



Improved histidinylated IPEI polyplexes for skeletal muscle cells transfection

Jean-Pierre Gomez, Guillaume Tresset, Chantal Pichon, Patrick Midoux

► To cite this version:

Jean-Pierre Gomez, Guillaume Tresset, Chantal Pichon, Patrick Midoux. Improved histidinylated IPEI polyplexes for skeletal muscle cells transfection. International Journal of Pharmaceutics, 2019, 559, pp.58-67. 10.1016/j.ijpharm.2019.01.003 . hal-02067297

HAL Id: hal-02067297

<https://hal.science/hal-02067297>

Submitted on 21 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Improved histidinylated IPEI polyplexes for skeletal muscle cells transfection

Jean-Pierre Gomez^{1#}, Guillaume Tresset², Chantal Pichon¹ and Patrick Midoux^{1*}

¹Centre de Biophysique Moléculaire, CNRS UPR4301, Inserm and University of Orléans, F-45071, Orléans cedex 02, France.

²Laboratoire de Physique des Solides, CNRS, Univ. Paris-Sud, Université Paris-Saclay, 91405 Orsay Cedex, France

*to whom correspondence should be addressed: Patrick.midoux@cnrs.fr

current address : University of Orléans, COST, BP 6749, F-45067 Orléans Cedex 2

Key words: DNA transfection; non-viral vector; polyethyleneimine; skeletal muscle; myoblasts

ABSTRACT

Linear Polyethylenimine (IPEI) is an efficient cationic polymer for transfecting cells, both *in vitro* and *in vivo*, but poses concerns regarding cytotoxicity. Histidinylated IPEI (His-IPEI) exhibits also high transfection efficiency but lower cytotoxicity than IPEI. For the first time, we tested polyfection efficiency of polyplexes comprising both IPEI and His-IPEI. A series of pDNA polyplexes was prepared with mixtures of IPEI and His-IPEI and the amount of each polymer within His-IPEI/IPEI polyplexes was determined by flow cytometry. We show that His-IPEI/IPEI polyplexes exhibit properties similar to IPEI polyplexes in terms of size, morphology, assembly with pDNA, and polyplex stability while His-IPEI/IPEI polyplexes exhibit properties similar to His-IPEI polyplexes in terms of buffering capacity. Compared to polyplexes consisting only of IPEI or His-IPEI, the transfection profile reveals that His-IPEI/IPEI polyplexes containing 30% to 57% IPEI strongly increase polyfection efficiency of NIH3T3 fibroblasts and murine, as well as human skeletal muscle cell lines without cytotoxicity. Importantly, improved transfection of human dystrophin deficient skeletal muscle cell lines was obtained. These results indicate that His-IPEI/IPEI polyplexes are an improved non-viral vector for efficient transfection of dystrophin deficient skeletal muscle cell lines that should be tested on animals.

1. Introduction

Gene therapy is an innovative therapeutic approach for treatment of genetic diseases, whether or not they are inheritable (Thorne et al., 2017). Among the different strategies available for gene delivery, recombinant viruses are powerful vectors that have significant clinical success in various pathologies, such as genetic immune severe combined immunodeficiency (X-SCID), Hemophilia or blinding retinal diseases, such as Leber congenital amaurosis (Dalkara and Sahel, 2014; Nienhuis et al., 2017). However several obstacles still limit their use, including immune responses against recombinant viruses, limited length for the transgene (which is particularly true for AAV, adeno-associated virus), and the expensive cost of their large-scale industrial production for human application. Chemical vectors, such as cationic polymers and cationic lipids complexed with plasmid DNA (pDNA) encoding the therapeutic gene, are a safer approach with higher pDNA loading capacity, lower immunogenicity and cheaper production, although still less efficient (Donkuru et al., 2010; Jafari et al., 2012; Junquera and Aicart, 2016; Rezaee et al., 2016; Tros de Ilarduya et al., 2010). Over the last 30 years, cationic polymers have chemically evolved but no molecular structure has emerged as a new leader (Lachelt and Wagner, 2015). Nowadays, linear polyethylenimine (LPEI) of 22 kDa is the “gold standard” cationic polymer for transferring pDNA owing to its unique high property to absorb protons in an acidic medium (Pandey and Sawant, 2016). This buffering capacity, due to the numerous secondary amino groups present on the polymer backbone, mediates a proton sponge effect in endosomes allowing escape of pDNA into the cytosol, thus limiting its lysosomal degradation and enhancing the transfection efficiency (Boussif et al., 1995; Neuberg and Kichler, 2014). Although LPEI is generally less toxic and exhibits higher transfection efficiency than its branched (bPEI) counterpart, there is still a certain amount of cytotoxicity (Castan et al., 2018; Demeneix and Behr, 2005; Gomez et al., 2013; Lungwitz et al., 2005; Lv et al., 2006). Covalent or non-covalent attachments of various molecules on LPEI

