



[biblio.ugent.be](http://biblio.ugent.be)

The UGent Institutional Repository is the electronic archiving and dissemination platform for all UGent research publications. Ghent University has implemented a mandate stipulating that all academic publications of UGent researchers should be deposited and archived in this repository. Except for items where current copyright restrictions apply, these papers are available in Open Access.

This item is the archived peer-reviewed author-version of: Decationized polyplexes as stable and safe carrier systems for improved biodistribution in systemic gene therapy

Authors: Novo L., Rizzo L.Y., Golombek S.K., Dakwar G.R., Lou B., Remaut K., Mastrobattista E., van Norstrum C.F., Jahnen-Dechent W., Kiessling F., Braeckmans K., Lammers T., Hennink W.E.

In: Journal of Controlled Release 2014, 195: 162-175

**To refer to or to cite this work, please use the citation to the published version:**

Novo L., Rizzo L.Y., Golombek S.K., Dakwar G.R., Lou B., Remaut K., Mastrobattista E., van Norstrum C.F., Jahnen-Dechent W., Kiessling F., Braeckmans K., Lammers T., Hennink W.E. (2014)

Decationized polyplexes as stable and safe carrier systems for improved biodistribution in systemic gene therapy. Journal of Controlled Release 195 162-175 DOI:

10.1016/j.jconrel.2014.08.028



1 **Decationized polyplexes as stable and safe carrier systems for**  
2 **improved biodistribution in systemic gene therapy**

3  
4 Luís Novo<sup>a,1</sup>, Larissa Y. Rizzo<sup>b,1</sup>, Susanne K. Golombek<sup>b</sup>, George R. Dakwar<sup>c</sup>, Bo Lou<sup>a</sup>,  
5 Katrien Remaut<sup>c</sup>, Enrico Mastrobattista<sup>a</sup>, Cornelus F. van Nostrum<sup>a</sup>, Wilhelm Jahnen-  
6 Dechent<sup>d</sup>, Fabian Kiessling<sup>b</sup>, Kevin Braeckmans<sup>c,f</sup>, Twan Lammers<sup>a,b,e</sup>, Wim E. Hennink<sup>a\*</sup>  
7  
8

9 <sup>a</sup> Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University,  
10 3584 CG Utrecht, The Netherlands

11 <sup>b</sup> Nanomedicines and Theranostics, Department for Experimental Molecular Imaging, University  
12 Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

13 <sup>c</sup> Laboratory for General Biochemistry and Physical Pharmacy, Faculty of Pharmacy, Ghent  
14 University, Ghent Research Group on Nanomedicines, Harelbekestraat 72, 9000 Ghent, Belgium

15 <sup>d</sup> Helmholtz Institute for Biomedical Engineering, Biointerface Laboratory, RWTH Aachen University,  
16 Aachen, Germany

17 <sup>f</sup> Centre for Nano- and Biophotonics, Ghent University, Harelbekestraat 72, 9000 Ghent, Belgium

18 <sup>e</sup> Department of Targeted Therapeutics, MIRA Institute for Biomedical Technology and Technical  
19 Medicine, University of Twente, Enschede, The Netherlands  
20

21 <sup>1</sup> Authors contributed equally to this work.  
22  
23

24 **Abstract**

25 Many polycation-based gene delivery vectors show high transfection *in vitro*, but their  
26 cationic nature generally leads to significant toxicity and poor *in vivo* performance which  
27 significantly hampers their clinical applicability. Unlike conventional polycation-based  
28 systems, decationized polyplexes are based on hydrophilic and neutral polymers. They are  
29 obtained by a 3-step process: charge-driven condensation followed by disulfide crosslinking  
30 stabilization and finally polyplex decationization. They consist of a disulfide-crosslinked  
31 poly(hydroxypropyl methacrylamide) (pHPMA) core stably entrapping plasmid DNA (pDNA),  
32 surrounded by a shell of poly(ethylene glycol) (PEG). In the present paper the applicability  
33 of decationized polyplexes for systemic administration was evaluated. Cy5-labeled  
34 decationized polyplexes were evaluated for stability in plasma by fluorescence single  
35 particle tracking (fSPT), which technique showed stable size distribution for 48 h **unlike its**  
36 **cationic counterpart**. Upon incubation of the polymers used for the formation of polyplexes  
37 with HUVEC cells, MTT assay showed excellent cytocompatibility of the neutral polymers.

1 The safety was further demonstrated by a remarkable low teratogenicity and mortality  
2 activity of the polymers in a zebrafish assay, in great contrast with their cationic counterpart.  
3 Near infrared (NIR) dye-labeled polyplexes were evaluated for biodistribution and tumor  
4 accumulation by noninvasive optical imaging when administered systemically in tumor  
5 bearing mice. Decationized polyplexes exhibited an increased circulation time and higher  
6 tumor accumulation, when compared to their cationic precursors. Histology of tumors  
7 sections showed that decationized polyplexes induced reporter transgene expression *in*  
8 *vivo*. In conclusion, decationized polyplexes are a platform for safer polymeric vectors with  
9 improved biodistribution properties when systemically administered.

10  
11 **KEYWORDS** Gene delivery, polymer, nanoparticle, biocompatibility, biodistribution, EPR  
12  
13

## 14 **1. INTRODUCTION**

15 Gene therapy has generated great interest to be used as a therapeutic tool to solve unmet  
16 medical needs [1, 2] and relies on the development of safe and efficient vectors. Nonviral  
17 vectors, such as cationic polymers, lipids or peptides, have been investigated in depth  
18 because of their flexibility and easiness of preparation when compared to viral vectors [3-9].

19 Polyplexes are normally formed by adding an excess of polycations to pDNA to yield  
20 positively charged and small-sized polyplexes which can, however, lead to significant toxicity  
21 *in vivo* [10, 11]. Toxicity of polycation-based vectors was especially evident for the “first-  
22 generation” homopolymer systems. Upon intravenous administration, cationic polyplexes  
23 show great instability *in vivo*, since they interact with negatively charged blood components  
24 (e.g. proteins, erythrocytes), followed by the formation of aggregates, leading to severe *in*  
25 *vivo* toxicity (lung embolism and/or liver necrosis) and uncontrollable biodistribution and off-  
26 targeting transfection [10-12]. Furthermore, cationic polymers/dendrimers might induce  
27 immunogenicity, complement activation and even blood coagulation [12-14].

28 Several strategies have been proposed and investigated to improve the *in vivo* behavior  
29 of polyplexes. Coating of polycation-based systems with PEG, effectively avoids formation  
30 of aggregates and reduces protein binding, resulting in improvements of the circulation  
31 kinetics and tumor accumulation *in vivo* [12, 15, 16]. Another strategy to improve polyplex  
32 stability consists of the incorporation of disulfide crosslinks into the polyplex core. Disulfide  
33 crosslinks stabilize the polyplex structure in the bloodstream, avoiding unwanted  
34 disassembly in circulation, but these bonds can be rapidly cleaved in the intracellular

1 reducing milieu [17-19]. *In vivo*, introduction of disulfide crosslinks, have demonstrated to  
2 improve circulation kinetics [20], as well as to improve tumor accumulation and transfection  
3 upon systemic administration [21]. However, further improvements on the blood circulation  
4 kinetics and biodistribution are of utmost importance to achieve the desired selective  
5 accumulation into target tissues. Even when shielded with PEG, the cationic groups of  
6 conventional polyplexes can also lead to unspecific cell binding in highly vascularized  
7 organs and, consequently, to poor *in vivo* biodistribution and insufficient blood circulation  
8 half-life [22, 23].

9 The major challenge for the clinical applicability of polymeric vectors is to achieve  
10 therapeutic efficacy with minimal toxicity and side effects upon systemic administration.  
11 Toxicity arising from polycations occurs not only on the systemic level but also at the cellular  
12 basis. Introduction of biodegradability into cationic polymers lead to the development of safer  
13 systems [5, 24]. However, during the development of new gene delivery polymer, toxicity is  
14 measured with cell viability assays in limited concentrations and exposure times, likely  
15 leading to underestimation of the toxicity problem. Toxicity is a complex process and  
16 requires deeper analysis at both acute and long term basis. When cationic polyplexes are  
17 taken up by cells, they will firstly compromise the cell membrane integrity [25-27].  
18 Polycations can also disrupt the cell homeostasis by interaction with cellular polyanions (e.g.  
19 cell receptors, enzymes, mRNA or genomic DNA) [28]. Polycations can change the genomic  
20 expression profile [29-31] and induce activation of oncogenes or even apoptosis [26, 32].  
21 Such consequences are directly related to the polycationic nature of synthetic vectors and  
22 have a substantial impact on the safety of such systems. Accordingly, neutral polymer based  
23 systems are a logical step to obtain not only improved blood circulation kinetics and  
24 biodistribution but also to accomplish the necessary safety requirements.

25 In previous work the development of decationized polyplexes was reported [33, 34].  
26 These polyplexes constitute of a core of disulfide-crosslinked pHPMA, surrounded by a PEG  
27 shell. Complexation with DNA to form polyplexes occurs by the transient presence of cationic  
28 charge in the core of the polyplexes. After structure stabilization by interchain disulfide  
29 crosslinking, the cationic groups are removed by hydrolysis, leading to a disulfide  
30 crosslinked polyplex based on a neutral polymer. As a result, entrapment of pDNA is  
31 exclusively based on disulfide crosslinks, providing an intracellularly triggered release  
32 mechanism. This means that pDNA release from decationized polyplexes occurs exclusively  
33 in a reducing environment, such as the intracellular milieu [17-19]. Importantly, the  
34 decationized polyplexes, in contrast to their cationic precursor, showed a low degree of

1 nonspecific uptake, which is thought to be an important advantage for improved blood  
2 circulation and higher target tissue accumulation exploiting the enhanced permeation and  
3 retention (EPR) effect [35].

4 In this study, we evaluated the stability, safety and *in vivo* biodistribution as well as the  
5 ability of decationized polyplexes for tumor targeting applications. The stability in biological  
6 fluids was evaluated by fSPT [36], as well as the safety by the teratogenicity and mortality  
7 potential in zebrafish embryo assay in parallel with cytotoxicity tests *in vitro* [37], and  
8 biodistribution and tumor accumulation in a A431 tumor-bearing mice by noninvasive optical  
9 imaging based on the combination micro-computed tomography ( $\mu$ CT) and fluorescence  
10 molecular tomography (FMT) [38]. Finally, transgene expression was also assessed by  
11 histological analysis of tumor cryosections.

## 12 13 14 **2. MATERIALS AND METHODS**

### 15 **2.1. Materials**

16 *N*-hydroxysuccinimide (NHS) ester functionalized dyes Cyanine5 NHS ester (Cy5-NHS)  
17 and Cyanine7 NHS ester (Cy7-NHS) were obtained from Lumiprobe (Hannover, Germany).  
18 Carbonyldiimidazole (CDI) activation of *N,N'*-dimethylaminoethanol (DMAE) was performed  
19 as previously described to yield DMAE-Cl [39]. N-[2-(2-pyridyldithio)]ethyl methacrylamide  
20 (PDTEMA) was synthesized as previously described [33]. N-(3-  
21 aminopropyl)methacrylamide hydrochloride (APMA) was obtained from Polysciences  
22 (Eppenheim, Germany). The synthesis and characterization of (mPEG)<sub>2</sub>-ABCPA (4,4'-  
23 azobis(4-cyanovaleric acid)) macroinitiator was done as previously described [40, 41]. 2,4,6-  
24 Trinitrobenzene sulfonic acid (TNBSA) was obtained from ThermoScientific (Etten-Leur, The  
25 Netherlands). pCMV\_EGFP plasmid, encoding for enhanced green fluorescent protein  
26 (EGFP) with human cytomegalovirus promoter (CMV), was amplified with competent *E. coli*  
27 DH5 $\alpha$  and purified with NucleoBond® (Macherey-Nagel, Bioke, Leiden, The Netherlands).  
28 pCMV\_EGFP construction was described by van Gaal et al. [42]. MTT Cell Proliferation Kit  
29 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Roche  
30 (Basel, Switzerland). Zebrafish medium was prepared in-house [37]. All other chemicals and  
31 reagents were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The following  
32 buffer systems were used: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for  
33 buffering at pH 6.8-8.2; 3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-  
34 sulfonic acid (TAPS) for buffering at pH pH 7.7-9.1.

## 1 **2.2. Polymer synthesis**

### 2 *2.2.1 Synthesis of p(HPMA-co-PDTEMA-co-APMA)-b-PEG*

3 Free radical polymerization using 5 kDa PEG bi-functionalized azo macroinitiator (PEG)<sub>2</sub>-  
4 ABCPA was performed to synthesize p(HPMA-co-PDTEMA-co-APMA)-b-PEG (Scheme 1).  
5 The polymers were synthesized using a monomer to initiator ratio (M/I) of 220 (mol/mol).  
6 The feed ratio HPMA/PDTEMA/APMA was 1/0.2/0.01 (mol/mol/mol). The polymerization  
7 was carried at 70 °C for 24 h in DMSO under an N<sub>2</sub> atmosphere, using 5 μmol macroinitiator  
8 and a monomer concentration of 0.4 M. After polymerization, the obtained product was  
9 precipitated in diethyl ether and collected by centrifugation. After removing ether under  
10 vacuum, the product was dissolved in 2.5 mM NH<sub>4</sub>OAc pH 5 buffer and dialyzed for 2 days  
11 at 4 °C (MWCO 6000–8000 Da) against the same buffer. Finally, the product was collected  
12 by freeze-drying.

13 Unreacted PEG was removed by precipitation in cold EtOH (5 mg/mL of solids) followed  
14 by centrifugation and filtration over a 0.2 μm filter. The product, solubilized in EtOH, was  
15 collected after EtOH evaporation, dissolution in water and freeze-drying.

