of **P2** (N/P 5) and **P3** (N/P 10) were prepared using pKMyC pDNA, which
241 was labeled with YOYO-1 as described above at a final pDNA concentration of 1.5 μg mL⁻¹
242 per well. For live cell imaging, the cells were stained with CellMask™ Deep Red membrane
243 stain (CMDR), Thermo Fisher Scientific, 1× working solution in HBSS buffer prepared from
244 1000x concentrated stain solution). For staining, the supernatant was removed, the cells were
245 washed once with 500 μL PBS buffer, and 100 μL of the staining solution was added per well.
246 After 8 min of incubation, the staining solution was removed, and the cells were washed three
247 times with PBS buffer, followed by addition of 250 μL fresh D10 per well. For treatment, 50 μL
248 of the polyplex solution was diluted with 200 μL D10 and added to the cells to obtain a final
249 volume of 500 μL per well. After incubation and live cell imaging (100 min), the cells were
250 washed three times with PBS, and cells treated with **P3** polyplex were fixed with 4 %
251 paraformaldehyde (PFA) solution in water by 10 min incubation at 37 °C. The cells were then
252 washed three times with PBS, 500 μL PBS was added per well and the cells were stored in the
253 fridge overnight before z-resolved measurements. For measurements of fixed cells treated with
254 **P2** polyplex, the medium was changed to 450 μL fresh D10 1 h prior to treatment.
255 Subsequently, 50 μL of **P2** polyplex solution was added per well and incubated with the cells
256 for 15 min, followed by CMDR staining and fixation with PFA as described above, and
257 immediate transfer to the microscope.

258 Two-channel images (CMDR, YOYO-1) were recorded using simultaneous excitation (488 nm
259 (YOYO-1), 650 nm (CMDR); white light laser, Leica) and the internal monochromators. For
260 all data, except the spectrally resolved YOYO-1 data, the signals were detected in the spectral
261 ranges 503 - 628 nm and 665 - 800 nm, respectively. For spectrally resolved YOYO-1 data, the
262 excitation wavelength was set to 483 nm and detection was performed in increments of 5 nm
263 and with detection windows of 5 nm. The pinhole for confocal operation was opened to account
264 for 1 airy disk in all measurements. Detailed parameters for every measurement are part of the
265 SI.

266

267 **Sample preparation for imaging of cellular uptake of P2 and P3 polyplexes in L-929 cells**
268 **by transmission electron microscopy (TEM).** For uptake studies *via* TEM, L-929 cells were
269 seeded into 6-well cell culture plates at a density of 0.5×10^6 cells mL⁻¹ in 2 mL D10 and
270 incubated for 24 h. Medium was changed to 1.8 mL fresh D10 1 h prior to treatment. Polyplexes
271 of **P2** (N/P 5) and **P3** (N/P 10) were prepared using pKMyC pDNA at a final pDNA
272 concentration of 4.5 µg mL⁻¹ per well and 200 µL of the sample was added per well. After
273 incubation for 1 h, the cells were detached by trypsin-EDTA and resuspended in 450 µL D10.
274 For embedding, the cells were washed three times with PBS and centrifuged at 100 g for
275 2 min.⁴⁷ The cells were fixated on ice for 1 h in an 2.5 % glutaryl aldehyde, 1 % osmium
276 tetroxide PBS solution. To remove any residues, the fixed cells were subsequently washed twice
277 for 15 min with PBS. Dehydration of the cells was reached by replacing the buffer solution
278 stepwise (50 %, 70 %, 90 %, 100 %) with ethanol (EtOH). 18 µL 2,4,6-
279 tri(dimethylaminomethyl) phenol solution (DMP-30) were added per mL to a previously
280 prepared Embed812 (EMbed 812, dodecyl succinic anhydride (DDSA) and methyl-5-
281 norbornene-2,3-dicarboxylic anhydride (NMA) in the ratio 1:1.1:0.25) solution. In a first step,
282 the embedding solution was added to the cells for 1 h with a ratio of 2:1 with EtOH.
283 Subsequently, the cells were centrifuged at 100 g for 8 min and the residual embedding solution

284 was removed followed by adding pure Embed812/DMP-30 solution, incubation at RT
285 overnight and centrifugation again. After the removal of the residual embedding solution,
286 freshly prepared Embed812/DMP-30 solution was added and incubated for 2 h at RT.
287 Afterwards the cells were transferred to BEEM® capsules and centrifuged again. Residual
288 embedding solution was removed before fresh Embed812/DMP-30 solution was added, and the
289 samples were dried at 60 °C overnight in the drying oven. The dried sample was cut in 100 nm
290 slices. Each slice was stained with 4 % uranyl acetate solution for 20 min and subsequently
291 with lead citrate for 5 min. The slices were transferred onto Quantifoil® carbon supported film
292 grids (Cu 300 mesh) and subjected to TEM measurements. For cryo-TEM investigations, 9 µL
293 of the particle solutions were deposited on a Quantifoil grid (R2/2) in a Vitrobot Mark IV.
294 Samples were blotted at a blot force of -1 for 1 s and rapidly immersed into liquid ethane serving
295 as the cryogen. Grids were transferred into the cryo-holder (Gatan 626) utilizing the Gatan cryo
296 transfer stage. During the entire process after vitrification in ethane, the temperature was
297 maintained at temperatures lower than 175 °C. Images were acquired on a side-entry CCD
298 camera (MegaView, Olympus Soft Imaging Solutions).

299

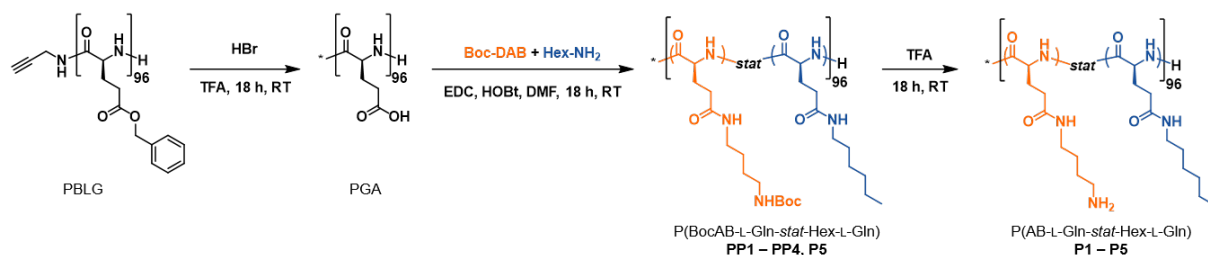
300 **Statistical analysis.** Statistically significant differences within transfection and uptake
301 experiments measured by flow cytometry were determined by analyzing multiple groups by
302 two-way mixed analysis of variance (two-way mixed ANOVA) and a Bonferroni's post-hoc
303 test. Statistical significance is indicated as follows: */# $p < 0.05$, **/## $p < 0.01$, and ***/###
304 $p < 0.001$ and analysis was conducted using OriginPro2021 software.

305

306 **RESULTS AND DISCUSSION**

307 **Polymer synthesis and characterization.** Cationic functionalities are necessary to enable the
308 complexation of pDNA and the formation of a polyplex. Therefore, a small library consisting
309 of the pure polycation P(AB-L-Gln) P1, the amphiphilic polypeptides P(AB-L-Gln-*stat*-Hex-L-

310 Gln) P2 - P4 and the hydrophobic P(Hex-L-Gln) P5 were prepared at room temperature (RT)
 311 by amidation of PGA and deprotection of the Boc-AB-L-Gln units of the protected polymers
 312 (PP1 - PP4), Scheme 1. PGA was obtained by classical ester hydrolysis of the starting material
 313 PBLG ⁴².



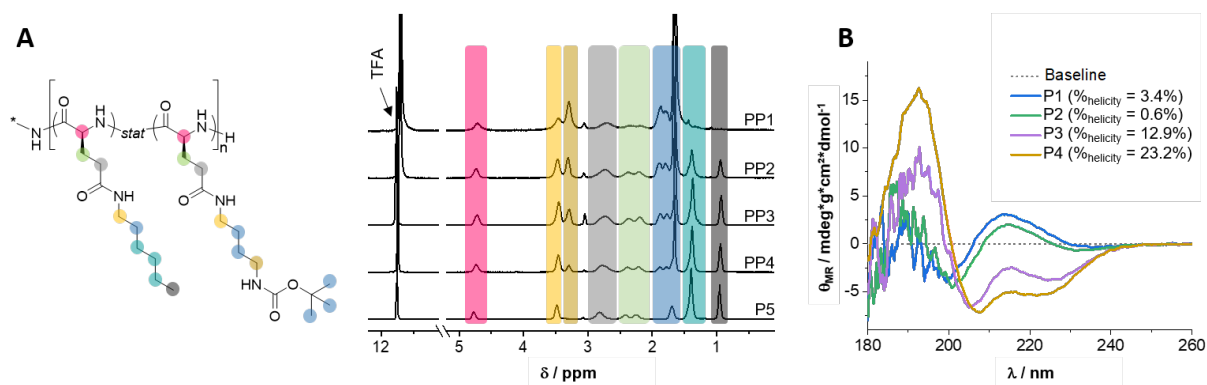
314
 315 **Scheme 1:** Schematic representation of the synthesis route of P(AB-L-Gln-*stat*-Hex-L-Gln) **P1**
 316 (100 mol% AB-L-Gln : 0 mol% Hex-L-Gln), **P2** (84 mol% : 16 mol%), **P3** (60 mol% :
 317 40 mol%), **P4** (30 mol% : 70 mol%) and **P5** (0 mol% : 100 mol%).