enhance its biocompatibility or allow targeting of specific cell populations. In this respect, poly(ethylene glycol) (PEG), some hydrophobic molecules, pluronic polymers, poly(N-(2-hydroxypropyl)methacrylamide) derived copolymers, RGD peptides, folic acid, transferrin, antibodies, saccharides, cyclodextrins, and chitosan/poly(lactic acid) nanoparticles have been tested (Hobel and Aigner, 2013; Kichler, 2004; Kircheis et al., 2001; Lai et al., 2014; Pandey and Sawant, 2016). The cytotoxicity was reduced and the transfection efficiency enhanced by the formation of polyplexes with two polymers, such as γ -polyglutamic acid (γ -PGA) with bPEI (Kurosaki et al., 2009), bPEI with anionic glycopolymers (Ahmed and Narain, 2013), polyethylenimine-poly(l-lysine)-poly(l-glutamic acid) (PELG) with hyaluronic acid (He et al., 2013; Tian et al., 2014; Wang et al., 2011), polyaspartamide with poly(L-lysine) (Sanjoh et al., 2010), poly (b-amino ester) (PbAE) with carboxymethyl poly (L-histidine) (CM-PLH) (Gu et al., 2014; Gu et al., 2012), bPEI with poly(methacryloyl sulfadimethoxine) (PSD)-block-PEG (PSD-b-PEG) (Sethuraman et al., 2006), PEI with chitosan (Jiang et al., 2008; Zhao et al., 2008), and b-PEI with poly(2-(dimethylamino) ethylmethacrylate (PDMAEMA) (Lo et al., 2015). Moreover, mixtures of three different polymers, such as poly-l-lysine (PLL) or Dendrigrraft poly-L-lysine (DGL) with poly-l-histidine (PLH) and γ -polyglutamic acid (γ -PGA) were used to formulate pDNA (Kodama, 2016; Kodama et al., 2015). Multilayered polyplexes formed by covering (i) DNA/protamine condensate with DNA and finally with PEI (Qu et al., 2012), (ii) pDNA/PEI or pDNA/poly (N-(8-aminooctyl)-acrylamide) (P8Am) with poly (acrylic acid) (PAA) and then with bPEI or P8Am (Ke and Young, 2010), enhanced cell uptake and endosomal escape.

In this work, we examined whether the transfection efficiency of histidinylated lPEI (His-lPEI) polyplexes would increase in the presence of another efficient cationic polymer, such as lPEI, the closest cationic polymer. His-lPEI exhibits high transfection efficiency, low cytotoxicity and significant trans-endothelial passage capacity both in normal or inflammatory

conditions (Bertrand et al., 2011; Billiet et al., 2012; Gomez et al., 2017; Gomez et al., 2013). The combination of these powerful cationic polymers has not yet been reported for the preparation of DNA polyplexes. Remarkably, we found that the combination of His-IPEI and IPEI gave more efficient polyplexes to transfect fibroblasts and skeletal muscle cells lines than those consisting only of IPEI or His-IPEI.

2. Materials and Methods

All reagents were purchased from Sigma (St. Quentin Fallavier, France) unless otherwise stated.

2.1. Plasmid

p3NFCMVLuc3NF (5556 bp) is a plasmid DNA (pDNA) encoding the firefly luciferase gene under the control of the human cytomegalovirus (CMV) promoter, with two optimized and extended NF kappaB DNA binding sequences (3NF) promoting pDNA nuclear import (Breuzard et al., 2008; Goncalves et al., 2009). Supercoiled plasmid DNA was isolated from *Escherichia coli* DH5 α TM supercompetent bacteria (Invitrogen, Cergy Pontoise, France) by alkaline lysis and purification was performed by using QIAGEN EndoFree Plasmid Mega Kit (Qiagen, Courtaboeuf, France).