16

### 17 *2.2.2. Synthesis of p(HPMA-DMAE-co-PDTEMA-co-Cy5/Cy7)-b-PEG*

18 Firstly, a solution of p(HPMA-co-PDTEMA-co-APMA)-b-PEG·TFA) (M<sub>w</sub> = 44.1 kDa; 10  
19 mg, 46.75 nmol NH<sub>2</sub>/mg polymer, 1 eq.) was prepared in 100 μL DMSO. Next, the polymer  
20 solution was slowly added to a 0.01 M Cy7-NHS or Cy5-NHS solution in 93.5 μL DMSO  
21 (935 nmol, 2 eq), containing 2.3 μmol (5 eq.) triethylamine (Scheme 2). The reaction was  
22 performed for 36 h (in the dark) under stirring and N<sub>2</sub> atmosphere.

23 To determine the coupling efficiency, the crude product was diluted to a final polymer  
24 concentration of 1 mg/mL in DMF containing 10 mM LiCl and analyzed by GPC equipped  
25 with a UV detector set at 646 nm for Cy5 and 700 nm for Cy7 detection. The coupling  
26 efficiency was determined by analyzing the area under the curve (AUC) of the polymer and  
27 unreacted dye peaks. AUC was determined using GraphPad Prism 5 (GraphPad Software  
28 Inc., La Jolla, California, USA).

29 A 1 M DMAE-Cl solution in DMSO (451.5 μmol), corresponding to a 10-fold excess to  
30 HPMA reactive groups of the polymer, was added to the reaction mixture. The reaction was  
31 performed at room temperature for 24h, in the dark and N<sub>2</sub> atmosphere to yield p(HPMA-  
32 DMAE-co-PDTEMA-co-Cy5/Cy7)-b-PEG (Scheme 2). After completion of the reaction, 1 M  
33 acetic acid was added to adjust the pH 5. Removal of free dye was performed by dialysis  
34 against a mixture of EtOH/water 10 mM NH<sub>4</sub>Ac pH 5 (50/50) for 2 days. The polymer was

1 collected by freeze-drying after buffer exchange to 5 mM NH<sub>4</sub>Ac pH 5 and desalting using  
2 PD-10 (GE Healthcare Life Sciences) columns following the supplier's protocol.

### 3 4 2.2.3. Preparation of p(HPMA-co-PDTEMA)-b-PEG

5 To prepare p(HPMA-co-PDTEMA)-b-PEG (pHP-PEG), 5 mg of p(HPMA-DMAE-co-  
6 PDTEMA)-b-PEG (pHDP-PEG) was dissolved in 2.5 mL 10 mM HEPES 10 mM TAPS pH  
7 8.5 buffer and hydrolyzed for 6 h at 37 °C. After hydrolysis the polymer was purified with a  
8 PD-10 column following the supplier's protocol and collected by freeze-drying.

### 9 10 2.3. <sup>1</sup>H NMR characterization of the polymers

11 The composition of the polymers was determined by <sup>1</sup>H NMR analysis performed with a  
12 400 MHz Agilent 400-MR NMR spectrometer (Agilent Technologies, Santa Clara, USA) in  
13 DMSO-d<sub>6</sub>. The ratio HPMA/PDTEMA was determined by comparison of the integrals at δ  
14 4.6 ppm (bs, CH<sub>2</sub>CHCH<sub>3</sub>O, HPMA) and the integral at δ8.5 ppm (bs, pyridyl group proton,  
15 PDTEMA) (∫δ<sub>4.6</sub>/∫δ<sub>8.5</sub>). The integral ratios between δ4.2 ppm (bs, OCH<sub>2</sub>CH<sub>2</sub>), HPMA-DMAE)  
16 and δ4.6 ppm (bs, CH<sub>2</sub>CHCH<sub>3</sub>O, HPMA-DMAE) was used to verify reaction between DMAE-  
17 Cl and hydroxyl groups in the polymer from HPMA.

18 The number average molecular weight (M<sub>n</sub>) of the polymers was determined according  
19 to equation (1).

$$20$$
$$21 \quad M_n = (\int\delta_{4.6} \times M_{(HPMA/HPMA-DMAE)} + \int\delta_{8.5} \times M_{PDTEMA}) / (\int\delta_{3.5}/448) + 5000 \text{ (g/mol)} \quad (1)$$
$$22$$

23 where, ∫δ<sub>3.5</sub>, ∫δ<sub>4.6</sub> and ∫δ<sub>8.5</sub> are the integrals at 3.5, 4.6, and 8.5 ppm, respectively. M<sub>HPMA</sub>,  
24 M<sub>HPMA-DMAE</sub> and M<sub>PDTEMA</sub> are the molar masses of HPMA, HPMA-DMAE and PDTEMA,  
25 respectively. The number of protons for the 5000 Da PEG block, at ∫δ<sub>3.5</sub>, was set at 448.

### 26 27 2.4. UV spectroscopy characterization of the polymers

28 UV spectroscopy was performed on Shimadzu UV-2450 UV/VIS spectrophotometer ('s-  
29 Hertogenbosch, The Netherlands) to quantify the molarity of thiol reactive pyridyl disulfide  
30 (PDS) groups per weight of polymer. Polymer stock solutions of 1 mg/mL were prepared in  
31 20 mM HEPES pH 7.4 containing 50 mM tris(2-carboxyethyl)phosphine (TCEP). After  
32 incubation for 1 h at 37 °C the UV absorbance at 343 nm was measured to determine the  
33 released 2-mercaptopyridine (2-MP) [43]. Quantification was performed using a calibration  
34 curve with 2-MP standards.

1 To quantify the molarity of dye Cy7 or Cy5 per weight of polymer, polymer stocks of 1  
2 mg/mL in DMSO were prepared and the UV absorbance was measured at 646 nm for pHDP-  
3 Cy5-PEG or 750 nm for pHDP-Cy7-PEG. The quantification was done using a calibration  
4 curve of dye standards in DMSO.

## 6 **2.5. TNBSA assay**

7 In order to determine the molarity of free primary amines in p(HPMA-co-PDTEMA-co-  
8 APMA)-b-PEG, the TNBSA assay was performed [44]. Polymer solutions were prepared at  
9 1 mg/mL in 0.1M sodium bicarbonate buffer (pH 8.5) using glycine standards. The amine  
10 content was determined by detecting the absorbance at 420 nm.

## 12 **2.6. Gel permeation chromatography (GPC) characterization of the polymers**

13 GPC analysis of the polymers was performed using a Waters System (Waters Associates  
14 Inc., Milford, MA) with refractive index (RI) and UV detector using two serial Plgel 5  $\mu$ m  
15 MIXED-D columns (Polymer Laboratories) and DMF containing 10 mM LiCl as eluent. The  
16 flow rate was 1 mL/min (30 min run time) and the temperature was 60 °C. The average  
17 molecular weight ( $M_n$ ), weight average molecular weight ( $M_w$ ) and polydispersity (PDI,  
18  $M_w/M_n$ ) was determined by calibration with a series PEG calibration standards of different  
19 molecular weights and with narrow molecular weight distribution.

## 21 **2.7. Preparation of decationized polyplexes**

22 The preparation of Cy5/Cy7-labeled decationized polyplexes was adapted from the  
23 method previously described (Scheme 3) [33]. In order to prepare a high concentrated  
24 polyplex dispersion 400  $\mu$ g/mL pDNA, 80  $\mu$ L of aqueous solution of pHDP-PEG was mixed  
25 with 160  $\mu$ L pDNA (pCMV\_EGFP) (600  $\mu$ g/mL) in 10 mM HEPES 10 mM TAPS pH 8.5 buffer  
26 containing 5% glucose. The amount of polymer added to the pDNA solution corresponded  
27 to N/P=4 (N, molarity of positively charged amines from polymer; P, molarity of negatively  
28 charged phosphates from pDNA)). Subsequently, the formed polyplexes were crosslinked  
29 by addition of dithiothreitol (DTT) corresponding with a half molar equivalent to PDS groups  
30 of the polymer, in order to induce self-crosslinking of the polyplexes [45] for 1 h at pH 8.5 at  
31 room temperature.

32 To prepare decationized pHDP-PEG polyplexes, the cationic DMAE side groups were  
33 removed by hydrolysis by incubation of the polyplex dispersions at 37 °C in 10 mM HEPES  
34 10 mM TAPS, pH 8.5 for 6 h [33]. Next, the pH of the dispersion was adjusted to pH 7.4. For

1 cationic pHDP-PEG polyplexes the pH was adjusted to pH 7.4 immediately after completion  
2 of the crosslinking step. When polyplexes needed to be stored for long periods, the pH of  
3 the dispersions was adjusted to pH 5 and the dispersions were stored at 0-5 °C, with the pH  
4 being readjusted to pH 7.4 immediately before use. Comparative studies were always  
5 performed by dividing the same batch of polyplexes into pHDP-PEG cationic and pHP-PEG  
6 decationized polyplexes. Given the fact that the side products from cross-linking and  
7 decationization (2-mercaptopyridine and DMAE) have a high cellular tolerance [33, 45, 46],  
8 the polyplexes were directly used without purification procedures.

## 9 10 11 **2.8. Particle size and zeta potential determination**

12 The size of the polyplexes was measured with DLS on an ALV CGS-3 system (Malvern  
13 Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at  
14 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator with temperature  
15 controller set at 25 °C or 37 °C. Measurements were performed in HBS (20 mM HEPES,  
16 150 mM NaCl, pH 7.4) at a pDNA concentration of 40 µg/mL.

17 Size distribution and polydispersity of the polyplexes were also determined by  
18 nanoparticle tracking analysis (NTA) measurements on a NanoSight LM10SH (NanoSight,  
19 Amesbury, United Kingdom), equipped with a sample chamber with a 532-nm laser.  
20 Polyplexes were diluted in phosphate buffered saline (PBS) to a concentration of 0.1 µg/mL  
21 pDNA and measured for 160 s with manual shutter and gain adjustments. The captured  
22 videos were analyzed by the NTA 2.0 image analysis software (NanoSight, Amesbury, UK).  
23 The detection threshold was set to 2 and the minimum track length to 10. The mode and  
24 mean size and SD values were obtained by the NTA software.

25 The zeta potential ( $\zeta$ ) of the polyplexes was measured using a Malvern Zetasizer Nano-  
26 Z (Malvern Instruments, Malvern, UK) with universal ZEN 1002 'dip' cells and DTS (Nano)  
27 software (version 4.20) at 25 °C. Zeta potential measurements were performed in 20 mM  
28 HEPES pH 7.4 at a pDNA concentration of 10 µg/mL.

## 29 30 **2.9. Stability using fluorescence single particle tracking (fSPT)**

31 fSPT was performed to measure the stability of the cationic pHDP-PEG polyplexes and  
32 decationized pHP-PEG polyplexes in full human plasma. fSPT is a fluorescence microscopy  
33 technique that uses wide-field and a fast and sensitive CCD camera to record movies of

1 diffusing particles in biofluids. These movies are analyzed using in-house developed  
2 software, to obtain size distributions as previously described [36].

3 fSPT measurements were performed as follows: a volume of 5  $\mu$ L of sample of Cy5-  
4 labeled polyplex dispersion in HEPES buffer (20 mM pH 7.4) or in fresh plasma (10  $\mu$ g/mL  
5 final pDNA concentration) was placed between a microscope slide and cover glass with  
6 double-sided adhesive sticker, following incubation for different time points at 37 °C. Both  
7 the objective and the sample were kept at 37 °C during the measurements using an objective  
8 heater (Bioptechs, Butler, USA) and a sample heater (Linkam, Surrey, U.K.). Videos were  
9 recorded with the NIS Elements software (Nikon) driving the EMCCD camera (Cascade  
10 II:512, Roper Scientific, AZ, USA) and a TE2000 inverted microscope equipped with a 100\_  
11 NA1.4 oil immersion lens (Nikon). To convert SPT diffusion measurements to size  
12 distributions, the viscosity of human plasma at 37 °C was set to 1.35 cP [36]. Human plasma  
13 was obtained from a healthy donor at UMC Utrecht. Blood was collected in EDTA tubes  
14 which were cooled on ice and subsequently centrifuged at 4 °C, 2000  $\times$ g for 10 min and  
15 plasma was isolated and stored at -80 °C.

## 16 17 **2.10. Cell Culture**

18 HUVEC (human umbilical vein endothelial) cells were obtained from human umbilical  
19 cords and cultured in Endopan 3 (E3) medium (Pan Biotech, Germany), supplemented with  
20 1% penicillin/streptomycin. A431 epidermoid carcinoma cells (ATCC) were cultured in  
21 Dulbecco's modified Eagle's Medium (DMEM; Gibco, Invitrogen, Germany), supplemented  
22 with 10% fetal bovine serum (FBS) (Invitrogen, Germany) and with 1%  
23 penicillin/streptomycin. Cell lines were kept at 37 °C and 5% CO<sub>2</sub>, in a humidified  
24 atmosphere.

## 25 26 **2.11. MTT assay**

27 The cell viability upon incubation with decationized pHP-PEG and cationic pHDP-PEG  
28 polymers was evaluated via MTT assay, which measures the cellular metabolic activity of  
29 the cells. HUVECs were seeded into 96-well plates: 12,000 cells were seeded per well for  
30 24 h test and 1,950 cells per well were seeded for the 72 h test. After 24 h, the medium was  
31 refreshed and 30  $\mu$ L of polymer samples in PBS was added to each well corresponding to  
32 a final concentration of 0.01-3 mg/mL also containing 12% FBS. After 24 h or 72 h, the MTT  
33 assay (Roche) was performed according to the manufacturer's protocol. First, cells were  
34 washed with 200  $\mu$ L PBS and 100  $\mu$ L of cell culture medium was added per well. Next, MTT

1 labeling reagent was added to a final concentration of 0.5 mg/mL. After 4 h at 37 °C and 5%  
2 CO<sub>2</sub>, the medium was discarded and 100 µL of DMSO was added per well to dissolve the  
3 formed formazan crystals. The plate was protected from light and formazan crystals were  
4 dissolved overnight at room temperature. The supernatants (100 µL) were then transferred  
5 into another 96-well plate and the absorbance was measured at 570 nm using reference  
6 wavelength of 690 nm.