318 PBLG was synthesized *via* ROP from BLG-NCA. Propargyl amine was used as an initiator
 319 with a M/I of 100 to allow a simplified determination of the DP based on the isolated signal in
 320 the ¹H-NMR of the methylene protons at $\delta = 4.07$ ppm (Figure S2). The conversion of the
 321 monomer was monitored by IR spectroscopy. After four days, the complete disappearance of
 322 the characteristic NCA bands at $\tilde{\nu} = 1853$ cm⁻¹ and 1786 cm⁻¹ indicated complete conversion
 323 (Figure S1). PBLG is narrowly distributed with a polydispersity index (*D*) of 1.07 as determined
 324 by SEC (Figure S3, Table S1). Using the ¹H-NMR spectrum in Figure S2, a chain length of on
 325 average 96 monomer units was obtained by integrating the signal from the chiral proton in the
 326 polymer backbone at $\delta = 4.76$ ppm, relative to the two protons of the methylene group of the
 327 starting group at $\delta = 4.07$ ppm. Starting from PBLG, three modification steps were required to
 328 reach the targeted structures **P1** to **P4** (Scheme 1).

329 The cleavage of the benzyl ester of PBLG led to PGA. The Raman spectra of both compounds
 330 (Figure S5) show the complete disappearance of the aromatic CH stretching vibration at
 331 $\tilde{\nu} = 3065$ cm⁻¹, as well as the aromatic ring breathing mode at $\tilde{\nu} = 1004$ cm⁻¹ and the
 332 accompanying peak at $\tilde{\nu} = 1031$ cm⁻¹, which is typical for mono-substituted aromatic
 333 compounds and, thus, indicates complete conversion. Accordingly, IR spectra of PGA (Figure

334 S4) show the presence of carboxylic OH groups at $\tilde{\nu} \sim 1700 \text{ cm}^{-1}$ and the signal at
335 $\tilde{\nu} \sim 3300 \text{ cm}^{-1}$, which is partially obstructed by the broad NH stretching mode. The
336 disappearance of all benzylic protons in the $^1\text{H-NMR}$ spectra (Figure S2) further confirms the
337 successful hydrolysis of the ester side arms. Elemental analysis of PGA showed 1.79 % Br,
338 indicating the presence of some hydrobromides and potentially a partial HBr addition to the
339 triple bond. However, stoichiometric conversion with 2 eq. HBr could only account for 1.26 %
340 Br, and all intermediates (**PP1 - PP4**) and final products (**P1 - P5**) show the characteristic
341 resonance of $\text{C}\equiv\text{C}$ triple bonds at 2130 cm^{-1} in their Raman spectra (Figure S5), indicating that
342 addition of HBr in the first reaction step is a minor concern. Because of the lack of suitable,
343 *i.e.*, non-overlapping and constant peaks in $^1\text{H-NMR}$, quantitative statements about the DP of
344 PGA are difficult to make. SEC measurements of PGA (Figure S3B) show a tailing that can be
345 explained by interactions with the column. The high molar mass (M_n) and D of PGA compared
346 to PBLG as determined by SEC can be explained by the aqueous eluent and a different
347 calibration (Table S1).

348 After ester hydrolysis, the carboxylic acid functionalities were now available to couple amines
349 in various ratios by activation with EDC and HOBt activation. Therefore, the mono-protected
350 compound of DAB, *i.e.*, BocDAB, was used to prevent crosslinking when working with dual-
351 functional DAB. In addition, hydrophobic HexA was chosen to lower the toxicity of the
352 polycation while ensuring efficient complexation of pDNA and preventing random coil
353 formation as secondary structures. To investigate the changes in chemical, structural,
354 spectroscopic, and biological behaviors, two homopolymers (**PP1** and **P5**) and three
355 copolymers (**PP2 - PP4**) with different ratios of HexA and BocDAB were synthesized, Table
356 S2. **PP2** and **PP3** were synthesized with an excess of BocDAB compared to HexA, while a
357 substoichiometric amount of BocDAB was used for **PP4**. Figure 1 shows the $^1\text{H-NMR}$ spectra
358 of **PP1** to **PP4** and **P5**. The detection of the starting group is shown in Figure S12.



359

360 **Figure 1** A $^1\text{H-NMR}$ spectra of **PP1** to **PP4** and **P5** measured at 25 °C and 300 MHz in
 361 deuterated d-TFA containing traces of CDCl_3 . **B** Overlay circular dichroism (CD) spectra of
 362 **P1** – **P4** measured in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) ($c_{\text{pi}} = 0.50 \text{ mg mL}^{-1}$). Mean
 363 residual ellipticity θ_{MR} calculated with Equation S6 including $\%_{\text{helicity}}$ calculated with Equation
 364 S7.

365

366 Both, ester hydrolysis and EDC coupling do not change the total DP of 96, which was
 367 determined for PBLG and, thus, also applies to the intermediates. The content of BocDAB and
 368 HexA can be determined by correlating the characteristic signals of both moieties in their $^1\text{H-}$
 369 NMR spectrum.⁵ $^1\text{H-NMR}$ studies revealed the following ratios of BocAB-L-Gln and Hex-L-
 370 Gln: **PP1** 100 mol%:0 mol%, **PP2** 84 mol%:16 mol%, **PP3** 60 mol%:40 mol%, **PP4**
 371 30 mol%:70 mol% and **P5** 0 mol%:100 mol%, Table 1. The elugrams of **PP1** to **PP4** measured
 372 by SEC showed monomodal distributions in all cases with low D around 1.17 (Figure S3A). **P5**
 373 is insoluble in all common solvents except TFA, consequently, investigation with the given
 374 SEC systems was not possible. The M_n values determined by universal PS calibration are
 375 comparable to $M_{n,\text{theo}}$ calculated from the $^1\text{H-NMR}$ results, Table 1. The cleavage of the Boc
 376 protecting group from **PP1** to **PP4** in pure TFA resulted in **P1** to **P4**. The disappearance of the
 377 prominent singlet at $\delta = 1.6 \text{ ppm}$ in $^1\text{H-NMR}$ spectroscopy (Figure S9A) indicates a complete
 378 deprotection of the amines of **PP1** to **PP4**. The SEC studies in Figure S9B show that polymers
 379 **P1**, **P2** and **P3** are still intact. However, in the case of **P3**, a second population elutes with a
 380 lower elution volume, indicating a higher molar mass species. This could be caused by
 381 interactions of the polymer with the stationary phase of column material or by self-assembly of

382 the polymer in the eluent. SEC analysis was not possible for **P4** and **P5** due to their insolubility
 383 in the eluent used.

384 **Table 1:** Results of analytical investigations of **PP1** to **PP4** and **P5**.

	¹ H-NMR ^{a)}					SEC ^{b)}		Yield	ID
	M _{n,theo}	mol% _{BocAB}	DP	mol% _{Hex}	DP	M _n	Đ		
	kg mol ⁻¹					kg mol ⁻¹		%	deprot.
PP1	28.8	100	96	-	-	24.0	1.15	62	P1
PP2	27.5	84	81	16	15	24.7	1.13	51	P2
PP3	25.5	60	58	40	38	23.0	1.17	71	P3
PP4	22.9	30	29	70	67	21.5	1.21	68	P4
P5	20.4	-	-	100	96	-	-	69	P5

385 a) ¹H-NMR measured in d-TFA containing traces of CDCl₃ at 25 °C and 300 MHz. In each
 386 case, sum of DP_{BocAB} and DP_{Hex} gives a total DP of 96 (DP of PBLG). b) SEC
 387 measurements were determined by using DMAc/0.21 wt% LiCl as eluent, RI detector and a
 388 polystyrene (PS) calibration.

389

390 **Investigation of the secondary structures.** Previous studies have already shown that the
 391 secondary structure of a polypeptide has an impact on effective cell uptake through
 392 destabilization of the membrane.⁴⁸ In Figure S13A, IR spectra of solid **P1** to **P5** show the
 393 influence of the aliphatic side arms. With increasing content of HexA, the band of β-sheets also
 394 increased at $\tilde{\nu}_\beta \sim 1625 \text{ cm}^{-1}$, while the band for α-helices decreases at $\tilde{\nu}_\alpha \sim 1645 \text{ cm}^{-1}$. In
 395 addition, it can be suspected that less amount of random coil structures was formed
 396 ($\tilde{\nu}_{rc} \sim 1650 \text{ cm}^{-1}$ and $\tilde{\nu}_{rc} \sim 1538 \text{ cm}^{-1}$) with an increasing content of HexA. Therefore, in solid
 397 state, it can be assumed that at the transition from **P2** to **P4** the content of α-helices and β-sheets
 398 exceeds that of random coil formation. Merely the spectra of **P1** complicates the interpretation
 399 of the macrostructure of the compound. However, the shape of the curve may indicate
 400 overlapping bands of α-helices, β-sheets, and random coil formations. Therefore, it can be
 401 assumed that **P1** has the highest content of random coil formation compared to the other

402 compounds probably due to its purely cationic structure. To confirm the results of the solid-
403 state IR studies, CD measurements were performed on polymers **P1** to **P4** dissolved in HFIP, a
404 solvent suitable for CD spectroscopy.⁴⁹ The curves of **P1** and **P2** show the characteristic shape
405 of coil formation with a maximum at 215 nm (Figure 1B).⁵⁰ In contrast, polymers **P3** and **P4**
406 show two minima at 205 and 222 nm, which can be assigned to α -helix formation. In addition,
407 CD measurements were performed with polyplexes of **P1** to **P3** dissolved in phosphate buffered
408 saline (PBS, pH 7.4) (N/P 10), Figure S13B. Due to their insolubility, **P4** and **P5** were not
409 suitable for polyplex formulation. The results derived from the profiles of **P1**, **P2** and **P3** in
410 physiological environment are comparable to their behavior dissolved in HFIP. The spectra of
411 the polyplexes of **P1** indicate mainly random coil formation, whereas the shape of **P2** reveals a
412 mixture of random coil and α -helix formation.⁵¹ The curve of the polyplexes of **P3** suggest a
413 mixture of β -sheet and α -helix formation.