2.2. Cells and cell culture

Cell lines were cultivated at 37°C in a 5% CO₂-humidified atmosphere. The mouse lung epithelial cell line (MLE-12 cells; ATCC CRL-2110; Rockville MD, USA) was grown in HITES medium comprising Dulbecco's Modified Eagle's Medium DMEM/F12 supplemented with 5 µg/ml insulin, 10 µg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β -oestradiol, 10 mM HEPES, 2 mM L-Glutamine, 2 % non-heat inactivated fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The mouse embryo fibroblast cell line (NIH3T3 cells, ATCC CRL-1658) was cultured in DMEM 1 g/l glucose supplemented with 10 % heat-inactivated FBS, 100 units/mL penicillin and 100

107 units/ml streptomycin. The mouse myoblast - C2C12 (CRL1772; ATCC), H2K 2B4 (Muses
108 et al., 2011) and H2K mdx cell lines (RRID:CVCL_8120) (Morgan et al., 1994) were cultured
109 in DMEM 4.5 g/l glucose (PAA) supplemented with 10 % heat-inactivated FBS, 1 % L-
110 Glutamax, 100 units/mL penicillin and 100 units/ml streptomycin. The normal human
111 bronchial epithelial cell line (16HBE14o-) (Cozens et al., 1994) (generous gift from Dieter
112 Gruenert, San Francisco, CA, U.S.A.) was cultured in MEM with Earle's Salts supplemented
113 with Penicillin (40 Units/ml) and Streptomycin (40 µg/ml), 2mM L-Glutamine and 10% heat-
114 inactivated FBS. Tissue culture plastic wares were coated for 20-30 min at 37°C with
115 minimum essential medium (MEM) with Earle's Salts containing fibronectin (0.01 mg/ml),
116 collagen (0.03 mg/ml) and bovine serum albumin (BSA) (0.1 mg/ml). The culture medium
117 was changed every 2 days. The human embryo kidney 293T7 cell line (kindly given by Dr L.
118 Huang) (Brisson et al., 1999) was cultured in DMEM containing 10% FBS, 2 mM L-
119 glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, 100 units/ml streptomycin, and
120 400 µg/ml Geneticin. The human epithelial ovarian carcinoma cell line (HeLa; CCL2, ATCC)
121 was cultured in MEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 1%
122 of a 100X non-essential amino acid solution, 100 units/mL penicillin and 100 units/ml
123 streptomycin. The human normal myoblasts (HSkM) and myoblasts from Duchenne muscular
124 dystrophic patients (SKM DMD), kindly provided by Dr. V. Mouly (Institut de Myologie,
125 Paris, France) (Mamchaoui et al., 2011), were cultured in Skeletal muscle cell basal medium
126 (C-23160, Promocell GmbH, Heildelberg, DE) supplemented with 50 µg/ml bovine fetuin, 10
127 µg/ml human insulin, 10 ng/ml hEGF (human epidermal growth factor), 1 ng/ml hbFGF
128 (basic human Fibroblast growth factor), 0.4 µg/ml dexamethasone, 5 % heat-inactivated FBS
129 (Promocell), 100 units/mL penicillin and 100 units/ml streptomycin. All cell lines were tested
130 free of mycoplasma by using Mycoalert™ PLUS Mycoplasma Detection kit (Lonza, Basel,
131 Switzerland).

2.3. *Polymers*

Linear polyethylenimine (IPEI; Mw of 22 kDa) and His-IPEI (IPEI modified with 16% histidine residues *per* polymer molecule; Mw of 34.5 kDa) were prepared as described by (Bertrand et al., 2011). IPEI was labelled with fluorescein. Briefly, 10 mg IPEI dissolved in 1 ml PBS was reacted at room temperature overnight with 1 mg FITC (Invitrogen) dissolved in 0.5 ml DMSO. FITC-IPEI was purified by dialysis (MW 10kDa) against water and then freeze-dried.

2.4. *Polyplexes*

Polyplexes made with IPEI, His-IPEI or their mixtures were prepared at DNA/polymer weight ratio ($\mu\text{g}/\mu\text{g}$) of 1:6. IPEI, His-IPEI or (IPEI + His-IPEI) mixture (30 μg in 30 μl of 10 mM HEPES buffer, pH 7.4) was added to pDNA (5 μg in 93 μl of 10 mM HEPES buffer, pH 7.4) and mixed by up-down pipetting. After 4 s vortex mixing, the solution was kept for 30 min at 20°C before use. For transfection, the volume was adjusted to 1 ml with the cell-specific culture medium.