## 8 **2.12. *In vivo* toxicity using the zebrafish embryo assay**

9 The *in vivo* toxicity of decationized pHP-PEG and cationic pHDP-PEG polymers was  
10 evaluated using the zebrafish (*Danio rerio*) embryo assay based on the method previously  
11 described in detail by Rizzo et al. [37]. In short, fertilized eggs were transferred into round-  
12 bottom 96-well plates (16-cell stadium, 1 egg per well). Polymers were diluted in fish medium  
13 corresponding to polymer concentrations ranging from 0.1 to 3 mg/mL. As positive control,  
14 branched polyethylenimine (b-PEI) 25 kDa was tested at 0.1 mg/mL. The development and  
15 survival of the zebrafish embryos was evaluated for 72 h every 24 h post-fertilization using  
16 a Leica DMI 6000B inverted microscope.

## 18 **2.13. Animal experiments**

19 CD-1 nude female mice were fed chlorophyll-free food pellets and water *ad libitum*. Mice  
20 were housed in ventilated cages and clinically controlled rooms and atmosphere. CD-1 nude  
21 mice were inoculated with A431 tumor cells (4×10<sup>6</sup> cells/100 µL) subcutaneously into the  
22 left flank 15 days before experiment, which lead to the development of A431 tumor  
23 xenografts with an approximate size of 6-7 mm in width.

24 Decationized pHP-PEG polyplexes (group 1, n=3 and cationic pHDP-PEG polyplexes  
25 (group 2, n=3) were tested for their *in vivo* biodistribution and tumor accumulation. Polyplex  
26 dispersions labeled with Cy7 were injected (80 µL, 32 µg pDNA, 2.5 nmol Cy7) into mice via  
27 tail vein under anesthesia. Immediately after sample injection, µCT (Tomoscope DUO; CT  
28 Imaging, Erlangen, Germany) and 3D FMT imaging (FMT2500; PerkinElmer), were  
29 performed essentially as previously described [38]. Briefly, mice were placed in a multi-  
30 modal imaging cassette under anesthesia (2% of isoflurane) to be firstly scanned in a µCT.  
31 Images with an isotropic voxel size of 35 µm were reconstructed using a modified Feldkamp  
32 algorithm with a smooth kernel. Immediately after µCT procedure, the animals were placed  
33 into the FMT docking station under 2% isoflurane anesthesia. The excitation wavelength  
34 channel was set to 750 nm. Whole body images of the mice were captured using FRI to

1 allow the definition of the region of interest (ROI) and 3D scans were performed.  $\mu$ CT and  
2 3D FMT images were collected 15 min, 4 h, 24 h and 48 h post-injection.

3 The obtained  $\mu$ CT and FMT scans were fused. Based on the  $\mu$ CT data, liver, kidneys,  
4 lungs, heart, bladder and tumor were segmented, using an Imalytics Research Workstation  
5 software (Philips Technologie GmbH Innovative Technologies, Aachen, Germany). FMT  
6 reconstructed signals were overlapped onto respective organ-segmented  $\mu$ CT images, and  
7 the amount accumulated Cy7 in these organs was quantified. The percentage injected dose  
8 (% ID) was calculated based on the quantification obtained for each segmented organ and  
9 normalized to the organ volume. In parallel to the imaging protocol, blood and urine samples  
10 were also collected at relevant time 2 min, 15 min, 1 h and 4 h for blood and 1 h, 4 h and 24  
11 h for urine collection.

### 12 13 **2.15. Ex vivo analysis**

14 Mice were injected intravenously with rhodamine-labeled lectin (Vector Laboratories,  
15 LTD, UK), for staining of blood vessels, 15 min before sacrifice and 48h after sample  
16 injection. Tumors, liver, spleen, heart, lungs, uterus, intestines, muscle and skin were  
17 collected, weighted and analyzed by 2D Fluorescence reflectance imaging (FRI) at the FMT  
18 at the 750 nm channel. Tumors were preserved in Tissue-Tek<sup>®</sup> O.C.T<sup>™</sup> compound (Sakura,  
19 The Netherlands) at -80 °C for immunohistochemistry.

### 20 21 **2.16. Histological analysis**

22 Histological staining was performed to analyze the Cy7-labeled polyplex accumulation in  
23 tumors. Simultaneously, EGFP expression in the tissue was evaluated in order to determine  
24 the degree of transfection for both decationized pHP-PEG and cationic pHDP-PEG  
25 polyplexes. Frozen 8  $\mu$ m sections were prepared, where blood vessels were previously  
26 stained by rhodamine-lectin perfusion. Sections were mounted using Mowiol and  
27 fluorescence microscopy imaging was performed using an Axio Imager M2 microscope and  
28 a high-resolution AxioCam MRm Rev.3 camera, at magnification 40 $\times$ . Images of 3  
29 independent sections per animal were further post-processed using AxioVision Rel 4.8  
30 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) and analyzed for Cy7 signal  
31 (polyplex accumulation) and EGFP signal (transfection efficiency).

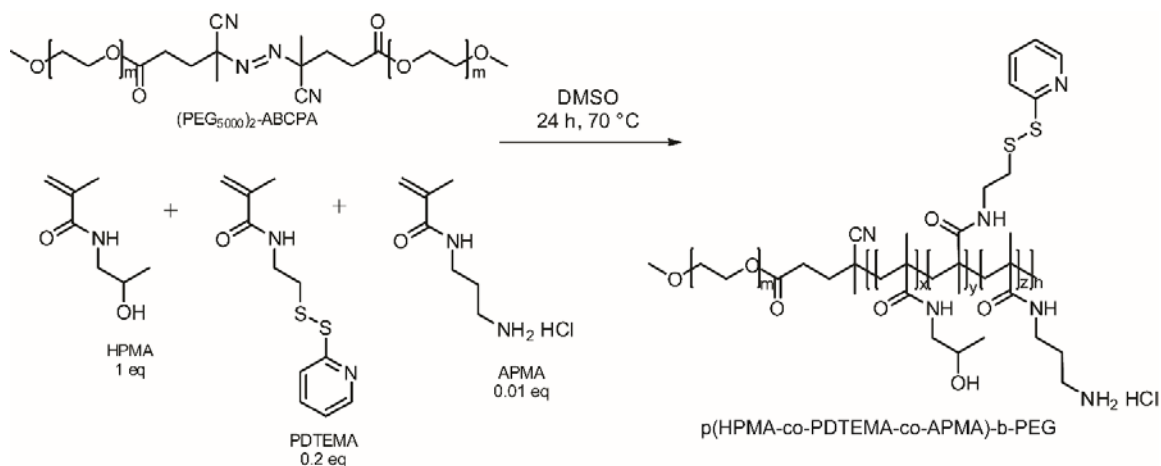
## 2.17. Statistical analysis

Statistical analysis for comparison between means were performed with the software GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California, USA) and a two-tailed paired Student's t-test was used, where  $p < 0.05$  was considered to represent statistical significance.

## 3. RESULTS AND DISCUSSION

### 3.1. Synthesis of fluorescently labeled p(HPMA-DMAE-co-PDTEMA)-b-PEG

The stability evaluation by fSPT and the *in vivo* evaluation by 3D-FMT of decationized polyplexes required the synthesis of Cy5- and Cy7-labeled pHDP-PEG, which was performed in 3 steps (Scheme 1). First, p(HPMA-co-PDTEMA-co-APMA)-b-PEG was synthesized via free-radical polymerization of HPMA, PDTEMA and APMA using (mPEG)<sub>2</sub>-ABCPA as a PEG<sub>5000</sub> bi-functionalized azo macroinitiator [40] (Scheme 1). The yield of polymerization was close to 75%. The results of the analysis of the polymer by <sup>1</sup>H NMR (Fig. 1), GPC and TNBSA assay are given in Table 1. The different monomers were incorporated in the polymer close to the feed ratio. The  $M_n$  calculated by <sup>1</sup>H NMR (44.0 kDa) is in good agreement with GPC (34.4 kDa, PDI=1.6). The incorporation of PDTEMA was confirmed by UV, and a value of  $821 \pm 43$  nmol per mg of polymer was found, which is very close to the value calculated by NMR (863 nmol per mg of polymer; thus around 40 units of PDTEMA were present per polymer chain). The TNBSA assay showed that approximately 2 units APMA per polymer chain were present.



**Scheme 1.** Synthesis of p(HPMA-co-PDTEMA-co-APMA)-b-PEG, by free-radical polymerization of HPMA, PDTEMA and APMA using ((mPEG<sub>5000</sub>)<sub>2</sub>-ABCPA) macroinitiator.

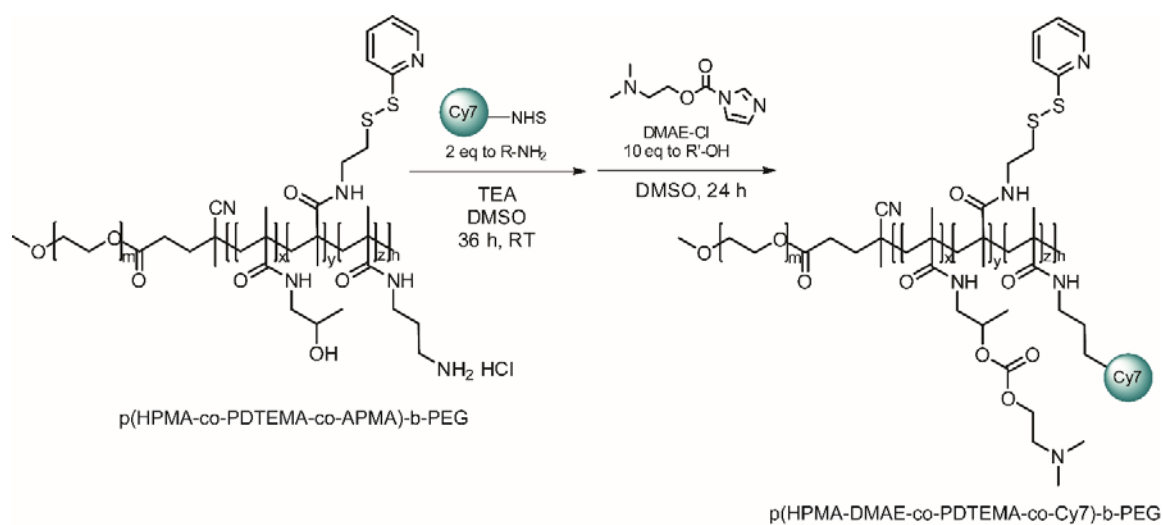
1 **Table 1.** p(HPMA-co-PDTEMA-co-APMA)-b-PEG characteristics as determined by GPC, <sup>1</sup>H NMR,  
 2 UV spectroscopy and TNBSA assay.

GPC		NMR		NMR, TNBSA	UV
M <sub>n</sub> (kDa)	PDI	M <sub>n</sub> (kDa)	Feed HPMA-DMAE/PDTEMA/APMA	Polymer HPMA-DMAE/PDTEMA/APMA	nmolPDS/mg <sub>polymer</sub>
34.4	1.6	44.0	1/0.20/0.01	1/0.19/0.01	821.0±43.3

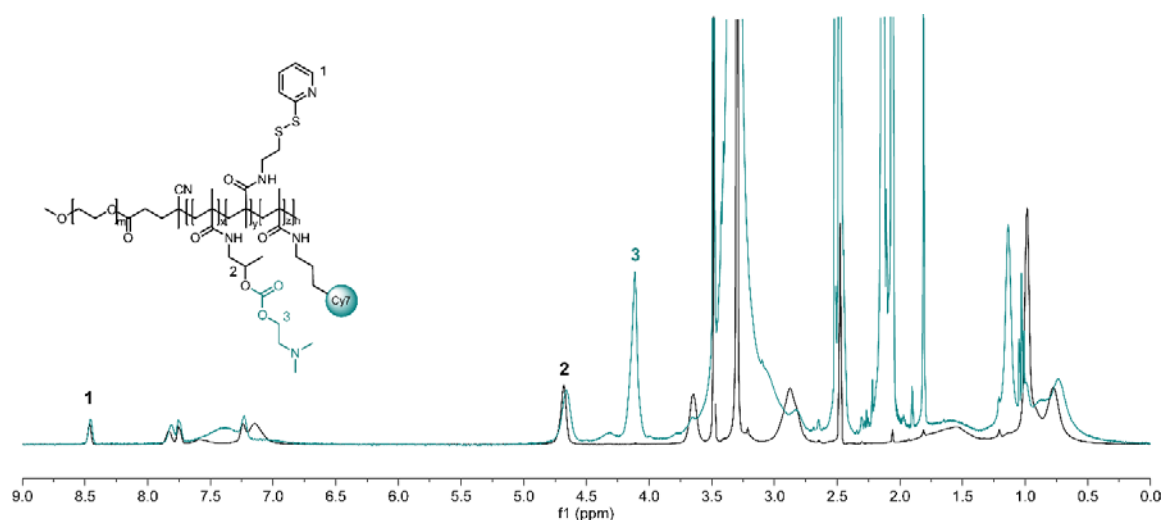
3  
4

5 NHS-functionalized dyes were conjugated to p(HPMA-co-PDTEMA-co-APMA)-b-PEG by  
 6 reaction with the primary amines of APMA for 36 h using a 2 molar excess of reactive dyes  
 7 (Scheme 2). After reaction, the crude product was analyzed with GPC, and the coupling  
 8 efficiency was determined by GPC analysis of the AUC of the polymer and unreacted dye  
 9 peaks (detection at 646 nm (Cy5 coupling) or 700 nm (Cy7 coupling)). The obtained  
 10 chromatograms revealed that the AUC for each peak was close to 50% for both reactions,  
 11 demonstrating that the amine groups of the polymers were quantitatively modified with dye  
 12 molecules. In Fig. 2a shows the GPC chromatogram of the crude product from Cy7 coupling  
 13 reaction. In the third step, the crude product (e.g. p(HPMA-co-PDTEMA-co-(Cy5/Cy7)-b-  
 14 PEG) was reacted with DMAE-Cl (Scheme 2) to yield the cationic polymer p(HPMA-DMAE-  
 15 co-PDTEMA-co-(Cy5/Cy7)-b-PEG. After purification of the polymer by extensive dialysis, <sup>1</sup>H  
 16 NMR (Fig. 1) showed that the integral ratio between peaks at δ4.2 ppm (bs, OCH<sub>2</sub>CH<sub>2</sub>),  
 17 HPMA-DMAE) and δ4.6 ppm (bs, CH<sub>2</sub>CHCH<sub>3</sub>O, HPMA-DMAE) was close to 2, confirming  
 18 a quantitative reaction of the OH groups of HPMA with DMAE-Cl. GPC analysis of the final  
 19 purified products showed complete removal of the unreacted dyes for both Cy5- and Cy7-  
 20 labeled polymer (e.g. Fig. 2b for Cy7-labeled polymer). The modification of HPMA groups  
 21 with the cationic DMAE groups after dye coupling was chosen over the direct polymerization  
 22 of HPMA-DMAE monomer [33, 34] because of the incompatibility of the carbonate ester  
 23 bond in HPMA-DMAE with primary amines.