414

415 **DNA binding and release.** To utilize the synthesized polypeptides as gene delivery vectors,
416 their pDNA binding and release capability was evaluated. First, the polypeptides were dissolved
417 in PBS (pH 7.4) or acetate buffer (0.2 M, pH 5.8) to test their solubility. **P1** and **P2** were highly
418 soluble in both buffers, while **P3** showed lower solubility in PBS. **P4** with the highest content
419 of hydrophobic HexA groups was not soluble in both buffers and was, therefore, excluded from
420 the study. pDNA binding was measured using the ethidium bromide binding assay (EBA).
421 Therefore, polyplexes were prepared with ethidium bromide (EtBr) labelled pDNA in PBS
422 (PBS polyplex) or a mixture of acetate buffer and HBG buffer (HBG polyplex) at various N/P
423 ratios (1 to 30), followed by monitoring of EtBr fluorescence (Figure S14A). All investigated
424 polypeptides showed pDNA binding at $N/P > 1$ independent of the buffer system used, as
425 indicated by the plateau reached in relative fluorescence intensity (rFI). These results clearly
426 show that for all polymers the N/P ratio and thus the total amount of primary amino groups
427 (AB-L-Gln) is the decisive factor for pDNA condensation, independent of the molar ratio of

428 AB-L-Gln and hydrophobic Hex-L-Gln within the statistical polymers. Differences were
429 observed in the level of the plateaus, with rFI decreasing to ~ 50 % for PBS and ~ 10 % for
430 HBG polyplexes, indicating a stronger binding in HBG. In comparison, PBS polyplexes might
431 be less charged either due to higher pH values or shielding of amine groups by phosphate ions
432 present in the solution. Nevertheless, in both buffer formulations all polypeptides (**P1 - P3**)
433 successfully bind pDNA at N/P ratios ≥ 5 . As pDNA further needs to be released within the
434 cell for successful gene delivery, dissociation of the polyplexes in the presence of heparin as
435 competing anion was studied by monitoring EtBr fluorescence upon addition of increasing
436 amounts of heparin (Figure S14B). All polyplexes showed pDNA release at heparin
437 concentrations above 9 U mL^{-1} . HBG polyplexes of all polypeptides completely released
438 pDNA at 20 U mL^{-1} . Release efficiencies in PBS showed the same tendencies but did not reach
439 100 % release in all cases. These results further support the assumption of pronounced
440 hydrophobic interactions in PBS, being less prone to displacement by highly negatively charged
441 heparin.^{52, 53} Overall, the polypeptides **P1** to **P3** showed good pDNA binding and release and
442 are therefore suitable candidates for gene delivery.

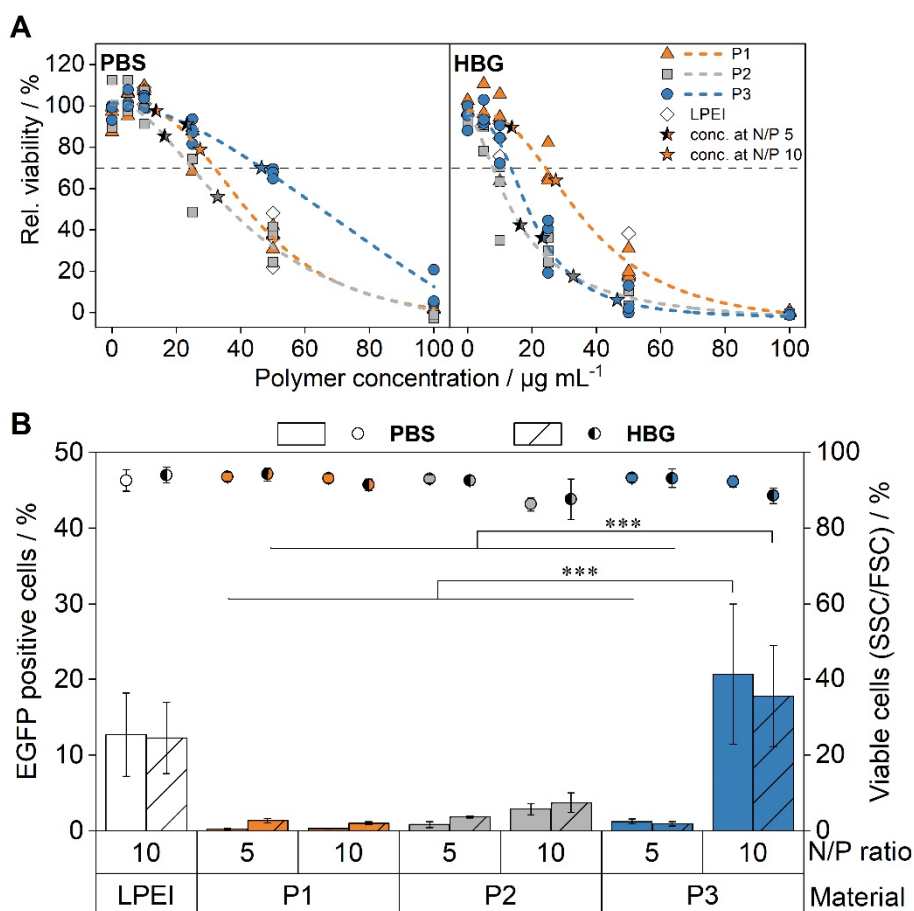
443 **Toxicity and transfection efficiency in L-929 cells.** A PrestoBlue™ assay was performed in
444 L-929 cells by incubating the cells with the polymers dissolved in PBS or acetate/HBG buffer
445 to evaluate their toxicity (Figure 2A). The L-929 cell line was chosen as standard cell line for
446 cytotoxicity testing recommended by the ISO 10993-5 guideline. Further, this cell line has been
447 intensively investigated with regards to its uptake mechanism and intracellular trafficking of
448 nanoparticles and polyplexes, rendering it an optimal system for the evaluation of novel gene
449 delivery systems and their transfection mechanism.^{54, 55} Substantial influence of the utilized
450 buffer system was observed. The cytotoxicity of **P1** is least influenced by the buffer
451 ($\text{IC}_{50, \text{PBS}} = 41.9 \text{ } \mu\text{g mL}^{-1}$, $\text{IC}_{50, \text{HBG}} = 33.6 \text{ } \mu\text{g mL}^{-1}$) followed by **P2** ($\text{IC}_{50, \text{PBS}} = 36.5 \text{ } \mu\text{g mL}^{-1}$;
452 $\text{IC}_{50, \text{HBG}} = 18.8 \text{ } \mu\text{g mL}^{-1}$). In comparison, **P3** is substantially less toxic in PBS buffer, revealing
453 the highest cell viability there ($\text{IC}_{50, \text{PBS}} = 65.1 \text{ } \mu\text{g mL}^{-1}$, $\text{IC}_{50, \text{HBG}} = 13.6 \text{ } \mu\text{g mL}^{-1}$) and showing

454 substantially lower toxicity than LPEI at similar concentrations. This could be attributed to a
455 higher charge density of **P3** in acetate/HBG buffer in combination with an increased proportion
456 of hydrophobic moieties leading to enhanced association with cellular membranes and thus to
457 cytotoxic effects.⁵⁶⁻⁵⁸ In contrast, it is assumed, that hydrophobic and cationic moieties are
458 balanced in PBS buffer and excess cationic charges are shielded by the presence of phosphate
459 ions leading to improved cytocompatibility.⁵⁹ To identify suitable conditions for evaluating the
460 influence of the polymer structure on transfection efficiency L-929 cells were incubated with
461 PBS or HBG polyplexes at various N/P ratios (2, 5, 10 and 20), pDNA concentrations (1.5, 3
462 and 4.5 $\mu\text{g mL}^{-1}$) and incubation times (4 + 20 h, 24 h and 24 + 24 h) (data not shown).
463 Considering these pretests, PBS and HBG polyplexes at N/P 5 and 10 and a pDNA
464 concentration of 4.5 $\mu\text{g mL}^{-1}$ were selected for the further study and incubated with L-929 cells
465 for 24 h, followed by further 24 h of incubation with fresh growth medium (24 + 24 h).
466 Transfection efficiency was measured *via* flow cytometry as percentage of viable cells
467 expressing EGFP (Figure 2B, S10, S15) and confirmed by fluorescence microscopy (Figure
468 S16, S17). Cell viability was monitored by the sideward and forward scattering (FSC/SSC) of
469 the cells (Figure S10).⁴⁴ In addition, cytotoxicity of polyplexes was evaluated *via* the
470 PrestoBlue™ assay after 24 h of incubation (Figure S18). The cells showed high viability
471 (> 85 % viable cells during transfection experiments (24 h + 24 h)). However, polyplexes
472 prepared in HBG caused higher cytotoxicity in comparison to PBS polyplexes, whereas at the
473 tested concentrations and N/P ratios no significant difference in transfection efficiency was
474 observed. The highest percentage of transfected cells was reached with **P3** at N/P 10 in both
475 buffers (20.7 % in PBS and 17.8 % in HBG), clearly outperforming **P1** and **P2** as well as the
476 commercial control LPEI at the same conditions (12.7 % in PBS and 12.3 % in HBG). **P1**
477 showed almost no transfection in PBS and HBG (N/P 10) (0.3 % and 1.0 % respectively),
478 whereas **P2** resulted in 2.9 % in PBS and 3.7 % in HBG (N/P 10). Among the polypeptides, **P3**
479 further resulted in the highest mean fluorescence intensity (MFI) (Figure S19) also visible by

480 fluorescence microscopy (Figure S16, S17). In contrast, hydrophobic cationic polypeptide
 481 systems previously investigated by our group showed much lower transfection efficiency
 482 compared to the commercial control LPEI.⁵ Thus, the best performing **P3** polypeptide in this
 483 study is a promising candidate for gene delivery not only because of its simpler synthesis and
 484 higher synthetic variability, but also due to its substantially better performance in comparison
 485 to LPEI.