2.5. *Flow cytometry analysis of fluorescent polyplexes*

The polymer composition of IPEI/His-IPEI polyplexes was determined by flow cytometry analysis by using BD LSR FORTRESSA X20 flow cytometer (Becton Dickinson). Polyplexes made with FITC-PEI/His-IPEI mixtures or FITC-IPEI/IPEI mixture were prepared as described above and their mean fluorescence intensity (MFI) was measured at 530 ± 30 nm upon excitation at 488 nm.

2.6. *Size and Zeta potential measurements*

Polyplexes made with 14 μg DNA were prepared in 1.4 ml of 10 mM HEPES buffer, pH 7.4. The size was measured at 25°C by quasi-elastic laser light scattering (QELS) with SZ-100 Analyser (Horiba Scientific, les Ulis, France), calibrated with 204 nm latex nanosphere size standards (Duke Scientific Corps Palo Alto, CA). The ζ potential was measured at 25°C by

electrophoretic mobility with SZ-100 Analyser, after calibration with DTS 1050 standard (Malvern).

2.7. *SYBR Green experiments and Dextran Sulphate competition assays*

SYBR Green nucleic acid gel stain (Invitrogen, Thermo Fisher Scientific) was added to pDNA (2 µg) in HEPES buffer (10 mM, pH 7.4) at 1:10000 dilution to obtain a pDNA/SYBR Green labelled solution. Intercalation-caused fluorescence intensity was quantified using a Victor I spectrophotometer (PerkinElmer, Courtaboeuf, France) at 520 nm emission and 488 nm excitation and expressed as a percentage of fluorescence in the absence of any polymer. In Dextran Sulphate (DS) competition assays, pDNA was first labelled with SYBR Green before polyplex formation, as described above. Dissociation of DNA complexes was then induced by adding DS (MW of 500 kDa). The fluorescence intensity was measured with a Victor I spectrophotometer and expressed as the ratio between polyplex fluorescence in the presence and absence of DS.

2.8. *CryoTEM*

Polyplexes were prepared at a final DNA concentration of 0.15 mg/mL in 10 mM HEPES, pH 7.4. A Quantifoil R2/2 carbon grid (Jena, Germany) was ionized by glow discharge and 3 µL of polyplexes was deposited onto it. The grid was then abruptly immersed in liquid ethane *via* a FEI Vitrobot cryoplunger and stored in liquid nitrogen until use. The frozen samples were introduced into a high resolution JEOL-2010 transmission electron microscope equipped with a 200-kV field emission gun via a Gatan 626 cryoholder. They were imaged at a magnification of 50,000x using a minimal dose system enabling film exposure while preserving the samples from beam damage. The images were collected with a Gatan Ultrascan 4K CCD camera with a defocus of 1 µm.

2.9. Transfection

Two days before transfection, cells were seeded in 24 well culture plates at a density of 1×10^5 to 1.5×10^5 cells/cm² depending on the cell lines used. At the time of transfection, the culture medium was discarded and the cells were incubated with 1 ml of polyplexes (5 µg/ml pDNA). After 4 h, the medium was removed and cells were cultured in their standard culture medium. The transfection efficiency was quantified after 48 h post-transfection by measuring the luciferase activity in cell lysates as follows. The culture medium was removed and cells were lysed at room temperature during 10 min in lysis buffer (1 % Triton X-100, 25 mM Tris-phosphate, 1 mM DTT, 1 mM EDTA, 15 % glycerol, 1 mM MgCl₂, pH 7.8). Luciferase substrate (Beetle luciferin, Promega, Madison, USA) was added to the lysate supernatant in the presence of 1 mM ATP. The luciferase activity was measured using a luminometer (Lumat LB 9507, Berthold, Thoiry, France) and expressed as RLU *per* mg of protein after protein normalization using the BCA colorimetric assay and bovine serum albumin.

2.10. Cytotoxicity

The cytotoxicity of polyplexes was measured after 48 h post-transfection by using the MTT assay (Mosmann, 1983). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100 µl of 5 mg/mL solution in PBS) was added *per* culture well. After 4 h incubation at 37°C, MTT converted in formazan was solubilized with acidic isopropanol. The absorbance at 570 nm was measured with Victor I spectrophotometer and cytotoxicity expressed as a percentage of absorbance relative to control cells (cells without any treatments).