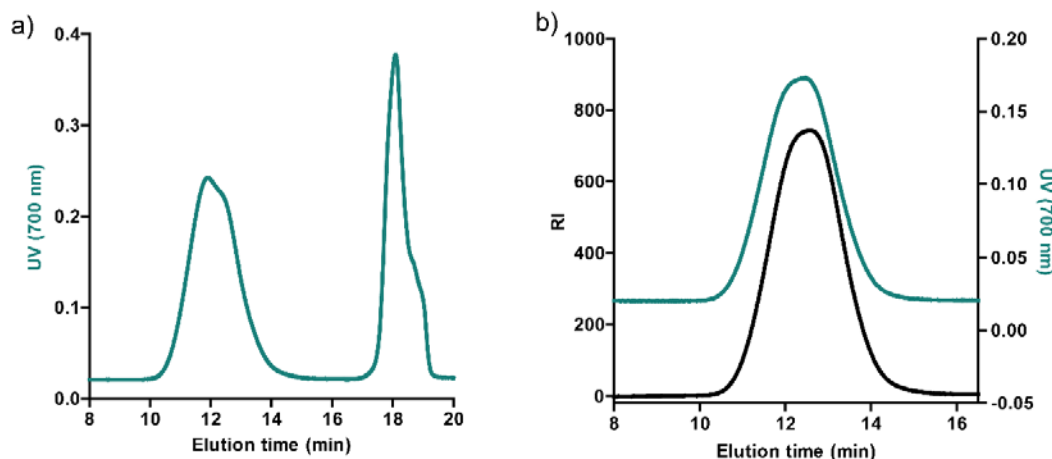
24  
25



1  
 2 **Scheme 2.** Synthesis of p(HPMA-DMAE-co-PDTEMA-co-Cy7)-b-PEG, by sequential coupling of  
 3 Cy7-NHS and DMAE-Cl to p(HPMA-co-PDTEMA-co-APMA)-b-PEG.  
 4



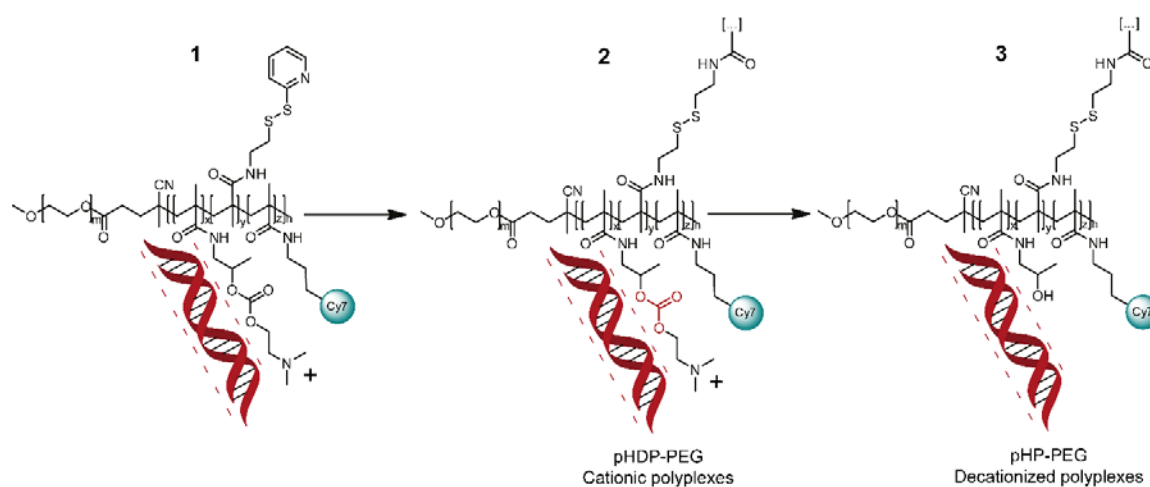
5  
 6 **Fig. 1.**  $^1\text{H}$ NMR spectra of p(HPMA-co-PDTEMA-co-APMA) and p(HPMA-DMAE-co-PDTEMA-co-Cy7)-  
 7 b-PEG in DMSO.  
 8  
 9



1  
2  
3 **Fig. 2.** GPC chromatograms (a) UV detection at 700 nm of the crude product from coupling of  
4 Cy7-NHS to p(HPMA-co-PDTEMA-co-APMA)-b-PEG, using a 2 molar equivalent excess of Cy7-NHS  
5 to primary amines in the polymer. (b) Refractive index signal (RI) and UV signal at 700 nm of the final  
6 purified p(HPMA-DMAE-co-PDTEMA-co-Cy7)-b-PEG polymer.

7  
8 **3.2. Preparation of fluorescently labeled decationized pHP-PEG polyplexes**

9 Polyplexes of pHP-PEG were formed through the 3-step process [33, 34] (Scheme 3).  
10 The preparation of Cy5 or Cy 7-labeled decationized polyplexes started with the  
11 complexation of the cationic block copolymer p(HPMA-DMAE-co-PDTEMA-co-Cy5/Cy7)-b-  
12 PEG (pHDP-PEG) with pDNA via electrostatic interactions. The interchain disulfide  
13 crosslinking of the polyplexes was performed via thiol-disulfide exchange reaction by  
14 addition of DTT corresponding to a 50% molar equivalent of the PDS groups of the polymer,  
15 to induce self-crosslinking of the polyplexes [45], yielding cationic pHDP-PEG polyplexes.  
16 To obtain decationized polyplexes from cationic precursors, decationization was performed  
17 by removal of the DMAE cationic side groups linked via carbonate ester bond to the HPMA  
18 backbone at pH 8.5 for 6.5 h. This process yields p(HPMA-co-PDTEMA-co-Cy5/Cy7)-b-PEG  
19 (pHP-PEG) decationized polyplexes. The structural stabilization and pDNA entrapment was  
20 solely dependent on the interchain disulfide crosslinks in the pHPMA-DMAE core.



1  
2 **Scheme 3.** Route for the preparation of interchain disulfide-crosslinked decationized polyplexes,  
3 through a 3-step process: 1. charge-driven condensation with nuclei acids; 2. stabilization through  
4 disulfide crosslinking; 3. decationization of cationic pHDP-PEG polyplexes, resulting in decationized  
5 pHP-PEG polyplexes (adapted from [33]).

6  
7 An overview of the biophysical properties of both the decationized pHP-PEG and cationic  
8 pHDP-PEG polyplexes by DLS and zeta potential measurements is shown in Table 2.  
9 Polyplexes were prepared by complexing the polymer with pDNA at an optimal N/P=4  
10 regarding their biophysical and *in vitro* transfection properties [33]. Firstly, pHDP-PEG  
11 formed nanosized particles with a diameter of  $140 \pm 5$  nm and a positive zeta potential of  
12  $+11 \pm 3$ . After decationization, pHP-PEG polyplexes had a size of  $142 \pm 8$  nm and a slightly  
13 negative zeta potential of  $-4 \pm 2$  mV. The decrease in zeta potential, from cationic to  
14 decationized arises from the loss of the cationic DMAE groups from the pHDP and from  
15 the entrapped pDNA in the core [33]. The loss of electrostatic interactions between the  
16 polymer and pDNA potentially leads to some hydration and thus slight swelling of the  
17 polyplex core, however by using a zero-length crosslinking agent such as DTT, this effect is  
18 limited. Size is a particularly critical property for the biodistribution of nanoparticles [47],  
19 however, here this variable is ruled out by evaluation polyplexes of very similar size.

20 Both decationized pHP-PEG and cationic pHDP-PEG polyplexes were complementarily  
21 analyzed by NTA, a technique that allows the simultaneous analysis of individual particles  
22 in a suspension and gives information of the true size distribution (Fig. 3) [48]. Similarly to  
23 DLS, decationized polyplexes and cationic polyplexes showed comparable average sizes  
24 (around 145-150 nm). Importantly, the size distribution measured by NTA displayed similar  
25 distribution profiles: for both particles, when prepared at high concentration for *in vivo*  
26 applications (400  $\mu\text{g/mL}$ ), around 90% of the polyplexes had a size below 210 nm. Size and

1 charge measurements demonstrate that introduction of cyanine fluorophores and  
2 preparation of polyplexes at high concentration did not affect significantly the polyplexes  
3 properties [33, 34].

4

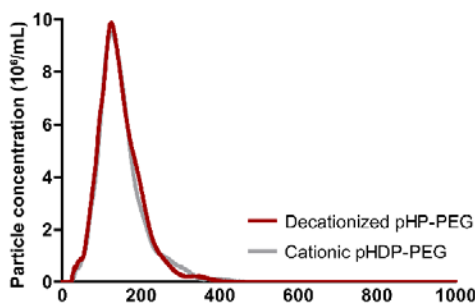
5 **Table 2.** Particle z-average diameter (Z-ave) and polydispersity index (PDI) determined by DLS, and  
6 particle charge ( $\zeta$  Pot) determined by zeta potential measurements, of decationized pHP-PEG and  
7 cationic pHDP-PEG polyplexes. Results are expressed as mean $\pm$ SD (n=3).

Polyplexes	DLS		Zetasizer
	Z-ave (nm)	PDI	$\zeta$ Pot (mV)
pHP-PEG	142 $\pm$ 8	0.20 $\pm$ 0.01	-4 $\pm$ 2
pHDP-PEG	140 $\pm$ 5	0.19 $\pm$ 0.01	11 $\pm$ 3

8

9

10



11

12

13

14

**Fig. 3.** Decationized pHP-PEG and cationic pHDP-PEG polyplexes size distribution determined by NTA in PBS at 25 °C.

15

### 3.3. Stability of decationized pHP-PEG polyplexes in human plasma

16

17

18

19

20

21

22

23

24

25

26

27

28

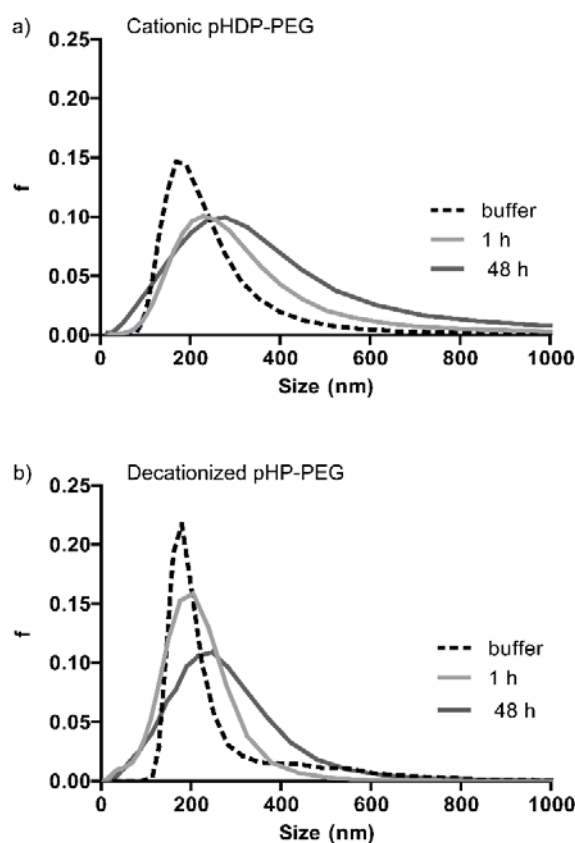
29

The stability of cationic pHDP-PEG and decationized pHP-PEG Cy5-labeled was evaluated by incubation at 37 °C in human plasma. The size distribution of the polyplexes in plasma was determined by fSPT and compared to the distributions obtained when polyplexes were diluted in HEPES buffer pH 7.4 (Fig. 4). The method is based on single particle tracking analysis of fluorescent particles which allows the determination of precise size distributions in media with intrinsic light scattering such as undiluted biological fluids [36]. The fSPT technique is especially important to gain information on the possible aggregation of nanoparticles. When Cy5-labeled polyplexes, both cationic and decationized, were measured in HEPES buffer by fSPT, comparable size distributions to the ones obtained by DLS and NTA were observed. The distribution was relatively narrow, with a peak around 200 nm. When the cationic pHDP-PEG polyplexes were incubated in human plasma for 1 h, a clear change in the distribution profile was observed and a higher average size (~300 nm) as well as polyplexes with >400 nm in diameter were clearly detected. Upon incubation for 48 h, the average size (560 nm) and the population of polyplexes with

1 diameter >400 nm was increased. The instability detected for crosslinked cationic  
2 polyplexes likely occurs is due to insufficient shielding of the positive charge of the  
3 polyplexes by PEG and consequent interaction of polyplexes with negatively charged  
4 proteins from plasma. Indeed, table 2 shows that the cationic pHDP-PEG polyplexes have  
5 a positive zeta-potential. Importantly, when pHP-PEG decationized polyplexes were  
6 incubated with human plasma for 1 h, only a slight change of the size distribution was  
7 observed, and the peak of the distribution remained unchanged. Consequently, crosslinked  
8 decationized polyplexes retained their integrity in biological fluids, and did not show  
9 aggregation (mediated e.g. by plasma proteins). For example, almost no polyplexes with a  
10 diameter of >400 nm were detected which is in great contrast with the cationic polyplexes.  
11 In line with this, also after incubation the polyplexes for 48 h the size distribution only  
12 changed slightly, without any aggregation detected. A slight increase in size after 48 h  
13 probably occurs due to protein adsorption to the polyplexes as a consequence of loss of  
14 PEG density due to continuous hydrolysis of the ester bond linking PEG chains with HPMA-  
15 co-PDTEMA block. PEG is essential to stabilize nanoparticles, avoid aggregation and  
16 protein adsorption in blood. This is quite evident for cationic particles [12, 15, 16], but  
17 essential for anionic particles as well [49, 50].