486 To get a deeper understanding of the transfection mechanism of the polypeptides, the following
 487 investigations were performed Since PBS polyplexes caused lower cytotoxicity in comparison
 488 to HBG polyplexes, without any substantial difference in transfection efficiency, the
 489 experiments were conducted exclusively with PBS polyplexes.

490



491

492 **Figure 2** Evaluation of cytotoxicity and transfection efficiency in L-929 cells. **A** Cytotoxicity
 493 of **P1**, **P2** and **P3** without pDNA in the respective buffer was measured by the PrestoBlue™

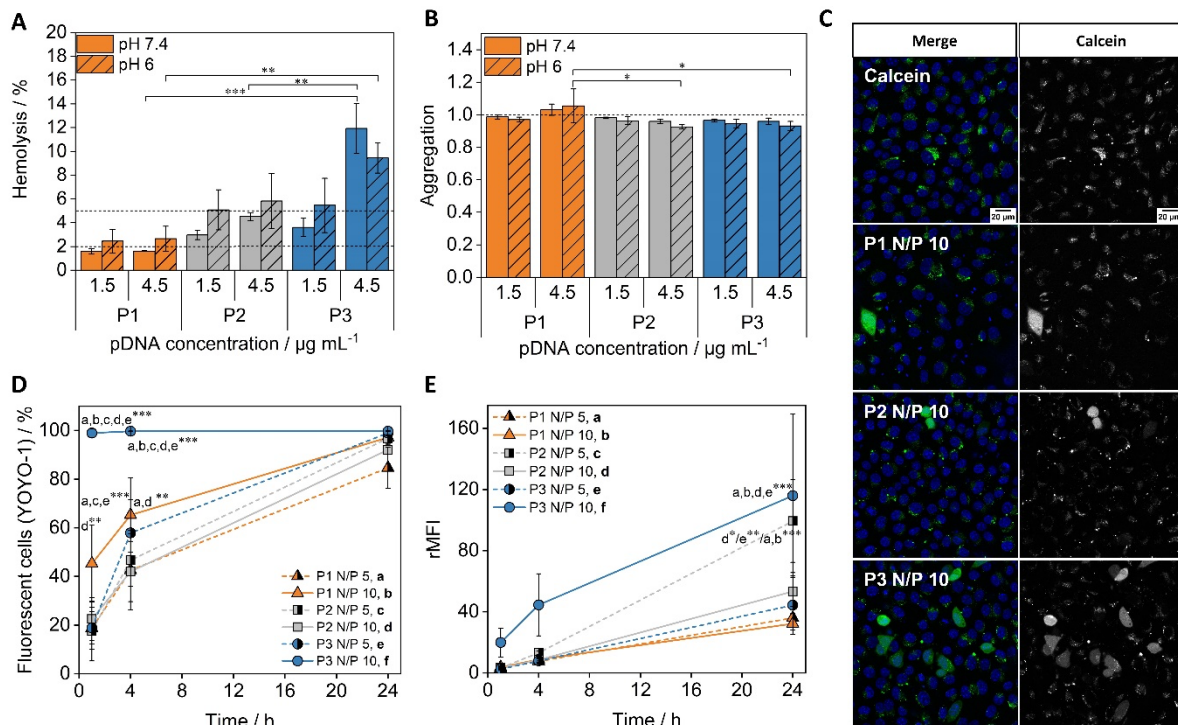
494 assay following incubation of the cells (with the polymer) for 24 h. The dashed line indicates
495 70 % cell viability and stars mark the polymer concentration required in the transfection studies
496 for the respective N/P ratio at a pDNA concentration of $4.5 \mu\text{g mL}^{-1}$ ($n \geq 3$). **B** Transfection
497 efficiency (EGFP expression) was investigated *via* flow cytometry after 24 h incubation with
498 the polyplexes (N/P 5 and 10, $4.5 \mu\text{g mL}^{-1}$ pDNA) followed by incubation with fresh growth
499 medium for further 24 h (24 h + 24 h). Transfection efficiency is displayed as percentage of
500 transfected cells in comparison to the control (polyplex with pKMyc pDNA) (mean \pm SD,
501 $n \geq 3$). Statistically significant differences between the polypeptides in the respective buffer are
502 denoted as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

503

504 **Transfection mechanism in L-929 cells. Membrane Interactions and Endosomal Escape.**

505 Membrane interactions and endosomal escape are well-known obstacles for non-viral gene
506 delivery and were studied by a hemolysis/aggregation and a calcein release assay. Erythrocytes
507 were incubated with PBS polyplexes at N/P 10 at various pDNA concentrations and pH values
508 to study membrane interactions at physiological (pH 7.4) and endosomal (pH 6) conditions.⁶⁰
509 Then, hemoglobin release was measured as indicator for membrane disruption (Figure 3A).
510 Additionally, erythrocyte aggregation was monitored, likewise an indicator for polymer-
511 membrane interactions (Figure 3B). An ascending hemolytic trend was observed at both pH
512 values, with **P3** showing the strongest hemolytic activity especially at high concentrations
513 (11.9 ± 2.1 %). An opposing trend emerged for aggregation, with **P1** resulting in slight
514 aggregation at high concentrations (1.03 at pH 7.4 and 1.06 at pH 6), whereas **P2** and **P3** did
515 not show any effect (aggregation < 1.0). This proposes a different mechanism of membrane
516 interaction for **P1**, showing merely membrane association, whereas **P2** and **P3** clearly lead to
517 membrane leakage events. This can be attributed to the increasing proportion of HexA groups
518 in **P2** and **P3** promoting additional hydrophobic membrane interactions, whereas the cationic
519 homopolymer **P1** mainly interacts electrostatically.^{57, 61} The formation of ordered secondary
520 structures might also contribute to the hemolytic activity of **P3** at both physiological and
521 endosomal pH values.^{19, 59} These results point towards a high cellular uptake as well as efficient
522 endosomal escape of **P3**, which could explain its good transfection performance.

523 To study endosomal release, a calcein release assay was performed. The cells were incubated
 524 simultaneously with the membrane impermeable dye calcein and the polyplexes at N/P 10 for
 525 1 h and subsequently imaged *via* CLSM (Figure 3C, Figure S20). Endosomal release indicated
 526 by homogeneous fluorescence of calcein within the cytosol was observed for all polypeptides,
 527 with **P3** showing the highest percentage of calcein releasing cells (8.2 ± 3.1 %, Figure S21),
 528 which is in accordance with the hemolytic activity at pH 6 (Figure 3A). However, differences
 529 within the polypeptides were rather small (4.9 ± 2 % for **P1** and 5.7 ± 3.8 % for **P2**). This
 530 indicates that the endosomal release efficiency may not be the main factor contributing to the
 531 superior transfection efficiency of **P3**.