2.11. Statistics

The results were expressed as the mean \pm standard deviation. The mean of each group was compared by the non-parametric Mann-Whitney statistical test. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Physicochemical characterization of IPEI / His-IPEI polyplexes

A series of polyplexes was prepared with pDNA and mixtures of IPEI and His-IPEI. The pDNA/polymer weight ratio was kept constant and equal to 1/6 (N/P of 4.88), a value which had demonstrated strong DNA condensation and high transfection efficiency in several cell lines (Bertrand et al., 2011; Goncalves et al., 2016). His-IPEI/IPEI polyplexes were prepared by varying the percentage of His-IPEI and IPEI from 0% IPEI (100% His-IPEI) to 100% IPEI (0% His-IPEI) at N/P ratios ranged from 4.88 to 9.18. First, polyplex formation was characterized by agarose gel electrophoresis shift assay. Independent of the nature of the polymer mixtures, no pDNA migrated, indicating its total complexation with the polymers and polymer mixtures (Fig. 1). The electrophoresis band in the well was more fluorescent with His-IPEI than with IPEI, revealing better pDNA condensation with the latter polymer than the former. The fluorescence intensity of wells corresponding to His-IPEI polyplexes containing IPEI was weak in line with an enhanced pDNA condensation (Fig. 1). To further characterize the polyplexes, their size was measured by DLS (Fig. 2). His-IPEI formed polyplexes of 90 ± 10 nm. The size of polyplexes with IPEI/His-IPEI mixture decreased as IPEI quantity increased in the mixture, from 83 ± 2.3 nm at N/P of 5.31 to 65.8 ± 2 nm at N/P of 8.75, a size value close to that of IPEI polyplexes (65 ± 1.4 nm). The ξ potential did not differ significantly between the various polymer mixtures and was in the range of 25 mV to 38 mV, similar to the polyplexes consisting only of IPEI (34.2 ± 8 mV) or His-IPEI (33.2 ± 0.6 mV) (Fig. 3).

3.2. Interaction strength between pDNA and His-IPEI, IPEI or His-IPEI/IPEI

As observed in agarose gel electrophoresis, pDNA condensation varied with the polymer mixture composition. Dye exclusion assay was performed to obtain more information. pDNA was stained with SYBR Green and then the fluorescence intensity was measured after

addition of IPEI or His-IPEI or their mixtures. The fluorescence intensity of IPEI/His-IPEI polyplexes decreased as the IPEI quantity increased, from 87 % at N/P of 5.31 to 95 % at N/P of 8.75, confirming an increased in pDNA condensation (Fig. 4). In contrast, fluorescence decrease in the presence of IPEI reached 98 % whereas it was only of 76 % in the presence of His-IPEI in line with agarose gel electrophoresis. These values corresponded to maximal and minimal pDNA condensation.

3.3. Polymer composition within polyplexes

At pH 7.4, IPEI and His-IPEI exhibit 102 and 85 cationic charges *per* polymer molecule, respectively (Bertrand et al., 2011). Therefore, IPEI interacts more strongly with pDNA than His-IPEI at the same concentration. Consequently, the percentages of IPEI and His-IPEI complexed with pDNA within the same polyplex cannot be the same as in the original polymer mixture. In order to determine the amount of IPEI and His-IPEI that are effectively associated with pDNA within polyplexes, the composition in polyplexes was assessed by flow cytometry analysis of polyplexes using FITC-IPEI (Fig. 5). First, the mean fluorescence intensity (MFI) of polyplexes made with FITC-IPEI/IPEI was measured as a function of the amount of FITC-IPEI and non-fluorescent IPEI. Figure 5A & 5B showed that MFI decreased linearly when the amount of non-fluorescent IPEI increased in the polymer mixture corresponding to a FITC-IPEI dilution effect by IPEI. This meant that interaction with pDNA of FITC-IPEI and non-fluorescent IPEI was identical. In contrast, the MFI of FITC-IPEI/His-IPEI polyplexes did not decrease linearly when the amount of non-fluorescent His-IPEI increased (Fig. 5B). This indicated that the interaction with pDNA of FITC-IPEI and non-fluorescent His-IPEI was different. As expected, IPEI interacts stronger than His-IPEI in line with its higher amount of cationic charges. The real amount of IPEI and His-IPEI within IPEI/His-IPEI polyplexes was determined from the FITC-IPEI/IPEI polyplex curve (Table 1). The results indicated that polyplexes contained 30% and 73% IPEI when the mixture

comprised 15% and 51% IPEI, respectively. Above 70% IPEI in the mixture, the polyplexes contained only IPEI.