18 The stability assessment is especially important for systemic administration of polyplexes.  
19 Firstly in terms of safety, cationic polymer based systems, when administered intravenously  
20 lead to the formation of aggregates by interactions with negatively charged blood  
21 components. These aggregates can be retained in the lungs causing embolism and  
22 consequent death [12]. Other severe consequences comprise liver necrosis and as  
23 uncontrollable distribution and expression [10]. The assessment of the stability is also  
24 important to ensure the success of these systems when applied intravenously. In order to  
25 target tumors, polyplexes should also possess prolonged circulation and maintain a stable  
26 size in the order of few hundreds of nanometers to reach and extravasate in the tissue leaky  
27 tumor vasculature, via EPR effect [51-53]. Decationization of polyplexes complemented by  
28 the shielding properties of PEG, significantly improves the stability of polyplexes when  
29 compared to their cationic polyplexes and therefore increasing the chances to target tumors  
30 via EPR.

31



1  
2 **Fig. 4.** The size distribution of cationic pHDP-PEG and decationized pHP-PEG polyplexes as  
3 determined by fSPT after incubation in 90% (v/v) human plasma at 37 °C for 1 h and 48 h. Size  
4 distribution was also determined in 20 mM HEPES pH 7.4.

### 6 3.4. *In vitro* and *in vivo* toxicity assessment

7 To assess the toxicity of the decationized pHP-PEG and cationic pHDP-PEG, the  
8 polymers were evaluated *in vitro* with HUVEC cells via MTT assay in an acute (24 h) and a  
9 long term toxicity test (Fig. 5a) and *in vivo* with a zebrafish embryo model (Figs. 5b and 5c)  
10 with different polymer concentrations (0.1 to 3 mg/mL). The cationic polymer b-PEI, a  
11 commonly used transfection agent, was used as a positive control for toxicity [37].

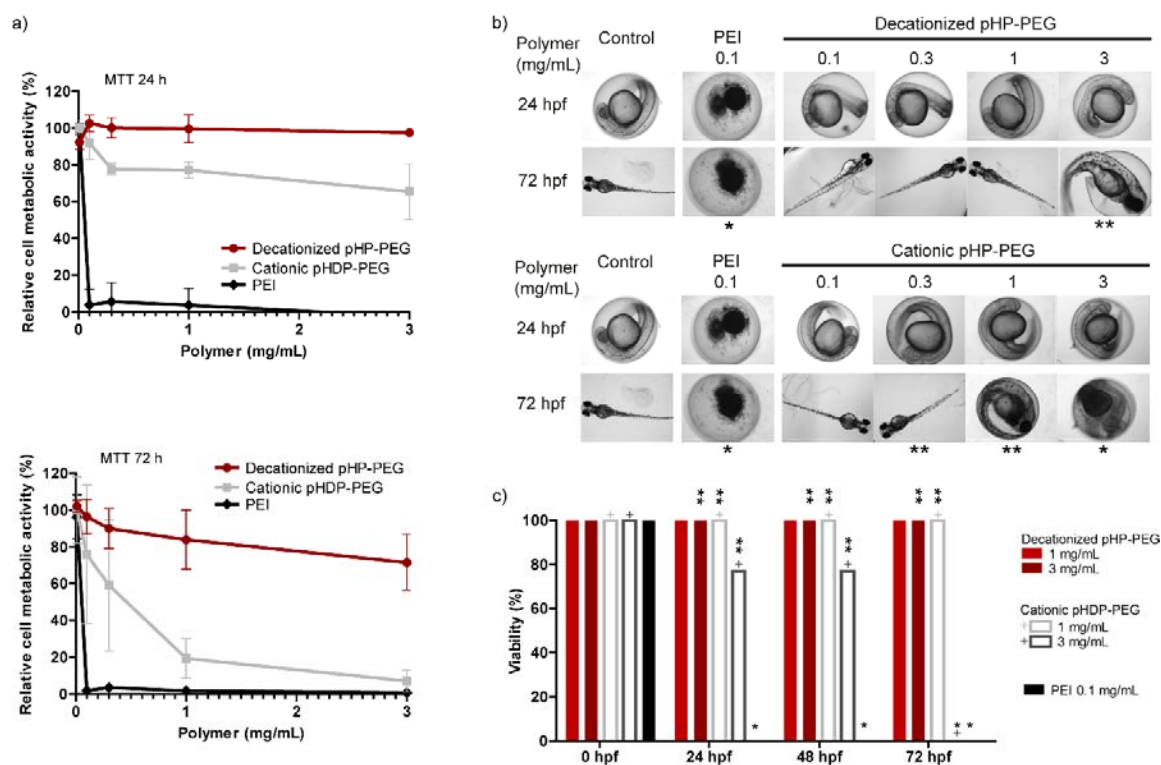
12 The *in vitro* toxicity was tested in HUVEC cells due their high sensitivity towards  
13 transfection agents [54]. The MTT test revealed that decationized pHP-PEG had no effect  
14 on the cell viability, in any of the concentrations tested, when incubated with the cells for 24  
15 h. Only a slight decrease on the cell viability was found in case incubation was prolonged  
16 for 72 h. The *in vitro* evaluation of the cationic pHDP-PEG polymer showed that the polymer  
17 lead to decrease in cell viability with increasing concentrations, to a level of 70% cell viability  
18 for 3 mg/mL when tested for acute toxicity. For long term toxicity test (72 h incubation) the

1 cytotoxic effects were even more pronounced and at 1 mg/mL already lead to decrease in  
2 cell viability to 20%. In the case of b-PEI extreme toxicity was observed, at the lowest  
3 concentration tested (0.1 mg/mL) b-PEI induced complete cell death in both acute and long  
4 term *in vitro* toxicity test.

5 The zebrafish eggs were incubated with increasing polymer concentrations, and  
6 teratogenic effect and the mortality potential on the different zebrafish embryonic stages  
7 were monitored microscopically for a period of 72 h. The neutral polymer pHP-PEG showed  
8 no effect on the fish viability for any of the concentrations tested. Developmental defects  
9 and delayed hatching were observed only for the highest concentration tested (3 mg/mL).  
10 For the cationic polymer pHDP-PEG a significant decrease on zebrafish viability was  
11 observed at 3 mg/mL, resulting in 100% fish death at 72 h. Mortality was also detected at a  
12 very early stage of the development (1 out of 6) together with a slower heartbeat in the  
13 surviving ones. Furthermore, the cationic pHDP-PEG also interfered significantly in the  
14 embryonic development of the fishes. A decrease in the pigmentation was observed from a  
15 concentration of 0.3 mg/mL onwards, no hatching was observed at 48 h for 1 mg/mL and at  
16 3 mg/mL all fish had a decreased heartbeat. In line with the *in vitro* test, b-PEI was again  
17 responsible for extreme toxicity, by inducing complete fish death at 0.1 mg/mL even at early  
18 stages of development.

19 Evident toxicity was induced by the biodegradable cationic pHDP-PEG polymer,  
20 particularly observed on long term (observed in HUVEC cells and zebrafish). Toxicity is  
21 obviously less than b-PEI, but by using a wide range of concentrations and exposure times  
22 we evidence that biodegradability is not sufficient for a completely safe gene delivery  
23 system. We demonstrate not only effects on cell and organism viability but also on the  
24 teratogenicity potential observed in zebrafish embryos at lower concentrations. In great  
25 contrast, a practically innocuous profile of the decationized pHP-PEG was observed in short  
26 and long term *in vitro* and and in the zebrafish assay. This contrast between decationized  
27 and cationic polymers proves the importance of developing gene delivery systems based on  
28 neutral polymers in order to build safe vectors.

29



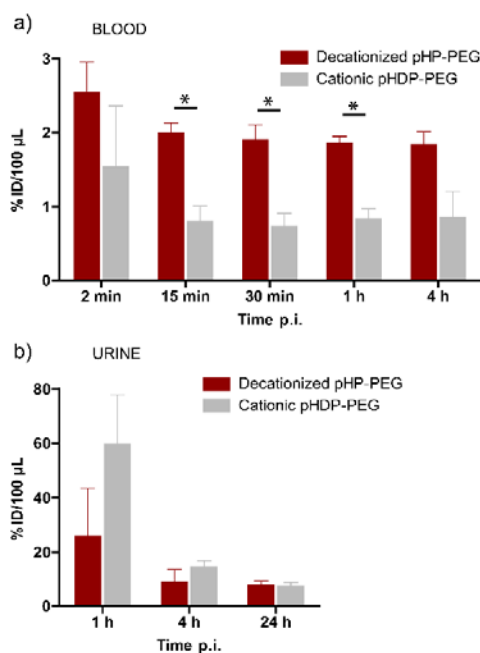
**Fig. 5.** Safety evaluation of decationized polyplexes (a) HUVEC cell relative viability relative upon exposure for 24 h or 72 h to decationized pHP-PEG, cationic pHDP-PEG and b-PEI from 0.1 mg/mL to 3 mg/mL. Results are expressed as mean±SD (n=4). (b) Representative images of zebrafish embryo development upon exposure to decationized pHP-PEG and to cationic pHDP-PEG at concentrations ranging from 0.1-3 mg/mL. \* significant mortality, \*\* significant developmental defects (i.e. decreased pigmentation, delayed hatching ratio and slower heartbeat). (c) zebrafish survival upon exposure to decationized pHP-PEG and cationic pHDP-PEG polymers in comparison with b-PEI (\* 100% mortality; n=6). hpf (hours post-fertilization).

### 3.5. *In vivo* biodistribution and tumor accumulation

Cy7-labeled pHP-PEG decationized and pHDP-PEG cationic polyplexes were intravenously injected into mice bearing subcutaneous A431 tumors, and their biodistribution and tumor accumulation were noninvasively monitored using 3D  $\mu$ CT-FMT. Hybrid  $\mu$ CT-FMT method allows the effective *in vivo* visualization of near-infrared (NIR) dye labeled nanomedicines, and the (semi-) quantification of their accumulation in tumors and healthy organs. By fusing and reconstructing 3D FMT data with  $\mu$ CT images, and by performing absorption pre-scans, a more accurate assignment of fluorescence signals to deep internal organs tissues can be performed [38, 55, 56]. Compared to the 2D FRI, 3D  $\mu$ CT-FMT is not limited to superficial visualization, animal sacrificing is not required.

1 In parallel with 3D  $\mu$ CT-FMT images, blood circulation kinetics was determined by 2D FRI  
 2 signals of blood and urine collected at different time points p.i (Fig. 6). Quantification of  
 3 polyplex signals in blood showed that there was a very rapid decrease of the %ID for both  
 4 cationic and decationized polyplexes which typical for polyplex systems when administered  
 5 intravenously [15, 16, 22, 57, 58] (Fig. 6a). Importantly, a significant higher blood circulation  
 6 time was observed for decationized pHP-PEG polyplexes. Decationized polyplexes showed  
 7 a higher signal in blood at all time points when compared to cationic pHDP-PEG. The  
 8 elimination rate via kidneys was determined by detecting 2D FRI signals from urine (Fig.  
 9 6b). High Cy7 %ID was detected in urine 15 min p.i. for both groups, followed by a rapid  
 10 decrease 4 h p.i. Most likely, this was due to a rapid glomerular filtration of low  $M_w$  polymer  
 11 fraction that is not built in the polyplexes. This fraction of polymers is the same for both  
 12 cationic and decationized polyplexes, as confirmed by the similar signals found for both  
 13 polyplexes.

14



15

16 **Fig. 6.** 2D FRI quantification as %ID (per 100 µL) of decationized pHP-PEG and cationic pHDP-PEG  
 17 Cy7-labeled polyplexes signals in (a) blood and (b) urine at different time points p.i. Results are  
 18 expressed as mean±SD (n=3). \*p<0.01 (t test).

19

20 As shown in Fig. 7, using  $\mu$ CT imaging, tumors, liver, heart, lungs, kidneys and bladder  
 21 were manually segmented, to assign the NIR fluorescence signals from the polyplexes at  
 22 different times points, as previously described [38]. Next, the accumulation of pHP-PEG  
 23 decationized and pHDP-PEG cationic polyplexes in tumors and different organs was

1 quantified. **Quantification in tumors** showed higher normalized %ID detected in tumors for  
2 decationized polyplexes when compared to their cationic counterpart 48 p.i. Also, for  
3 decationized polyplexes increasing accumulation in tumors was observed with the highest  
4 accumulation point at 48 h p.i. In contrast, for cationic polyplexes an accumulation plateau  
5 was rapidly reached (within 15 min), which have been previously observed for other cationic  
6 systems [22].