532
 533 **Figure 3** Investigation of the gene delivery process in L-929 cells. **A** Hemolytic activity was
 534 measured as release of hemoglobin from sheep erythrocytes after incubation with the
 535 polyplexes (N/P 10, 1.5 and 4.5 mg mL⁻¹ pDNA). Erythrocytes were incubated with the
 536 polyplexes at pH 7.4 and 6 to mimic conditions present in the blood and the endosomes,
 537 respectively (mean ± SD, n = 3). **B** Aggregation of erythrocytes incubated with the polyplexes
 538 was measured as light absorption of erythrocytes. 10 kDa branched poly(ethylenimine) was
 539 used as positive control and PBS as negative control (mean ± SD, n=3). Statistically significant
 540 differences at the respective concentration and pH value are denoted as follows: **p* < 0.05,
 541 ***p* < 0.01, and ****p* < 0.001. **C** Representative images of the endosomal escape of polyplexes
 542 analyzed *via* CLSM are shown. Cells were incubated simultaneously with the polyplexes
 543 (N/P 10, 4.5 µg mL⁻¹ pDNA) and the non-permeable dye calcein (green) for 1 h. After
 544 incubation, the cells were washed twice, and nuclei were further stained with Hoechst (blue).
 545 **D + E** Time-dependent uptake of polyplexes formulated in PBS was measured *via* flow

546 cytometry. L-929 cells were incubated with polyplexes (N/P 5 and 10, 1.5 $\mu\text{g mL}^{-1}$ pDNA) for
547 1, 4 or 24 h. Cellular uptake is plotted as **D** percent of cells showing increased fluorescence
548 compared to the control (YOYO-1 stained pDNA) and **E** relative mean fluorescence intensity
549 (rMFI) of viable single cells, calculated relative to the control (YOYO-1 stained pDNA)
550 (mean \pm SD, n = 3). Statistically significant differences at the respective timepoints are
551 indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

552
553

554 *Cellular Uptake via Flow Cytometry.* To study cellular uptake in a time-dependent manner,
555 L-929 cells were incubated with YOYO-1 labeled polyplexes up to 24 h. The percentage of
556 cells with increased YOYO-1 fluorescence (YOYO-1 positive cells) and the rMFI were
557 measured quantitatively *via* flow cytometry (Figure 3D, E, S22). Cellular uptake of all polymers
558 is time-dependent and reached > 90 % YOYO-1 positive cells after 24 h, whereas **P3** (N/P 10)
559 polyplexes required only 1 h for this (99.8 % YOYO-1 positive cells). The highest rMFI values
560 were found for **P2** N/P 5 and **P3** N/P 10 polyplexes after 24 h (99.4 and 116.0 respectively),
561 hence these were later examined in greater detail. The lowest rMFI was observed for **P1** even
562 after 24 h (35.9 at N/P5 and 32.2 at N/P 10), which is in accordance with its low transfection
563 efficiency. Investigation of uptake patterns measured *via* flow cytometry further underline the
564 differences within the used polypeptides (Figure S22). While the population of YOYO-1
565 positive cells homogeneously shifts to higher FI values for **P3** N/P 10 polyplexes over time,
566 only partial and broader shifts are observed for **P1** and **P2**. This indicates a homogeneous uptake
567 of **P3** N/P 10 polyplexes, complementing the transfection results with a high number of
568 transfected cells with high rMFI.

569 As the polyplex size is a crucial parameter influencing cellular uptake, dynamic light scattering
570 (DLS) measurements were conducted (Figure S23, Table S4). **P3** N/P 10 polyplexes had a
571 diameter (z-average) of 140 nm and a PDI of 0.16, while all other polypeptides and **P3** N/P 5
572 showed diameters > 1000 nm, a PDI of > 0.38 and inappropriate correlation coefficients (Figure
573 S23), indicating the formation of larger aggregates within the samples, which might be
574 overestimated in DLS measurements and, thus, rendering this characterization method

575 unsuitable for these samples. Therefore, more detailed size measurements of selected samples
576 were performed, employing nanoparticle tracking analysis (NTA) and imaging methods
577 including cryogenic transmission electron microscopy (cryo-TEM), fluorescence microscopy
578 (*chapter 3.5.3.*), and TEM measurements after incubation with cells (*chapter 3.5.4.*), all
579 identifying nano-sized particles as well. Nanoparticle tracking analysis (NTA) measurements
580 were conducted for **P3** N/P 10 and **P2** N/P 5, both showing the highest uptake in L-929 cells
581 (Figure S24, Table S5), with measurements of **P3** N/P 10 complementing the DLS results with
582 a mean diameter of 196 nm. Aggregate formation for **P2** N/P 5 was confirmed, however after
583 separation of the aggregates by centrifugation, nanostructures with a mean diameter of 258 nm
584 were measured, which were not detectable by DLS in the presence of aggregates. For potential
585 *in vivo* applications, such aggregates can be separated by additional processing steps, *e.g.* by
586 centrifugation or filtration. These differences in polyplex sizes and aggregation behavior may
587 explain the differences in uptake levels in particular at shorter incubation times as particle sizes
588 below 200 nm are promoting controlled cellular uptake by endocytosis, while aggregation
589 results in lower or at least slower and less controlled uptake by cells.⁶² This might influence
590 transfection efficiencies, with **P3** N/P 10 polyplexes being taken up in a controlled and
591 homogeneous manner, whereas only the nanosized portion of **P2** N/P 5 structures shows
592 controlled uptake, and the portion of large aggregates present in the sample is either not taken
593 up within short time periods or only occasionally by few cells.⁶³ Cryo-TEM measurements of
594 **P3** N/P 10 polyplexes showed nanostructures in the size range of approx. ≤ 100 nm in diameter
595 with slight aggregation (Figure S25). This size range is slightly smaller than intensity-weighted
596 diameters within DLS measurements but is complementing the number-weighted diameters.
597 The polyplexes appeared as particles with an uneven boundary, thus representing a structure
598 and morphology in between classical polymer nanoparticle and hydrophilic polyplexes.^{64, 65}
599 The observation of such structures is in line with a previous study of us exploring amphiphilic
600 methacrylates for gene delivery.⁶⁶ Besides particle size and morphology, the surface charge

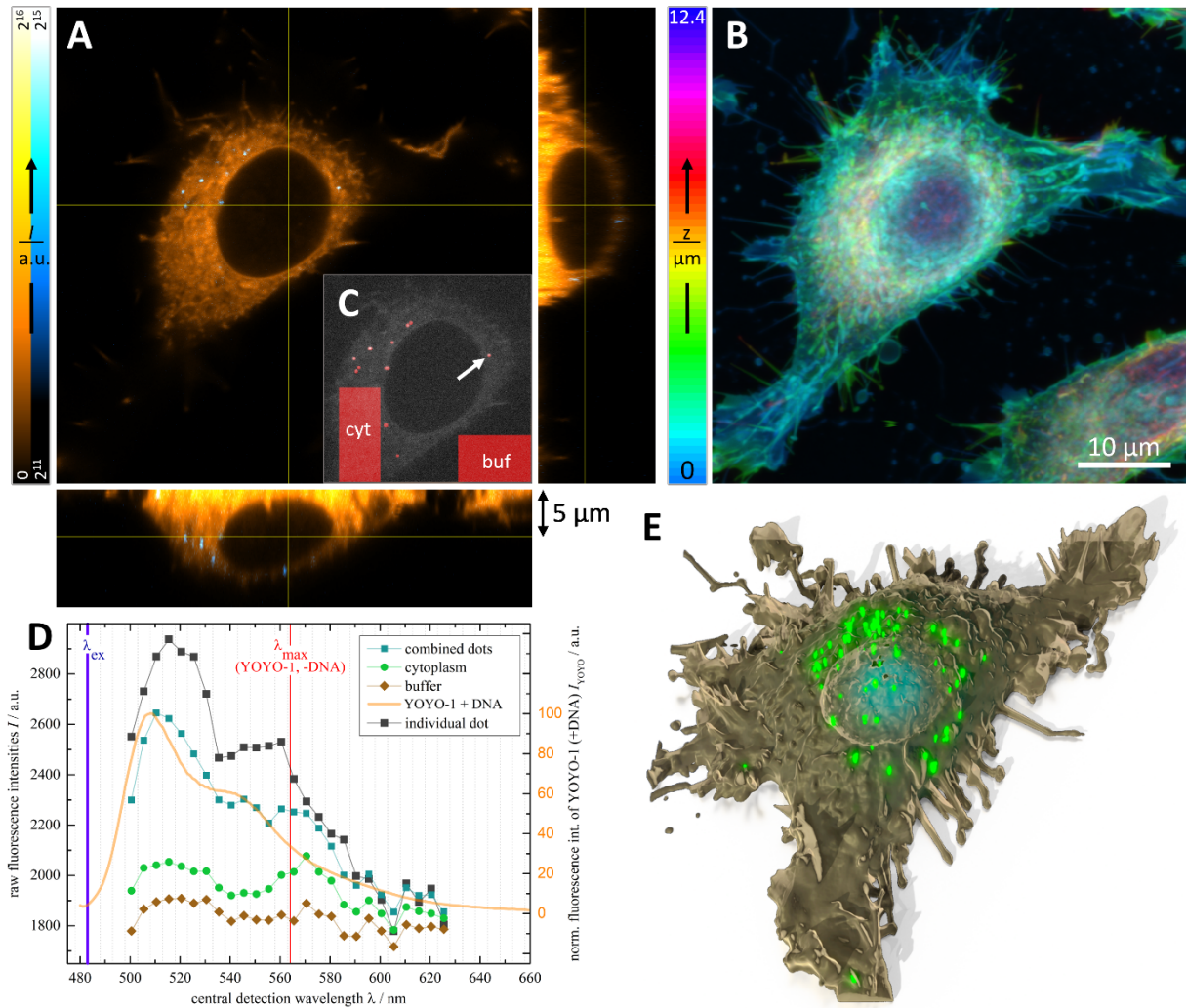
601 plays a crucial role in cellular uptake and transfection efficiency.^{67, 68} Therefore, zeta potential
602 measurement of polyplexes were conducted (Table S4). The polyplexes had a similar positive
603 zeta potential independent of the N/P ratio and polymer (+ 21.2 to 22.8 mV). It is thus assumed
604 that the surface charge is no decisive factor for the cellular uptake of the polyplexes within this
605 study.