3.4. Buffering capacity

Endosome destabilization by IPEI and His-IPEI is a common property of these two polymers. Thus, the buffering capacities of the HisIPEI/IPEI mixture (molar ratio of 72/28; %/%) estimated by measuring the change in pH of the polymer solution upon titration with 0.1N HCl solution was compared to that of IPEI and His-IPEI and (Fig. 6). The buffering capacity is proportional to the reciprocal slope of the titration plot over a pH range. The titration curve of IPEI over the pH range of 4 to 8 presents one phase with a reciprocal slope of 47.6 ml. In contrast, there are two phases in the titration curve of His-IPEI and the mixture. The buffering capacity of His-IPEI was 36.7 ml in the pH range of 4 to 6.3, which was slightly lower than that of IPEI. In contrast, it was higher (135 ml) than that of IPEI in the pH range of 6 to 7.4 related to the pH range of endosomes. The buffering capacity of the mixture in the pH range of 4 to 6.5 (29.5 ml) was slightly lower than that of His-IPEI in the same pH range whereas it was closer to that of His-IPEI in the pH range of endosomes (109 ml). Compared to His-IPEI, the reduced buffering capacity of the mixture in the two pH ranges was due to the presence of IPEI.

3.5. Polyplex stability

The stability of the polyplexes was tested by measuring their fluorescence intensity in the presence of DS, a polyanion of high molecular weight (500 kDa) (Fig. 7). The addition of 2 μ M DS drastically increased the intensity of SYBR Green fluorescence from a ratio of 1 to 4 for IPEI polyplexes and His-IPEI/IPEI polyplexes containing 30% and 57% IPEI, indicating that decondensation and/or release of pDNA occurred. In contrast, the fluorescence intensity of His-IPEI polyplexes did not change whatever the DS concentration, indicating a greater stability of these polyplexes compared to those consisting only of IPEI.

3.6. *Polyplex morphology*

The supramolecular structures of His-IPEI/IPEI polyplexes made with 72% of His-IPEI and 28% IPEI were investigated by cryo-transmission electron microscopy (cryo-TEM). His-IPEI/IPEI polyplexes micrographs showed the characteristic toroidal wrapping of DNA with a clear short-range organization (Fig. 8a & 8b). This organization was similar to that of polyplexes consisting only of IPEI (Fig. 8c) but contrast with that of polyplexes consisting of only His-IPEI that did not show any DNA organization, as previously reported (Fig. 8d) (Maury et al., 2014).

3.7. *Transfection efficiency of IPEI / His-IPEI polyplexes*

The transfection efficiency of IPEI/His-IPEI polyplexes was assessed in various cell lines with pDNA encoding the luciferase reporter gene. In NIH 3T3 cells, the luciferase expression upon transfection with His-IPEI polyplexes was 3.9×10^5 RLU per mg of protein (Fig. 9). The luciferase activity increased progressively upon transfection with IPEI/His-IPEI polyplexes containing an increasing amount of IPEI and reached a value 200-fold higher than that obtained with His-IPEI polyplexes. When polyplexes contained 27% His-IPEI and 73% IPEI, the luciferase activity reached a maximum of 8.5×10^7 RLU/mg of protein, a value close to that obtained with IPEI polyplexes (4.5×10^7 RLU/mg of protein).

The transfection efficiency profile of H2K mdx cells was different (Fig. 10). In the first place, His-IPEI polyplexes were more efficient than IPEI polyplexes (3.7×10^6 versus 1.1×10^5 RLU/mg protein). On the other hand, the efficiency of His-IPEI/IPEI polyplexes containing 30% IPEI and 70% His-IPEI was ~12-fold higher compared to His-IPEI polyplexes. The addition of a higher proportion of IPEI did not offer any additional benefit, since the transfection efficiency gradually decreased to reach a value (3.5×10^5 RLU/mg protein) close to that of IPEI polyplexes, in line with the fact that above 70% IPEI, polyplexes contained only IPEI. When assessed by MTT assay, IPEI polyplexes displayed high cytotoxicity ($77.5 \pm$