7 Both polyplexes showed a high accumulation in healthy organs, mainly in liver and  
8 kidneys 15 min p.i. However, in the case of decationized polyplexes there was a rapid signal  
9 decrease at 4 h p.i. resulting in an **apparent** lower accumulation of decationized polyplexes  
10 in liver and kidneys, when compared to cationic polyplexes. Most likely decationized  
11 polyplexes were able to maintain a population of polyplexes in the circulation or rapidly  
12 accumulated in the different organs, but due to their **improved** ability to maintain small and  
13 stable size **in biological fluids (Fig. 4)** together with their low unspecific uptake properties  
14 [33, 34], polyplexes can reenter the blood circulation **and accumulate in tumor tissues**. This  
15 behavior been previously described for other nanoparticulate systems [59, 60].

16 The major organ of polyplex accumulation of polyplexes was the liver, which contains  
17 Kupffer cells as part of the mononuclear phagocyte system (MPS) which are responsible for  
18 phagocytic activity and rapid clearance of nanoparticles *in vivo* [60-62]. Liver accumulation  
19 is very critical for PEGylated polymer-DNA complexes [15, 22, 57]. The ability of  
20 decationized polyplexes to escape from liver and remain stable in circulation more efficiently  
21 determines their higher circulation *in vivo*. As hypothesized PEG is not able to completely  
22 shield cationic particles, leading to unspecific uptake or opsonization, which in turn leads to  
23 decreased circulation time when compared to the decationized polyplexes.

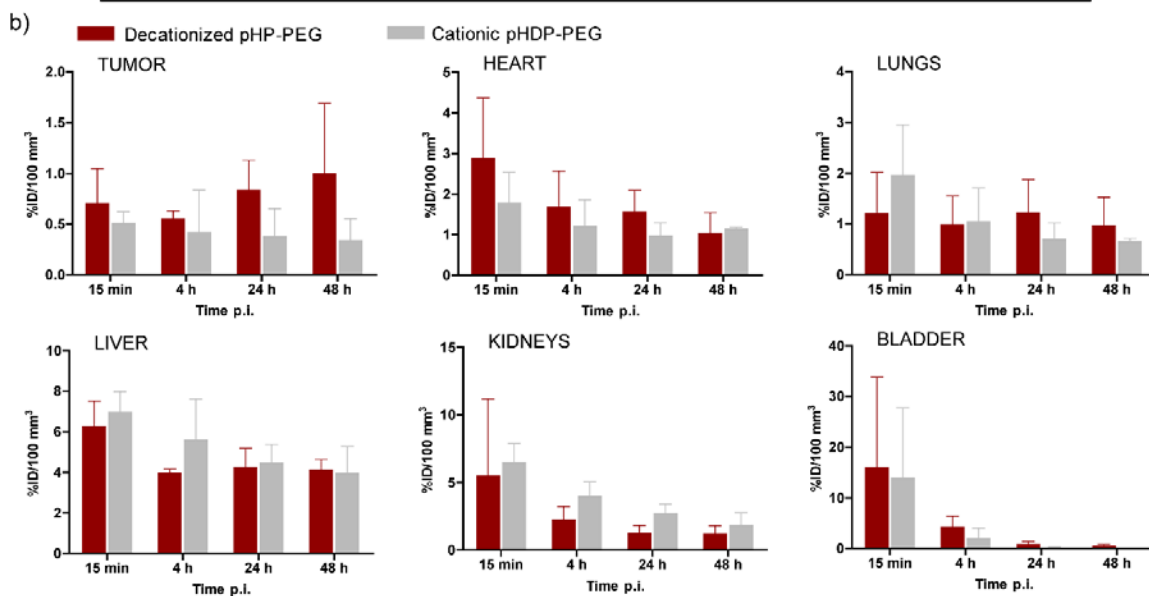
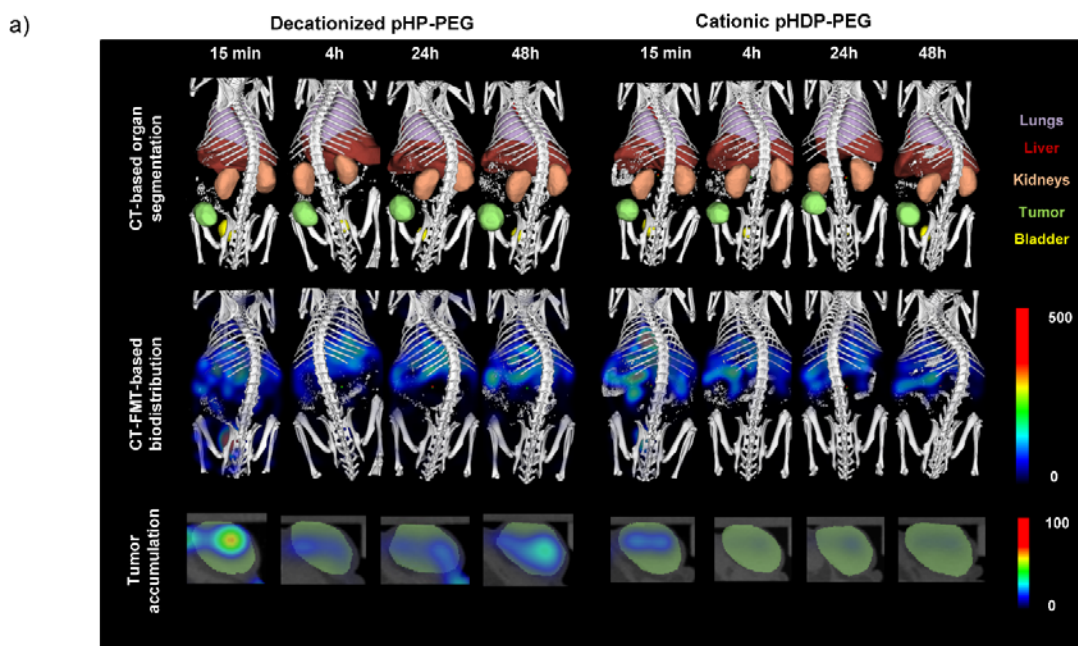
24 Significant NIR signal was noticed in the bladder 15 min p.i. for both groups with similar  
25 degrees and with a rapid drop of signal 4 h p.i. The signals profile are in line with the results  
26 determined by 2D FRI in urine and as quantified, the initial elimination into the bladder  
27 corresponds to 15-20 total %ID.

28 Accumulation in kidneys was also pronounced, **with a tendency** of higher accumulation  
29 of cationic polyplexes. Kidney accumulation has been previously found for polymeric vectors  
30 [57, 63, 64]. Kidney retention can be particularly critical for polycation-based systems. The  
31 nanoparticles within the kidney will have pass through the glomerulus which is in close  
32 contact with glomerular basement membrane (GBM), a 300- to 350-nm-thick basal lamina  
33 rich in negatively charged proteoglycans, to which particles of around 100 nm can access

1 [65]. Ultimately, particle retention in the GBM is driven by the surface charge, leading to  
 2 cationic particles to be retained [66].

3 It is also important to point out that no significant lung deposition was observed for both  
 4 systems. Lung capillaries are among the smallest blood vessels and sieving might occur in  
 5 case aggregates are formed [60], which is normally associated with acute toxicity or off-  
 6 targeting transfection [10, 12]. Our results demonstrate that both polyplexes do not induce  
 7 acute aggregation *in vivo* in line with the results shown in Fig.4.

8



9

1 **Fig. 7.** Noninvasive *in vivo* 3D CT-FMT imaging of the biodistribution and tumor accumulation of  
2 decationized pHP-PEG and cationic pHDP-PEG Cy7-labeled polyplexes. (a) Principle of 3D CT-FMT  
3 imaging: anatomical information obtained using  $\mu$ CT is used to assign the Cy7 signals coming from  
4 polyplexes to a specific organ or tissue of interest. The images were obtained at 15 min, 4 h, 24 h  
5 and 48 h p.i. and show Cy7 localization mainly in liver (red) and kidney (orange). Tumor accumulation  
6 was more prominent for decationized polyplexes. (b) Quantification of the tumor accumulation and  
7 biodistribution of Cy7-labeled decationized pHP-PEG and cationic pHDP-PEG polyplexes in tumors,  
8 liver, lungs, kidney, bladder and heart, expressed as %ID per 100 mm<sup>3</sup> tissue. Results are presented  
9 as mean $\pm$ SD (n=3).

### 11 **3.6. Ex vivo for probe accumulation and in vivo transfection**

12 After the 3D  $\mu$ CT-FMT procedure at 48 p.i., mice were sacrificed and tumors, lungs,  
13 spleen, kidneys, heart skin, intestines and muscle were analyzed using 2D FRI (Figs. 8a  
14 and b). The NIR signals from tumors and several physiologically relevant healthy organs  
15 (liver, spleen, lungs and kidneys) were quantified and compared for both Cy7-labeled  
16 decationized pHP-PEG and cationic pHDP-PEG polyplexes. 2D FRI quantification  
17 determined *ex vivo* supports and further validates the results of the 3D  $\mu$ CT-FMT analysis.  
18 In the case of 2D FRI measurements, a significantly higher accumulation in the tumors was  
19 observed for decationized polyplexes. As determined by 3D  $\mu$ CT-FMT, it was also found 3  
20 times higher accumulation for decationized polyplexes. The tendency of a lower  
21 accumulation in healthy organs was also observed for decationized polyplexes with 2D FRI.  
22 Similar accumulation in liver was observed for both polyplexes 48 p.i., but in the case of  
23 spleen and kidneys higher accumulation was observed for cationic polyplexes. The spleen,  
24 together with liver constitutes the MPS. In the spleen nanoparticles are sieved and  
25 subsequently taken up by macrophages or splenocytes [60, 61]. The apparent lower  
26 retention of decationized polyplexes in the spleen further demonstrates the advantages of  
27 decationized polyplexes.

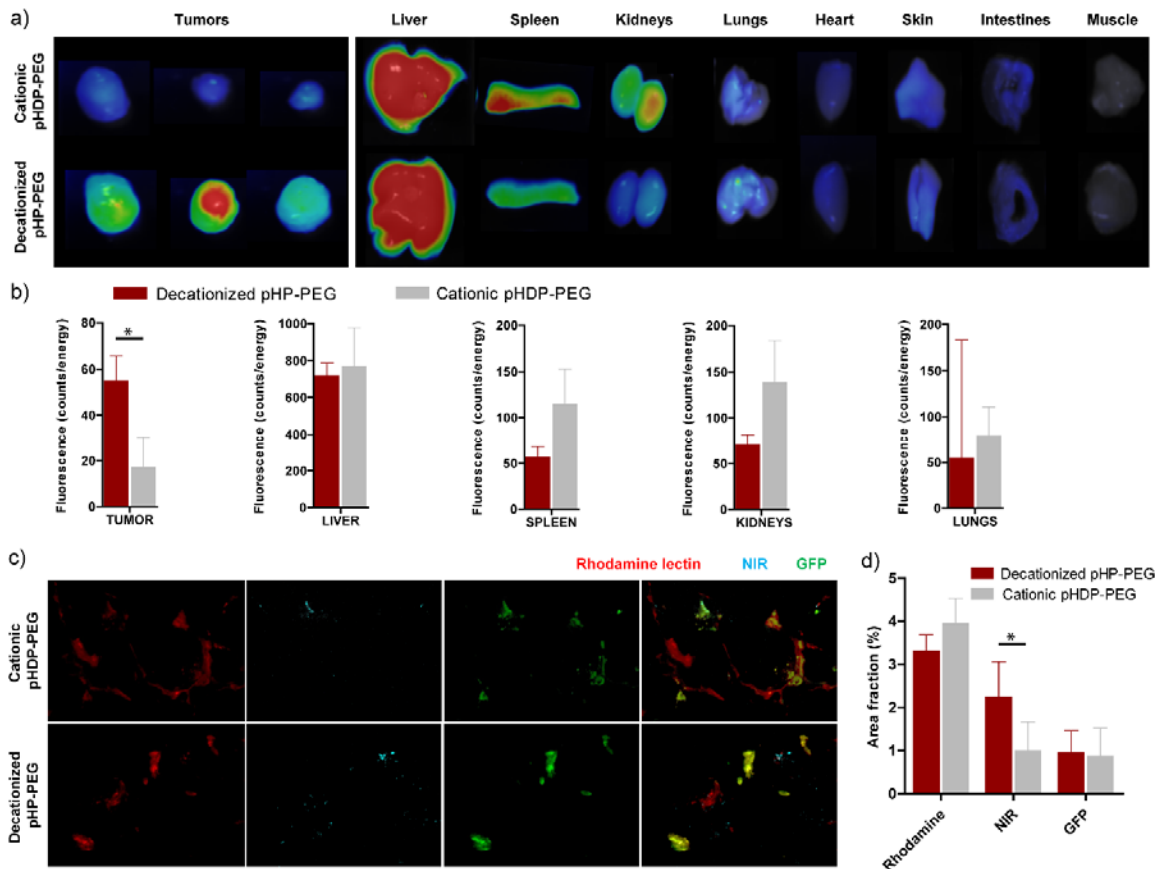
28 Cryosections of tumors collected 48 p.i. were analyzed using fluorescence microscopy,  
29 to validate the *in vivo* and *ex vivo* Cy7-labeled polyplex accumulation results (Figs. 8c and  
30 d). By quantifying the Cy7 signals on tumor sections of both groups, 2 times higher signal  
31 was observed for decationized polyplexes with statistical significance. Microscopy was  
32 also used to determine the *in vivo* tumor transfection potential of both decationized pHP-  
33 PEG and cationic pHDP-PEG polyplexes containing EGFP encoding pDNA (Figs. 8c and  
34 d). The EGFP signal, which allows to infer on the transfection ability of polyplexes, was  
35 similar for both systems, even though significant differences were found in NIR tumor

1 accumulation. Transfection requires polyplexes' (extensive) uptake by the target cells. The  
2 lower transfection ability of decationized polyplexes is therefore likely attributed to its low  
3 degree of unspecific uptake. We also showed that uptake can be triggered by introduction  
4 of a targeting ligand at the surface of decationized polyplexes [33, 34]. Furthermore,  
5 introduction of targeting ligands is important to accelerate the rate of cellular uptake of  
6 polyplexes and prevent premature reduction of disulfide crosslinks before cell entry due to  
7 the presence of secreted and cell membrane thiols [67]. Cationic polyplexes, in contrast, are  
8 known to be taken up extensively by electrostatic interaction with cell membrane anionic  
9 components [3, 68, 69]. The transfection ability of cationic pHDP-PEG polyplexes is in line  
10 with previous findings of pHPMA-DMAE to induce *in vivo* transfection in tumors [70].