606
607 *Details of Cellular Uptake Visualized by Fluorescence Microscopy.* To analyze the uptake
608 mechanism in more detail, the most promising polyplexes, **P2** N/P 5 and **P3** N/P 10, were
609 further investigated in L-929 cells using fluorescence microscopy. For this purpose, polyplexes
610 labeled with intercalated YOYO-1, in conjunction with cell membrane staining by CDMR,
611 were used. The dyes are spectrally fully separated with regard to both their excitation and
612 emission, enabling simultaneous imaging. No qualitative differences regarding polyplex uptake
613 and distribution could be detected after incubation with **P3** or **P2** polyplexes in a series of z-
614 resolved (Video S1, Figure 4, S26-30) and time-resolved measurements of L-929 cells (Videos
615 S2-S4, Figure S31-40). Thereby, Video S4 shows a **P3** polyplex settling to the substrate surface,
616 being subsequently ‘found’ by a lamellipodium and transported to the cell, before being
617 encapsulated and endocytically taken up (marked by a white arrow), representing an interesting
618 observation of the initial interaction of the polyplexes with the cells. A detailed discussion of
619 this interaction and approaching of the polyplexes to the cell surface together with their uptake
620 dynamics is provided below. Interestingly, fixated **P3**-incubated cells, which were stored in the
621 fridge overnight after fixation showed signs of polyplex disintegration as evidenced by diffuse
622 fluorescence from several locations, predominantly in areas of former hotspots of polyplex
623 uptake (Figure S28). Contrastingly, polyplexes in the main cell body seemed to be unaffected
624 (Figure S27). As already mentioned, we did not observe qualitative differences in the uptake
625 behavior and also the initial interaction of the cells with the polyplexes in time- and z-resolved
626 measurements between **P2** and **P3** polyplexes, but a slight focus drift in the time-resolved **P3**

627 measurements limited their analysis and, hence, a more detailed discussion of the **P2**
628 measurements is given in the following.

629 High resolution overview images of fixated, **P2**-incubated cells in the bottom-most layer, *i.e.*,
630 the glass-cell interface, and 2.4 μm above were recorded (Figure S41-S43). These illustrate the
631 cell structures and organization, particularly the dense network of filopodia emerging from each
632 cell and interconnecting it with its neighboring cells (Figure S41). In the YOYO-1 channel
633 (Figure S42, 43), bright dots with an apparent size (full width at half maximum of point spread
634 function $(\text{FWHM})_{\text{PSF},xy}(488 \text{ nm}, 1\text{AU}, 1.4 \text{ NA}) \approx 178 \text{ nm}$) of approximately 250 nm to 400 nm
635 located in the cytoplasm of some, but not all, cells indicate that uptake took place (0–12 dots
636 per cell, avg.: 2.4, $n = 26$). These observations complement the NTA measurements of
637 centrifuged **P2** polyplexes confirming the presence of nanoparticles of approx. 300 nm in
638 diameter besides larger aggregates (Figure S24 Table S5). However, due to the small particle
639 size in combination with a relatively high z-resolution, these data do not allow for reliable
640 quantitative statements about the number of dots (meaning polyplexes) per cell, because that
641 would depend strongly on the imaged plane. For individual cells and small groups of cells this
642 can be circumvented by z-resolved measurements. Figure 4A and Video S1 provide an example
643 of this approach. While in any given plane in this cell, the maximum number of bright dots we
644 were able to observe was 12, a 3D reconstruction (Figure 4E) revealed a total of 77 bright dots
645 that are scattered mostly around the nucleus. Additionally, a multitude of filopodia and intricate
646 intracellular features are visible, contextualizing the positional information (Figure 4B). The
647 time requirements for this procedure, however, are prohibitive for studying larger numbers of
648 cells, and this microscopic approach, therefore, requires statistically more powerful techniques
649 like the above discussed flow cytometry to complement it. To confirm the observed particles'
650 identification as YOYO-1 containing polyplexes, spectrally resolved measurements (Figure 4C,
651 4D) were taken in the plane indicated in Figure 4A. The combined emission spectra of all
652 11 dots closely resembles the fluorescence spectrum of DNA-intercalated YOYO-1,⁶⁹

653 confirming the identification of these dots as polyplexes. Slight deviations at about 570 nm may
 654 be due to free YOYO-1. Noteworthy, for some of the more intense and thus less noisy dots, it
 655 is possible to obtain meaningful spectra of individual polyplexes, as exemplified in Figure 4D.



656

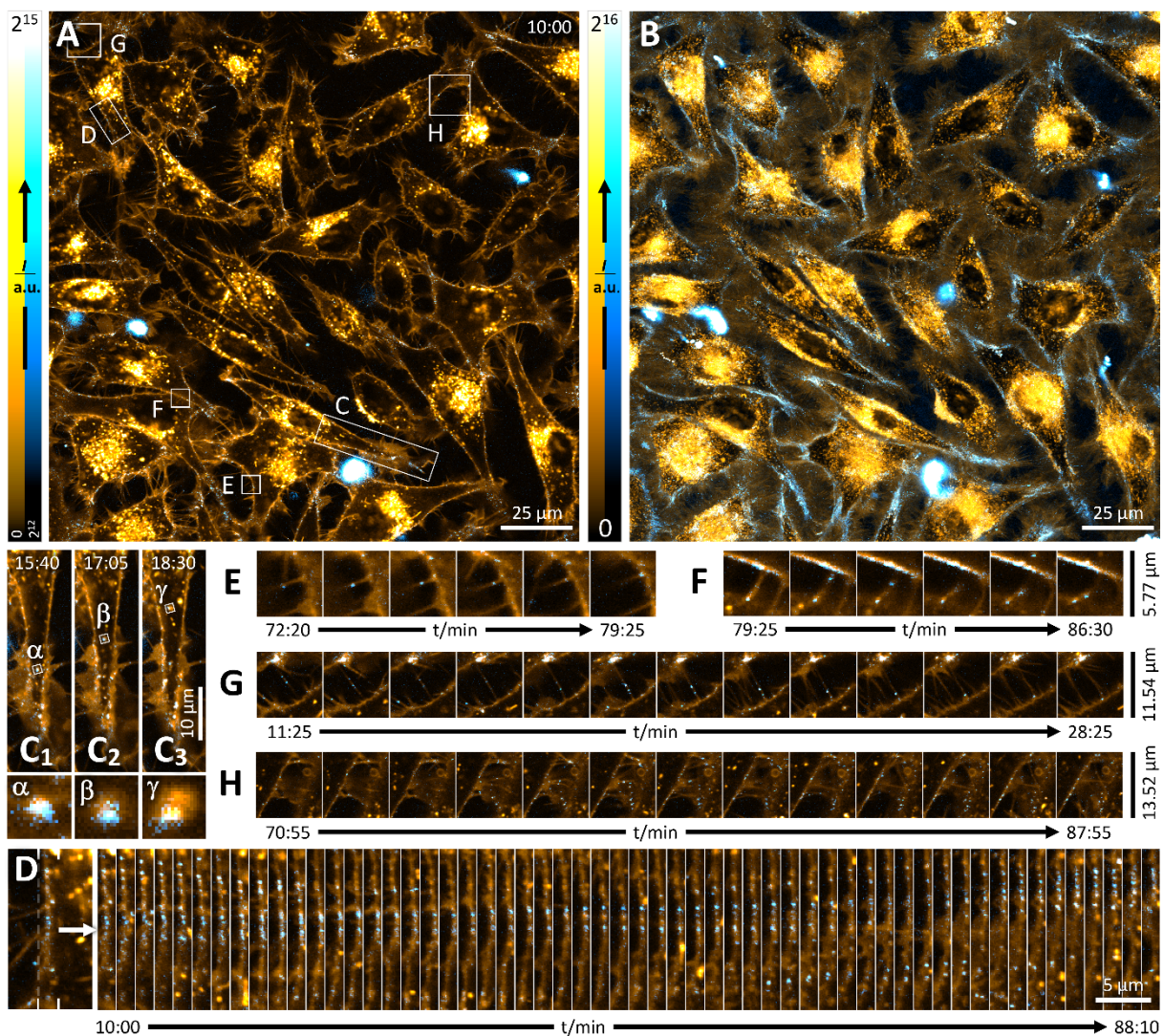
657 **Figure 4** z-Resolved and λ -resolved fluorescence microscopy images of L-929 cells after
 658 incubation with P2 N/P 5 polyplexes (15 min) and PFA fixation. **A** z-Stack with orthogonal
 659 views at positions indicated by yellow lines. The central image depicts the plane 5 μm
 660 above the microscope slide in which the subsequent λ -scan was performed. Orange: CDMR
 661 fluorescence, blue-cyan: YOYO-1 fluorescence indicating polyplexes. Intensities are scaled
 662 linearly along the indicated color bars. **B** Maximum intensity projection (MIP) of the CDMR
 663 channel of the same z-stack after intensity adjustment ($I_{\text{new}} = (1 + 0.075z)I_{\text{old}}$ with $z = 0 \dots 62$
 664 being the slice number) and color coding along the z-axis. **C** MIP along the λ -axis of an
 665 emission scan ($\lambda_{\text{ex}} = 483 \text{ nm}$, $\Delta\lambda_{\text{det}} = 5 \text{ nm}$). Eleven polyplexes were manually identified (red
 666 overlay). **D** Emission spectra of cytoplasm, PBS, and polyplexes, averaged over all respectively
 667 labeled pixels in C, alongside an individual polyplex (marked by white arrow in) spectrum and
 668 a reference spectrum of YOYO-1+DNA, normalized to the average polyplex spectrum
 669 (YOYO-1+DNA spectrum obtained from Thermo Fisher Scientific). Purple line: excitation
 670 wavelength, dotted grid lines: detection window boundaries, red line: emission maximum of
 671 free YOYO-1. **E** 3D reconstruction of the respective cell with polyplexes marked as green dots,