1 %) on H2K mdx cells in contrast to His-IPEI polyplexes (20 ± 2 %) (Fig. 11). The cytotoxicity of His-IPEI/IPEI polyplexes appeared when His-IPEI/IPEI polyplexes contained 73% IPEI and increased progressively to reach a cytotoxicity close to that of polyplexes consisting of only IPEI. The IC₅₀% of cytotoxicity of IPEI was 30, 6.5, 20 and 6.5 μ g/ml on HeLa, HEK293T7, C2C12 and Σ CFTE cells, respectively whereas it was $\gg 100$ μ g/ml for His-IPEI (Bertrand et al., 2011). Accordingly, in His-IPEI/IPEI polyplexes made with polymer mixtures containing less than 70% IPEI, a large amount of IPEI was associated with pDNA, thus contributing to a reduction in cytotoxicity. Above 70% IPEI, however, the polyplex solution contained mostly free IPEI, which was responsible for the cytotoxicity. In summary, His-IPEI/IPEI polyplexes containing less than 70% IPEI presented a benefit in terms of transfection efficiency and viability of H2K mdx cells.

Next, the transfection efficiency of His-IPEI/IPEI polyplexes was evaluated on various murine and human cell lines (Tables 2 and 3). There was no beneficial effect in the mouse lung epithelial MLE-12 cells (Table 2). Conversely, His-IPEI/IPEI polyplexes were more effective than His-IPEI and IPEI polyplexes in mouse muscular cells. Transfection increased 6.5- to 6.7-fold in normal myoblasts (C2C12 cells) and immortal satellite cell-derived from myofibres (H2K 2B4 cells) with His-IPEI/IPEI polyplexes containing 30 to 57 % IPEI and 11.6-fold in dystrophic cells (H2K mdx cells). A similar transfection profile was observed in human cell lines (Table 3). There was no benefit for transfection from His-IPEI/IPEI polyplexes in non-muscular cells, such as the epithelial ovarian carcinoma cells (HeLa cells) and the human embryo kidney cells (293T7 cells). A weak benefit (1.24-fold) was observed in human bronchial epithelial cells (16HBE14o- cells) in the presence of 30% IPEI. However, His-IPEI/IPEI polyplexes containing 30 % IPEI enhanced ~3-fold the transfection of normal (SKMH cells) and dystrophic (SKM DMD cells) myoblasts.

4. Discussion

In the present study, the transfection efficiency of polyplexes consisting of both His-IPEI and IPEI demonstrated superiority in NIH3T3 cells and skeletal muscle cells in comparison to polyplexes consisting only of IPEI or His-IPEI. The presence of IPEI within His-IPEI polyplexes increased pDNA condensation in line with its higher amount of cationic charges providing stronger interaction with pDNA rather than His-IPEI. This higher condensation was observed by agarose gel electrophoresis showing lower dye inclusion in pDNA and by dynamic light scattering showing hydrodynamic size reduction of His-IPEI polyplexes in the presence of IPEI. The ζ potential of His-IPEI/IPEI polyplexes was not modified and was in the range of 24.8 mV to 38.4 mV, close to that of polyplexes consisting only of His-IPEI or IPEI. The presence of IPEI within His-IPEI polyplexes decreased polyplex stability suggesting that the supramolecular assembly was different from that of His-IPEI polyplexes. DNA compaction varies with the type of cationic polymers, the number of cationic charges *per* polymer molecule, and the nature of the chemical group bearing the cationic charges, leading to structurally different supramolecular assemblies with pDNA. High-resolution cryo-TEM images previously showed that histidinylation of IPEI had a crucial influence on polyplex morphology. His-IPEI polyplexes exhibited a spherical shape and amorphous character indicating that the binding energy of partners was weaker than in the ordered globular structures of IPEI polyplexes (Maury et al., 2014). The same results were obtained by Cryo-TEM analyses in the present study. Here, Cryo-TEM of polyplexes consisting of 72% His-IPEI and 28% IPEI showed that their well-ordered structure was similar to that of IPEI polyplexes. DNA condensation by cationic polymers is reversible in the presence of high molecular weight polyanions (Sun et al., 2011a; Sun et al., 2011b). When tested, DS of high molecular weight allowed dissociation of IPEI polyplexes and His-IPEI/IPEI polyplexes but not His-IPEI polyplexes. While DNA compaction or packaging is a relatively slow process,