11 It should be noticed that the ability of cationic polyplexes to transfect cells more efficiently  
12 has counterproductive effects because it also leads to an increased probability of off-  
13 targeting expression, especially when high accumulation is observed in liver, spleen and  
14 kidneys. Unwanted transfection in healthy organs has been previously observed particularly  
15 in liver and lungs [16, 22, 71]. In recent years, several strategies have been developed to  
16 introduce target transfection with encouraging results [58, 63, 64, 72]. Decationized  
17 polyplexes can potentiate further specific transfection in tumors specially when a targeting  
18 ligand is introduced [34]. Furthermore, due to its low toxic and low teratogenic potential,  
19 decationized polyplexes are less limited to dose and repeated administration restrictions,  
20 resulting in a promising and attractive system for further optimization strategies and testing.

21 Future studies have to be focused on deeper evaluation of transfection ability of optimized  
22 formulations of decationized polyplexes. Optimization for a better *in vivo* performance can  
23 be done by using targeted systems or by optimizing the polymer structure, for example by  
24 determining the best PEG block molecular weight and density [73, 74] or by introduction of  
25 hydrophobic groups in the core of polyplexes groups into polyplexes [75]. In a next step,  
26 therapeutic genes should be used to determine the therapeutic potential of decationized  
27 polyplexes.

1



2

3 **Fig. 8.** *Ex vivo* analysis. (a) Representative *ex vivo* 2D FRI assessment of the tumor accumulation  
4 and biodistribution of decationized pHP-PEG and cationic pHDP-PEG Cy7-labeled polyplexes at 48  
5 h p.i. (b) Quantification of polyplex accumulation in tumors and healthy organs. Results are expressed  
6 as mean±SD (n=3). (c-d) Fluorescence microscopy imaging (c) and quantification (d) of decationized  
7 pHP-PEG and cationic pHDP-PEG Cy7-labeled polyplexes (blue) accumulating in tumors at 48 h p.i.  
8 and inducing EGFP expression (green). Blood vessels are labeled using rhodamine-lectin (red).  
9 Results are expressed as mean±SD (n=3). \*p<0.05 (t test).

10

11

## 12 4. CONCLUSIONS

13 Decationized polyplexes, unlike conventional polycationic polymeric gene delivery systems,  
14 are based on neutral polymers. In the present study, we demonstrate that the neutral  
15 polymer pHP-PEG when tested for toxicity *in vitro* with HUVEC cells showed a very good  
16 cytocompatibility in both acute and long term toxicity test. Furthermore, an *in vivo* zebrafish  
17 toxicity assay revealed that pHP-PEG did not induce fish mortality and, importantly, showed  
18 a much lower teratogenicity potential when compared with its cationic counterpart. **This**

1 apparent safe polymer formed polyplexes with a high stability in biological fluids, as  
2 determined by fSPT, which validated its applicability *in vivo* applicability for systemic  
3 administration. Using noninvasive optical imaging (3D  $\mu$ CT-FMT) and complementary *ex*  
4 *vivo* analysis we demonstrated that by removing the cationic groups of polyplexes, we  
5 obtained a system with higher tumor accumulation, most likely due to their longer blood  
6 circulation and apparent decreased accumulation in healthy organs. *Ex vivo* analysis  
7 validated the optical imaging results and histology in tumor cryosections and showed that  
8 decationized polyplexes induced transgene expression *in vivo*.

9 The described strategy for preparing decationized polyplexes and the results reported are  
10 an important contribution to take into consideration to develop safer and more efficient  
11 nonviral gene delivery systems.

### 12 **Acknowledgement**

14 The authors gratefully acknowledge financial support by the Portuguese Foundation for Science and  
15 Technology (FCT) (grant SFRH/BD/47085/2008), by the German Academic Exchange Service  
16 (DAAD; 290084/2011-3), by the DFG (LA 2937/1-2), by the European Union (COST – Action  
17 TD1004), and by the European Research Council (ERC Starting Grant 309495: NeoNaNo).

### 18 **References**

- 19 [1] L.W. Seymour, A.J. Thrasher, Gene therapy matures in the clinic, *Nat. Biotechnol.*, 30 (2012) 588-  
20 593.  
21 [2] T. Wirth, N. Parker, S. Ylä-Herttuala, History of gene therapy, *Gene*, 525 (2013) 162-169.  
22 [3] M.A. Mintzer, E.E. Simanek, Nonviral vectors for gene delivery, *Chem. Rev.*, 109 (2008) 259-302.  
23 [4] W. Li, F.C. Szoka, Jr., Lipid-based nanoparticles for nucleic acid delivery, *Pharm. Res.*, 24 (2007)  
24 438-449.  
25 [5] J. Lutén, C.F. van Nostrum, S.C. De Smedt, W.E. Hennink, Biodegradable polymers as non-viral  
26 carriers for plasmid DNA delivery, *J. Control. Release*, 126 (2008) 97-110.  
27 [6] S.Y. Wong, J.M. Pelet, D. Putnam, Polymer systems for gene delivery—Past, present, and future,  
28 *Progress in Polymer Science*, 32 (2007) 799-837.  
29 [7] E. Mastrobattista, M.A.E.M. van der Aa, W.E. Hennink, D.J.A. Crommelin, Artificial viruses: a  
30 nanotechnological approach to gene delivery, *Nat. Rev. Drug Discov.*, 5 (2006) 115-121.  
31 [8] A. Yousefi, G. Storm, R. Schifflers, E. Mastrobattista, Trends in polymeric delivery of nucleic  
32 acids to tumors, *J. Control. Release*, 170 (2013) 209-218.  
33 [9] P. Midoux, C. Pichon, J.-J. Yaouanc, P.-A. Jaffrès, Chemical vectors for gene delivery: a current  
34 review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers, *Br.*  
35 *J. Pharmacol.*, 157 (2009) 166-178.  
36 [10] P. Chollet, M.C. Favrot, A. Hurbin, J.-L. Coll, Side-effects of a systemic injection of linear  
37 polyethylenimine–DNA complexes, *J. Gene Med.*, 4 (2002) 84-91.  
38 [11] C.M. Ward, M.L. Read, L.W. Seymour, Systemic circulation of poly(L-lysine)/DNA vectors is  
39 influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats  
40 and the implications for human gene therapy, *Blood*, 97 (2001) 2221-2229.  
41 [12] M. Ogris, S. Brunner, S. Schuller, R. Kircheis, E. Wagner, PEGylated DNA/transferrin-PEI  
42 complexes: reduced interaction with blood components, extended circulation in blood and potential  
43 for systemic gene delivery, *Gene Ther.*, 6 (1999) 595-605.  
44 [13] R. Duncan, The dawning era of polymer therapeutics, *Nat. Rev. Drug Discov.*, 2 (2003) 347-360.

- 1 [14] C.F. Jones, R.A. Campbell, A.E. Brooks, S. Assemi, S. Tadjiki, G. Thiagarajan, C. Mulcock, A.S.  
2 Weyrich, B.D. Brooks, H. Ghandehari, D.W. Grainger, Cationic PAMAM dendrimers aggressively  
3 initiate blood clot formation, *ACS Nano*, 6 (2012) 9900-9910.
- 4 [15] F.J. Verbaan, C. Oussoren, C.J. Snel, D.J.A. Crommelin, W.E. Hennink, G. Storm, Steric  
5 stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged  
6 circulation and tumor targeting in mice, *J. Gene Med.*, 6 (2004) 64-75.
- 7 [16] M. Harada-Shiba, K. Yamauchi, A. Harada, I. Takamisawa, K. Shimokado, K. Kataoka, Polyion  
8 complex micelles as vectors in gene therapy – pharmacokinetics and in vivo gene transfer, *Gene  
9 Ther.*, 9 (2002) 407-414.
- 10 [17] M.H. Lee, Z. Yang, C.W. Lim, Y.H. Lee, S. Dongbang, C. Kang, J.S. Kim, Disulfide-cleavage-  
11 triggered chemosensors and their biological applications, *Chem. Rev.*, 113 (2013) 5071-5109.
- 12 [18] F. Meng, W.E. Hennink, Z. Zhong, Reduction-sensitive polymers and bioconjugates for  
13 biomedical applications, *Biomaterials*, 30 (2009) 2180-2198.
- 14 [19] D.S. Manickam, J. Li, D.A. Putt, Q.-H. Zhou, C. Wu, L.H. Lash, D. Oupický, Effect of innate  
15 glutathione levels on activity of redox-responsive gene delivery vectors, *J. Control. Release*, 141  
16 (2010) 77-84.
- 17 [20] D. Oupický, R.C. Carlisle, L.W. Seymour, Triggered intracellular activation of disulfide crosslinked  
18 polyelectrolyte gene delivery complexes with extended systemic circulation in vivo, *Gene Ther.*, 8  
19 (2001) 713-724.
- 20 [21] Y. Vachutinsky, M. Oba, K. Miyata, S. Hiki, M.R. Kano, N. Nishiyama, H. Koyama, K. Miyazono,  
21 K. Kataoka, Antiangiogenic gene therapy of experimental pancreatic tumor by sFlt-1 plasmid DNA  
22 carried by RGD-modified crosslinked polyplex micelles, *J. Control. Release*, 149 (2011) 51-57.
- 23 [22] H.K. de Wolf, C.J. Snel, F.J. Verbaan, R.M. Schiffelers, W.E. Hennink, G. Storm, Effect of cationic  
24 carriers on the pharmacokinetics and tumor localization of nucleic acids after intravenous  
25 administration, *Int. J. Pharm.*, 331 (2007) 167-175.
- 26 [23] D. Oupický, M. Ogris, K.A. Howard, P.R. Dash, K. Ulbrich, L.W. Seymour, Importance of lateral  
27 and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation, *Mol.  
28 Ther.*, 5 (2002) 463-472.
- 29 [24] R.J. Fields, C.J. Cheng, E. Quijano, C. Weller, N. Kristofik, N. Duong, C. Hoimes, M.E. Egan,  
30 W.M. Saltzman, Surface modified poly( $\beta$  amino ester)-containing nanoparticles for plasmid DNA  
31 delivery, *J. Control. Release*, 164 (2012) 41-48.
- 32 [25] D. Fischer, Y. Li, B. Ahlemeyer, J. Kriegelstein, T. Kissel, In vitro cytotoxicity testing of polycations:  
33 influence of polymer structure on cell viability and hemolysis, *Biomaterials*, 24 (2003) 1121-1131.
- 34 [26] S.M. Moghimi, P. Symonds, J.C. Murray, A.C. Hunter, G. Debska, A. Szewczyk, A two-stage  
35 poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy, *Mol. Ther.*, 11 (2005)  
36 990-995.
- 37 [27] S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, M. Hashida, In vitro cytotoxicity of  
38 macromolecules in different cell culture systems, *J. Control. Release*, 34 (1995) 233-241.
- 39 [28] C. Loney, M. Vandenbranden, J.-M. Ruysschaert, Cationic lipids activate intracellular signaling  
40 pathways, *Adv. Drug Deliv. Rev.*, 64 (2012) 1749-1758.
- 41 [29] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethylenimine)-mediated gene delivery affects  
42 endothelial cell function and viability, *Biomaterials*, 22 (2001) 471-480.
- 43 [30] K. Masago, K. Itaka, N. Nishiyama, U.-i. Chung, K. Kataoka, Gene delivery with biocompatible  
44 cationic polymer: Pharmacogenomic analysis on cell bioactivity, *Biomaterials*, 28 (2007) 5169-5175.
- 45 [31] O.M. Merkel, A. Beyerle, B.M. Beckmann, M. Zheng, R.K. Hartmann, T. Stöger, T.H. Kissel,  
46 Polymer-related off-target effects in non-viral siRNA delivery, *Biomaterials*, 32 (2011) 2388-2398.
- 47 [32] K. Regnström, E.G.E. Ragnarsson, M. Köping-Höggård, E. Torstensson, H. Nyblom, P.  
48 Artursson, PEI – a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell  
49 response and FasL-mediated cell death in mice, *Gene Ther.*, 10 (2003) 1575-1583.
- 50 [33] L. Novo, E.V.B. van Gaal, E. Mastrobattista, C.F. van Nostrum, W.E. Hennink, Decationized  
51 crosslinked polyplexes for redox-triggered gene delivery, *J. Control. Release*, 169 (2013) 246-256.
- 52 [34] L. Novo, E. Mastrobattista, C.F. van Nostrum, W.E. Hennink, Targeted decationized polyplexes  
53 for cell specific gene delivery, *Bioconjug. Chem.*, 25 (2014) 802-812.
- 54 [35] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR  
55 effect in macromolecular therapeutics: a review, *J. Control. Release*, 65 (2000) 271-284.