672 depicting their distribution around the nucleus. A total of 77 polyplexes was identified, notably,
673 none of which stuck to the outer cell membrane.
674

675 To monitor the uptake behavior of polyplexes and the approaching of the polyplexes to the cell
676 surface, a time series of living cells was recorded after addition of the polyplex solution (10 min
677 to 88 min after addition, Video S2). During the first 10 min after addition, the polyplexes could
678 be observed settling onto the glass substrate and cells (not shown), whereas no dots were visible
679 in the YOYO-1 channel before polyplex addition (Figure S31). Figure 5A shows the first frame
680 of the time series. Large numbers of polyplexes are stuck to the cell membranes but only some
681 cells have small numbers of polyplexes in the cytoplasm in the imaged plane. After 88 min, all
682 cells in the field of view show polyplexes in the cytoplasm, though the amounts vary from
683 below 10 to several dozens. In all cases, internalized polyplexes coincide with bright spots in
684 the CMDR channel, indicating encapsulation in membrane vesicles and endocytosis as uptake
685 mechanism. Illustrations emphasizing these effects are given in the SI, Figure S32-37. A
686 discrete uptake event is documented in Figure S38. The uptake appears to happen
687 predominantly, if not exclusively, at the tip of cell extrusions in the immediate vicinity of
688 lamellipodia. These are also the areas of highest polyplex density and strongest polyplex
689 agitation (Video S2), with the latter being a result of rapid filopodium and lamellipodium
690 movement. In combination with the relative flatness of those areas (as compared to the main
691 cell bodies), confining the polyplex movement mostly to two dimensions (*i.e.*, the imaging
692 plane), this leads to prominent hotspots in the lamellipodial/filopodial region in a maximum
693 intensity projection (MIP) along the time axis of the series (Figure 5B). The highly dynamic
694 movement of filopodia leads to a hazy glow around the mostly stationary cells in this
695 representation, showing that very few areas remain ‘unexplored’ by the cell cluster. Once taken
696 up, we observed the polyplex containing vesicles being rapidly transported towards the cell
697 center in most cases, as illustrated in Figure 5C. In linear approximation, the distance covered
698 from C1 to C2 and from C2 to C3 was 5.8 μm each, corresponding to a transport velocity of

699 4.09 $\mu\text{m min}^{-1}$. We did not observe discrete uptake events in regions other than the above
700 described, though it should be noted that high polyplex mobility on the cell membrane could
701 be seen, leading to them frequently drifting into and out of focus and making it difficult to
702 follow individual polyplexes for prolonged amounts of time. Figure 5D illustrates this drifting
703 motion within the imaging plane. It appears to be largely passive without strong directional
704 preferences. It can be assumed that this motion is not confined to two dimensions as individual
705 polyplex traces in Figure 5D get darker and lighter in an unpredictable manner with some
706 vanishing completely or suddenly appearing. This membrane drifting probably plays a role in
707 supplying the endocytically active lamellipodia regions with polyplexes ¹³.

708

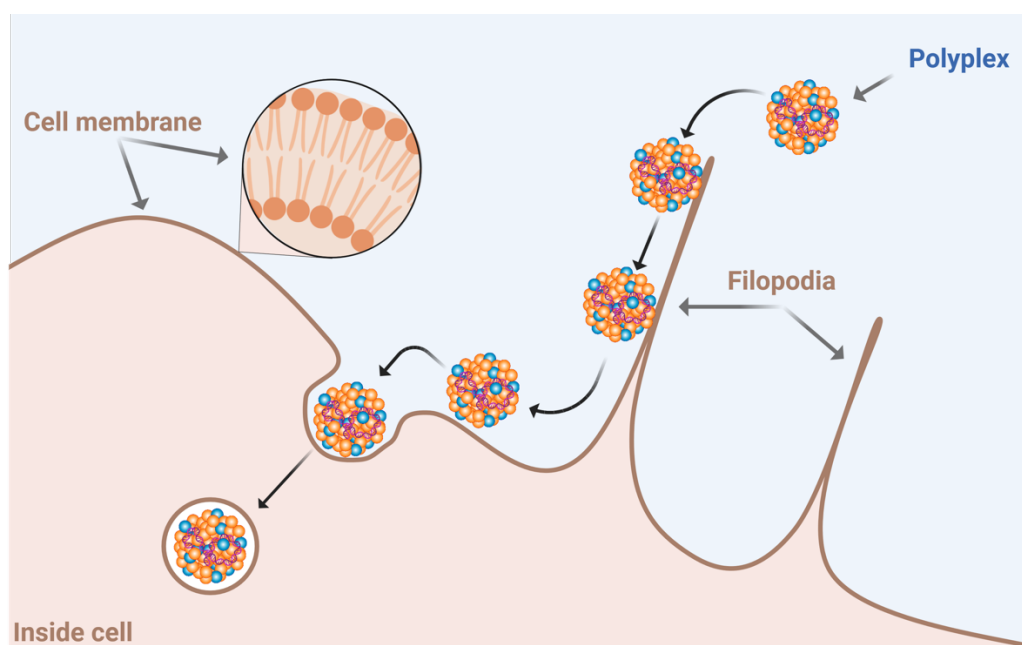


709 **Figure 5** Time series of fluorescence images (orange: CMDR, cyan-blue: YOYO-1) of L-929
710 cells after addition of polypeptide polyplex (P2). A First image ($t = 10$ min) of the series. Cell
711

712 membranes are clearly distinguishable by CMDR staining. Each cell has a multitude of
713 filopodia. Polyplexes have settled on the cells and stick to the membranes. **B** MIP of the series
714 along the time axis from 10 to 88 min. **C** Series of three consecutive frames showing the rapid
715 transport of a polyplex-containing vesicle from the lamellipodium towards the main cell body
716 after endocytosis. Inserts α - γ have side lengths of 1.8 μm . **D** Polyplexes passively sliding along
717 the cell membrane without strong directional preferences and with no cellular uptake occurring.
718 All 56 frames are depicted. **E** Filopodium surfing of a single polyplex towards the membrane
719 of the cell body. **F** A single polyplex is transferred from the membrane of one cell to that of
720 another *via* filopodium surfing. **G** Filopodium bridges between cells mediate polyplex
721 exchange. **H** Lamellipodium of one cell touches another cell's membrane. Large numbers of
722 polyplexes are transported *via* the gap in a relatively short time span.

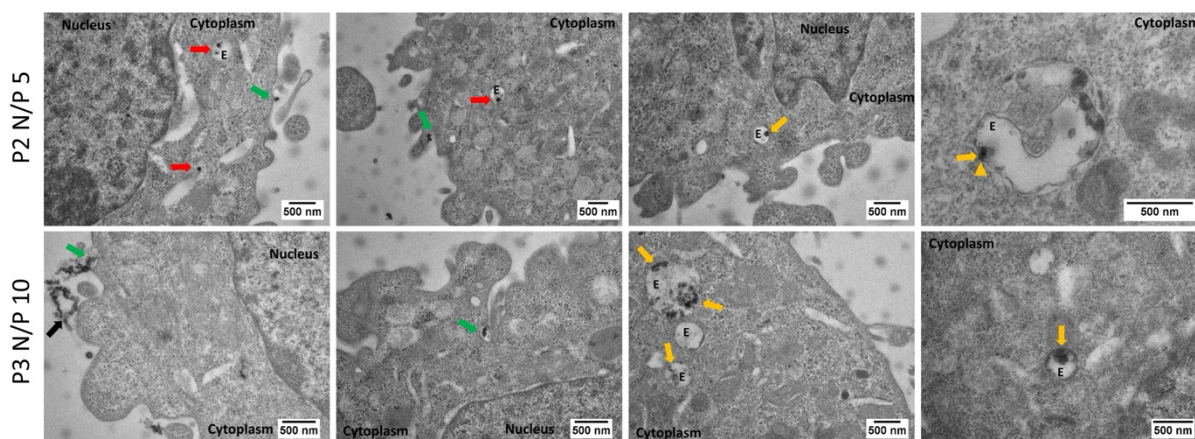
723
724 As mentioned above, an interesting observation is the mode of polyplex acquisition and initial
725 interaction with the cells. In contrast to those polyplexes, which initially settle on the cells,
726 others are actively transported from the vicinity towards the cells *via* filopodium surfing by a
727 large number of filopodia, constantly exploring the cells' surroundings.^{13, 70} One of the
728 numerous examples of this effect is depicted in Figure 5E. This surfing motion appears to be
729 independent of filopodium retraction,⁷¹ though in many cases these effects are difficult to
730 separate. Particularly interesting in this regard is the role of cell-bridging filopodia. In areas of
731 close contact, stretches of the cell membrane with more than 1 bridging filopodium per 1 μm
732 are not uncommon, often with unclear filopodium affiliation (compare also Figure S41). These
733 bridges can be brief contacts, lasting only for a few minutes, but also remarkably long-lived
734 with examples in the here discussed dataset lasting for almost 1 h. Polyplex transport along
735 those bridges occurs as for non-bridging filopodia (Figure 5G). While in many cases the
736 polyplex origin is unclear, in others, filopodia of one cell clearly detach polyplexes from
737 another cell's membrane and subsequently acquire it (Figure 5F). A similar transfer effect can
738 be observed where lamellipodia of one cell touch another cell's membrane (Figure 5H), though,
739 again, the polyplex' origin remains unclear in this example. These findings (regarding the role
740 of lamellipodia and filopodia in the initial contact of cells with particle like structures such as
741 polyplexes, their acquisition and transport by surfing on and retraction of filopodia and cell-
742 to-cell distribution) have been firstly shown for viruses attaching to the filopodia and surfing
743 towards the cell surface and main body where they are taken up by the cell.^{72, 73} Subsequent to

744 reaching the cell surface by help of the filopodia, the particle like structures are taken up by
745 several endocytic processes, *e.g.*, clathrin- and caveolae-mediated endocytosis and
746 macropinocytosis, ending up in the endolysosomal pathway (early endosomes), which is
747 usually desired for non-viral gene delivery systems.^{74, 75} The uptake thereby often occurs in the
748 endocytically active lamellipodia regions as also observed within this study.¹³ This mechanism
749 of acquisition followed by endocytic uptake can be probably attributed to the enrichment of
750 proteoglycan receptors such as syndecans on filopodia, known to facilitate interaction with
751 cationic particles such as cationic non-viral vectors.^{72, 76} However, so far little is known about
752 the initial interaction and approaching of non-viral gene delivery vectors to cells and the role
753 of this mechanism in the transfection process is largely unrecognized and underappreciated, as
754 only few studies investigated this mechanism for lipoplexes and hydrophilic polyplexes.^{13, 70} In
755 this study, it has now firstly been shown that this mechanism also plays a role for amphiphilic
756 polyplexes and their subsequent cellular internalization and is thus an important aspect and
757 promising approach for further optimizing the transfection efficiency of such polyplexes in
758 future studies. The proposed mechanism is shown in simplified form in Scheme 1.



759
760 **Scheme 1** Proposed mechanism of polyplex acquisition and their transport by surfing on
761 filopodia.

762 *Cellular Uptake Measured via Transmission Electron Microscopy.* In order to further evaluate
763 the uptake mechanism following polyplex acquisition by the lamellipodia and filopodia and the
764 intracellular polyplex location, TEM measurements of L-929 cells incubated with **P2** N/P 5 and
765 **P3** N/P 10 polyplexes for 1 h were conducted. It was possible to identify several cellular
766 substructures, *e.g.*, the nucleus and endolysosomal vesicles within the obtained TEM images.
767 Further structures with high electron density were observed, which were attributed to **P2** and
768 **P3** polyplexes that were stained with uranyl acetate.⁷⁷ In both samples, the polyplexes appear
769 as nearly spherical nanosized structures and for **P3** N/P 10, partial agglomeration of polyplexes
770 in the extracellular environment was observed, which is in line with the cryo-TEM
771 measurements (Figure S 25). Both samples show location of single or multiple nanosized
772 polyplexes in endolysosomal vesicles. These observations complement the NTA measurements
773 of centrifuged **P2** polyplexes and the fluorescence microscopy measurements, again confirming
774 the presence of nanosized polyplexes besides larger aggregates (Figure S24, Figure 5, Table
775 S5). Furthermore, these results illustrate that larger aggregates are not taken up by cells within
776 an hour, which correlates with the fluorescence microscopy measurements. Subsequent to
777 cellular uptake, within the endolysosomal vesicles, polyplexes are often associated with the
778 vesicle membranes and partially result in membrane deformation. These results confirm the
779 active endocytic uptake of **P2** and **P3** polyplexes into L-929 cells as observed by fluorescence
780 microscopy.^{78, 79} However, no qualitative differences between **P2** and **P3** regarding location or
781 membrane interaction were observed in TEM measurements following 1 h incubation.



782
 783 **Figure 6** TEM images of L-929 cells incubated with **P2** N/P 5 and **P3** N/P 10 polyplexes for
 784 1 h. Electron dense structures (black) were identified as polyplexes. Black arrows indicate
 785 extracellular accumulation of polyplex structures. Green arrows indicate polyplex-cell
 786 interaction or uptake events. The location of polyplexes in vesicles, *e.g.*, endosomes (E) is
 787 indicated by red arrows. Yellow arrows indicate interaction of polyplexes with vesicle
 788 membranes and the deformation of these membranes by polyplexes is indicated by yellow
 789 arrow heads.

790 In summary, a filopodia- and lamellipodia-based endocytic uptake mechanism was observed
 791 for both **P3** N/P 10 polyplexes and the nanosized portion of **P2** N/P 5 polyplexes. Quantitative
 792 flow cytometry measurements revealed faster and more homogeneous uptake kinetics for **P3**
 793 N/P 10 compared to **P2** N/P 5 polyplexes, but the uptake levels (percentage of cells, rMFI) after
 794 24 h were similar (Figure 3E). Notwithstanding, the transfection efficiency is substantially
 795 higher for **P3** N/P 10 after 24 h incubation. This might be explained by higher endosomal
 796 release efficiency observed after 1 h, facilitated by higher membrane interaction of the **P3**
 797 polymer and faster polyplex uptake resulting in high endosomal polyplex concentration.⁸⁰
 798 Nevertheless, the escape efficiency is rather low and not substantially higher than for **P1** and
 799 **P2**. This leads to the assumption that endosomal escape might take place over longer time
 800 periods, steadily releasing the polyplexes resulting in higher transfection efficiency compared
 801 to the other polypeptides.

802 CONCLUSION

803 A selected number of amphiphilic polypeptides containing different distributions of cationic
 804 AB-L-Gln (100, 84, 60, 30 and 0 mol%) and aliphatic Hex-L-Gln (0, 16, 40, 70 and 100 mol%)
 805 side chains were obtained *via* an efficient four-step synthesis. It was shown that the secondary

806 structure of P(AB-L-Gln-*stat*-Hex-L-Gln) can be influenced by varying the distribution of both
807 cationic and aliphatic units.

808 The water-soluble polypeptides **P1**, **P2** and **P3** were evaluated for their ability to complex
809 pDNA, showing sufficient binding and release capability. An intensive screening of the
810 transfection performance of the polyplexes in the mouse fibroblast cell line L-929 revealed **P3**
811 as clear outperformer over the other polypeptides and commercial LPEI. Polyplexes formed
812 with the **P3** polypeptide comprising the highest proportion of hydrophobic HexA groups and
813 highest amount of ordered secondary structures such as α - helical and β -sheet structures
814 resulted in the highest percentage of EGFP expressing cells in comparison to **P1** and **P2** at
815 similar N/P ratios and, thus, similar amount of cationic charges, while maintaining high cell
816 viability. In-depth investigation of the interaction with erythrocyte membranes as a model
817 system and with L-929 cells illustrated the highest membrane interaction and endosomal release
818 levels of **P3** polyplexes. Evaluation of cellular uptake by flow cytometry revealed that **P3**
819 polyplexes were taken up by almost all cells after 1 h, which was observed for **P2** polyplexes
820 only after a longer incubation period. This difference was attributed to the presence of larger
821 aggregates besides nanosized polyplexes in **P2**. Time- and z-resolved fluorescence microscopy
822 and TEM demonstrated an active filopodia- and lamellipodia-based initial acquisition of the
823 nanosized polyplexes present in **P2** and **P3** followed by endocytic uptake mechanisms for both
824 polypeptides, showing no qualitative difference within the polypeptides. In both cases, cells
825 were found to actively explore the surroundings with their filopodia and acquire the polyplexes
826 before transporting them towards the main cell body, predominantly by filopodium surfing,
827 possibly in combination with filopodium retraction. The polyplexes presented here exhibited
828 high mobility on the cell surface, where they can reside for prolonged amounts of time. Polyplex
829 uptake appears to happen in a strictly localized fashion in the very active lamellipodia regions,
830 followed by active transport to the cell interior. In combination with higher endosomal release

831 efficiency, facilitated by higher membrane interaction, the faster and more homogenous
832 polyplex uptake mediated by filopodia- and lamellipodia-based initial acquisition resulted in
833 substantially higher transfection efficiency of the **P3** polypeptide outperforming the other
834 polypeptides.

835 Taken together, these results give insight into the gene transfer process of amphiphilic
836 polypeptides including the mechanism of initial polyplex acquisition and internalization and
837 reveal the potential of the integration of aliphatic groups into cationic polypeptides to obtain
838 nanosized, effective, biocompatible, and potentially biodegradable gene delivery vectors. The
839 straightforward and versatile synthesis of polypeptides by post-polymerization
840 functionalization provides a promising platform for further optimization and fine-tuning in
841 terms of charge, hydrophobicity, secondary structure, and the introduction of shielding units
842 such as PEG in order to develop highly efficient amphipathic polypeptides that are capable of
843 overcoming the physiological barriers faced when delivering pDNA but also other cargos such
844 as siRNA and mRNA *in vitro* and *in vivo*. Imaging techniques such as fluorescence microscopy
845 and transmission electron microscopy help understanding the involved processes and
846 interactions on a subcellular level and in particular allow to study the so far largely
847 unrecognized and for amphiphilic polypeptides unstudied lamellipodia- and filopodia-based
848 mechanism of initial interaction of the with cells. This offers the possibility for further design
849 and optimization of tailor-made polypeptide-based vectors.

850 **ASSOCIATED CONTENT**

851 **Supporting information**

852 The datasets used and/or analyzed during the current study are available from the corresponding
853 author on reasonable request. Additional material, methods, and results, including Figure
854 S1 - S43, Tables S1 - S5, Videos S1 - S4, are collected in the Supplementary Information.

855

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864 **NOTES**

865 The authors declare that they have no competing interests. Scheme 1 was created using
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