- 1 [36] K. Braeckmans, K. Buyens, W. Bouquet, C. Vervaet, P. Joye, F.D. Vos, L. Plawinski, L.c.  
2 Doeuvre, E. Angles-Cano, N.N. Sanders, J. Demeester, S.C.D. Smedt, Sizing nanomatter in  
3 biological fluids by fluorescence single particle tracking, *Nano Lett.*, 10 (2010) 4435-4442.
- 4 [37] L.Y. Rizzo, S.K. Golombek, M.E. Mertens, Y. Pan, D. Laaf, J. Broda, J. Jayapaul, D. Mockel, V.  
5 Subr, W.E. Hennink, G. Storm, U. Simon, W. Jahnen-Dechent, F. Kiessling, T. Lammers, In vivo  
6 nanotoxicity testing using the zebrafish embryo assay, *J. Mater. Chem. B*, 1 (2013) 3918-3925.
- 7 [38] S. Kunjachan, F. Gremse, B. Theek, P. Koczera, R. Pola, M. Pechar, T. Etrych, K. Ulbrich, G.  
8 Storm, F. Kiessling, T. Lammers, Noninvasive optical imaging of nanomedicine biodistribution, *ACS*  
9 *Nano*, 7 (2012) 252-262.
- 10 [39] A. Funhoff, C.F. van Nostrum, A. Janssen, M. Fens, D. Crommelin, W.E. Hennink, Polymer side-  
11 chain degradation as a tool to control the destabilization of polyplexes, *Pharm. Res.*, 21 (2004) 170-  
12 176.
- 13 [40] D. Neradovic, C.F. van Nostrum, W.E. Hennink, Thermoresponsive polymeric micelles with  
14 controlled instability based on hydrolytically sensitive N-isopropylacrylamide copolymers,  
15 *Macromolecules*, 34 (2001) 7589-7591.
- 16 [41] M. Talelli, C.J.F. Rijcken, S. Oliveira, R. van der Meel, P.M.P. van Bergen en Henegouwen, T.  
17 Lammers, C.F. van Nostrum, G. Storm, W.E. Hennink, Nanobody – Shell functionalized  
18 thermosensitive core-crosslinked polymeric micelles for active drug targeting, *J. Control. Release*,  
19 151 (2011) 183-192.
- 20 [42] E.V.B. van Gaal, R. van Eijk, R.S. Oosting, R.J. Kok, W.E. Hennink, D.J.A. Crommelin, E.  
21 Mastrobattista, How to screen non-viral gene delivery systems in vitro?, *J. Control. Release*, 154  
22 (2011) 218-232.
- 23 [43] D.R. Grassetti, J.F. Murray, Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine,  
24 *Arch. Biochem. Biophys.*, 119 (1967) 41-49.
- 25 [44] A.W. York, F. Huang, C.L. McCormick, Rational design of targeted cancer therapeutics through  
26 the multiconjugation of folate and cleavable siRNA to RAFT-synthesized (HPMA-s-APMA)  
27 copolymers, *Biomacromolecules*, 11 (2010) 505-514.
- 28 [45] J.-H. Ryu, R.T. Chacko, S. Jiwanich, S. Bickerton, R.P. Babu, S. Thayumanavan, Self-cross-  
29 linked polymer nanogels: A versatile nanoscopic drug delivery platform, *J. Am. Chem. Soc.*, 132  
30 (2010) 17227-17235.
- 31 [46] G.T. Zugates, D.G. Anderson, S.R. Little, I.E.B. Lawhorn, R. Langer, Synthesis of poly( $\beta$ -amino  
32 ester)s with thiol-reactive side chains for DNA delivery, *J. Am. Chem. Soc.*, 128 (2006) 12726-12734.
- 33 [47] A. Schädlich, H. Caysa, T. Mueller, F. Tenambergen, C. Rose, A. Göpferich, J. Kuntsche, K.  
34 Mäder, Tumor accumulation of NIR fluorescent PEG-PLA nanoparticles: Impact of particle size and  
35 human xenograft tumor model, *ACS Nano*, 5 (2011) 8710-8720.
- 36 [48] V. Filipe, A. Hawe, W. Jiskoot, Critical evaluation of nanoparticle tracking analysis (NTA) by  
37 Nanosight for the measurement of nanoparticles and protein aggregates, *Pharm. Res.*, 27 (2010)  
38 796-810.
- 39 [49] C.D. Walkey, W.C.W. Chan, Understanding and controlling the interaction of nanomaterials with  
40 proteins in a physiological environment, *Chem. Soc. Rev.*, 41 (2012) 2780-2799.
- 41 [50] G.R. Dakwar, E. Zagato, J. Delanghe, S. Hobel, A. Aigner, H. Denys, K. Braeckmans, W. Ceelen,  
42 S.C. De Smedt, K. Remaut, Colloidal stability of nano-sized particles in the peritoneal fluid: Towards  
43 optimizing drug delivery systems for intraperitoneal therapy, *Acta Biomater.*, 10 (2014) 2965-2975.
- 44 [51] H. Maeda, Tumor-selective delivery of macromolecular drugs via the EPR effect: Background  
45 and future prospects, *Bioconjug. Chem.*, 21 (2010) 797-802.
- 46 [52] J. Fang, H. Nakamura, H. Maeda, The EPR effect: Unique features of tumor blood vessels for  
47 drug delivery, factors involved, and limitations and augmentation of the effect, *Adv. Drug Deliv. Rev.*,  
48 63 (2011) 136-151.
- 49 [53] T. Lammers, F. Kiessling, W.E. Hennink, G. Storm, Drug targeting to tumors: Principles, pitfalls  
50 and (pre-) clinical progress, *J. Control. Release*, 161 (2012) 175-187.
- 51 [54] Y. Lee, K. Miyata, M. Oba, T. Ishii, S. Fukushima, M. Han, H. Koyama, N. Nishiyama, K. Kataoka,  
52 Charge-conversion ternary polyplex with endosome disruption moiety: A technique for efficient and  
53 safe gene delivery, *Angew. Chem. Int. Ed. Engl.*, 47 (2008) 5163-5166.
- 54 [55] S. Kunjachan, R. Pola, F. Gremse, B. Theek, J. Ehling, D. Moeckel, B. Hermanns-Sachweh, M.  
55 Pechar, K. Ulbrich, W.E. Hennink, G. Storm, W. Lederle, F. Kiessling, T. Lammers, Passive versus

1 active tumor targeting using RGD- and NGR-modified polymeric nanomedicines, *Nano Lett.*, 14  
2 (2014) 972-981.

3 [56] F. Gremse, B. Theek, S. Kunjachan, W. Lederle, A. Pardo, S. Barth, T. Lammers, U. Naumann,  
4 F. Kiessling, Absorption reconstruction improves biodistribution assessment of fluorescent  
5 nanoprobes using hybrid fluorescence-mediated tomography, *Theranostics*, *In press* (2014).

6 [57] T. Merdan, K. Kunath, H. Petersen, U. Bakowsky, K.H. Voigt, J. Kopecek, T. Kissel, PEGylation  
7 of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a  
8 dose-dependent manner after intravenous injection into mice, *Bioconjug. Chem.*, 16 (2005) 785-792.

9 [58] Z. Ge, Q. Chen, K. Osada, X. Liu, T.A. Tockary, S. Uchida, A. Dirisala, T. Ishii, T. Nomoto, K.  
10 Toh, Y. Matsumoto, M. Oba, M.R. Kano, K. Itaka, K. Kataoka, Targeted gene delivery by polyplex  
11 micelles with crowded PEG palisade and cRGD moiety for systemic treatment of pancreatic tumors,  
12 *Biomaterials*, 35 (2014) 3416-3426.

13 [59] Y. Anraku, A. Kishimura, A. Kobayashi, M. Oba, K. Kataoka, Size-controlled long-circulating  
14 PICsome as a ruler to measure critical cut-off disposition size into normal and tumor tissues, *Chem.*  
15 *Commun.*, 47 (2011) 6054-6056.

16 [60] N. Bertrand, J.-C. Leroux, The journey of a drug-carrier in the body: An anatomo-physiological  
17 perspective, *J. Control. Release*, 161 (2012) 152-163.

18 [61] G. Storm, S.O. Belliot, T. Daemen, D.D. Lasic, Surface modification of nanoparticles to oppose  
19 uptake by the mononuclear phagocyte system, *Adv. Drug Deliv. Rev.*, 17 (1995) 31-48.

20 [62] A. Schädlich, C. Rose, J. Kuntsche, H. Caysa, T. Mueller, A. Göpferich, K. Mäder, How stealthy  
21 are PEG-PLA nanoparticles? An NIR *in vivo* study combined with detailed size measurements,  
22 *Pharm. Res.*, 28 (2011) 1995-2007.

23 [63] T. Nomoto, S. Fukushima, M. Kumagai, K. Machitani, Arnida, Y. Matsumoto, M. Oba, K. Miyata,  
24 K. Osada, N. Nishiyama, K. Kataoka, Three-layered polyplex micelle as a multifunctional nanocarrier  
25 platform for light-induced systemic gene transfer, *Nat. Commun.*, 5 (2014).

26 [64] U. Lächelt, P. Kos, F.M. Mickler, A. Herrmann, E.E. Salcher, W. Rödl, N. Badgujar, C. Bräuchle,  
27 E. Wagner, Fine-tuning of proton sponges by precise diaminoethanes and histidines in pDNA  
28 polyplexes, *Nanomedicine*, (2013).

29 [65] C.H.J. Choi, J.E. Zuckerman, P. Webster, M.E. Davis, Targeting kidney mesangium by  
30 nanoparticles of defined size, *Proc. Natl. Acad. Sci. U.S.A.*, 108 (2011) 6656-6661.

31 [66] J.E. Zuckerman, C.H.J. Choi, H. Han, M.E. Davis, Polycation-siRNA nanoparticles can  
32 disassemble at the kidney glomerular basement membrane, *Proc. Natl. Acad. Sci. U.S.A.*, 109 (2012)  
33 3137-3142.

34 [67] L. Brüllsauer, G. Valentino, S. Morinaga, K. Cam, J. Thostrup Bukrinski, M.A. Gauthier, J.-C.  
35 Leroux, Bio-reduction of redox-sensitive albumin conjugates in FcRn-expressing cells, *Angew. Chem.*  
36 *Int. Ed. Engl.*, 53 (2014) 8392-8396.

37 [68] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene  
38 transfer, *Proc. Natl. Acad. Sci. U.S.A.*, 93 (1996) 12349-12354.

39 [69] D. Vercauteren, J. Rejman, T.F. Martens, J. Demeester, S.C. De Smedt, K. Braeckmans, On the  
40 cellular processing of non-viral nanomedicines for nucleic acid delivery: Mechanisms and methods,  
41 *J. Control. Release*, 161 (2012) 566-581.

42 [70] H.K. de Wolf, J. Luten, C.J. Snel, G. Storm, W.E. Hennink, Biodegradable, cationic  
43 methacrylamide-based polymers for gene delivery to ovarian cancer cells in mice, *Mol.*  
44 *Pharmaceutics*, 5 (2008) 349-357.

45 [71] L. Wightman, R. Kircheis, V. Rössler, S. Carotta, R. Ruzicka, M. Kursa, E. Wagner, Different  
46 behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo, *J. Gene Med.*,  
47 3 (2001) 362-372.

48 [72] J. Zhou, J. Liu, C.J. Cheng, T.R. Patel, C.E. Weller, J.M. Piepmeier, Z. Jiang, W.M. Saltzman,  
49 Biodegradable poly(amine-co-ester) terpolymers for targeted gene delivery, *Nat. Mater.*, 11 (2012)  
50 82-90.

51 [73] Q. Yang, S.W. Jones, C.L. Parker, W.C. Zamboni, J.E. Bear, S.K. Lai, Evading immune cell  
52 uptake and clearance requires PEG grafting at densities substantially exceeding the minimum for  
53 brush conformation, *Mol. Pharmaceutics*, 11 (2014) 1250-1258.

54 [74] T.A. Tockary, K. Osada, Q. Chen, K. Machitani, A. Dirisala, S. Uchida, T. Nomoto, K. Toh, Y.  
55 Matsumoto, K. Itaka, K. Nitta, K. Nagayama, K. Kataoka, Tethered PEG crowdedness determining

1 shape and blood circulation profile of polyplex micelle gene carriers, *Macromolecules*, 46 (2013)  
2 6585-6592.

3 [75] C.E. Nelson, J.R. Kintzing, A. Hanna, J.M. Shannon, M.K. Gupta, C.L. Duvall, Balancing cationic  
4 and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability,  
5 blood circulation time, and bioactivity in vivo, *ACS Nano*, 7 (2013) 8870-8880.  
6  
7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

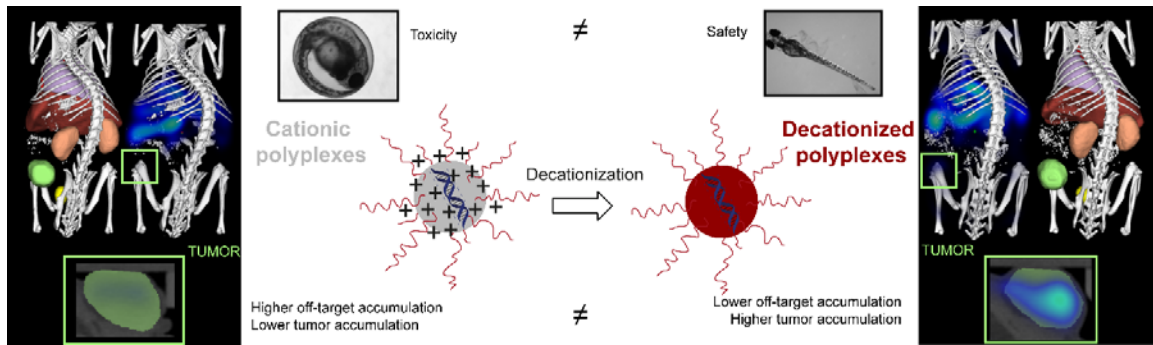
32

33

---

34 **Graphical abstract**

35



1  
2  
3  
4