357 DNA decondensation is a rapid process occurring in a few seconds upon its initialization
358 (Widom and Baldwin, 1980). Therefore, decondensation of the well-ordered structure
359 required lower amounts of polyanion than in unordered structures. Compared to polyplexes
360 consisting only of His-IPEI, the presence of IPEI within His-IPEI polyplexes decreased their
361 stability in the presence of DS, suggesting that IPEI could control the assembly of polyplexes
362 containing both polymers. This confirms the Cryo-TEM images, which show organizational
363 similarity between His-IPEI/IPEI/DNA complexes and IPEI/DNA complexes. Here, the
364 polyplexes were formed by mixing pDNA with the mixture of two polymers at different
365 percentages. Interaction of pDNA with IPEI is greater compared to His-IPEI due to its higher
366 number of cationic charges. Flow cytometry allowed the determination of the amount of each
367 polymer really associated with the polyplexes. This determination was essential and
368 confirmed that competition occurred between the two polymers. The preparation of His-
369 IPEI/IPEI polyplexes was different to that of multi-layered polyplexes, where the polyplexes
370 were formed with a first polymer before addition of the second polymer interacting with the
371 polyplex surface. Here, the interactions of His-IPEI and IPEI with the nucleic acid occur at the
372 same time and form an ordered assembly probably without polymer multilayers.

373 The impact of His-IPEI/IPEI polyplexes on transfection was assessed in various murine and
374 human cell lines. Transfection was performed in NIH3T3 mouse fibroblasts because a
375 superior transgene expression (~10-fold) was reported in this cell line transfected with
376 polyplexes made with a PDMAEMA/PEI mixture at a ratio 1:9 compared to polyplexes
377 containing PDMAEMA or PEI (Lo et al., 2015). IPEI/His-IPEI polyplexes revealed also better
378 transfection efficiency (up to 200-fold) than polyplexes consisting only of IPEI or His-IPEI in
379 NIH3T3 mouse fibroblasts. The transfection was one order of magnitude higher than with
380 PDMAEMA/PEI polyplexes. Transfections of bronchial epithelial cells and skeletal muscle
381 cells were evaluated because these cell lines are relevant in the Cystic Fibrosis and Duchenne

Muscular Dystrophy gene therapy field. His-IPEI/IPEI polyplexes improved the transfection of murine or human skeletal muscle cell lines by 4.3- to 11.5- fold in C2C12 and H2K mdx cell lines and by 3-fold in SKM and SKM DMD cell lines. These results are interesting since proliferating myoblasts are usually difficult to transfect with non-viral systems, such as calcium phosphate precipitation, cationic lipid reagents and many cationic polymers. Transfection efficiency typically varies between 10 and 30% (Jackson et al., 2013). Improvements of the transgene expression in C2C12 cells have been reported with polyplexes made with hyperbranched poly(ester amine)s (PEAs) (Wang et al., 2012) and with PEI and the amphipathic peptide KALA (Min et al., 2006). We did not observe significant improvement in the other cell lines including bronchial epithelial cells. At this stage, it is unclear why His-IPEI/IPEI polyplexes transfected fibroblasts and skeletal muscle cells more efficiently than the other cell lines. The fact that fibroblasts are convertible into cells of the skeletal muscle lineage (Bar-Nur et al., 2018) and C2C12 or NIH/3T3 cells can differentiate into chondrogenic and osteogenic lineages in vitro (Li et al., 2005) could suggest common cell behavior.

5. Conclusion

The combination of the His-IPEI and l-PEI polymers for DNA polyplexes preparation has not previously been reported. Remarkably, we found that some lPEI/His-IPEI combinations lead to performant and non-cytotoxic polyplexes for fibroblast and skeletal muscle cell transfection. His-IPEI/IPEI polyplexes exhibited properties similar to lPEI polyplexes in terms of size, morphology, assembly with pDNA and stability, while His-IPEI/IPEI polyplexes exhibited properties similar to His-IPEI polyplexes in terms of buffering capacity. At this stage, it is unclear why His-IPEI/IPEI polyplexes transfected fibroblasts and skeletal muscle cells more efficiently than the other cell lines. Deeper investigations are required to determine

which specific properties of His-IPEI/IPEI polyplexes contribute to the improved effect observed with these cells. The evaluation of other cationic polymers could determine whether this effect is restricted to the His-IPEI/PEI couple. IPEI/His-IPEI polyplexes that showed particular efficacy in dystrophic skeletal muscle cells should be tested to transfer the dystrophin gene in mdx animals.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Virginie Malard for buffering capacity measurements, David Gosset and the “Cytometry and Cell Imaging” P@CYFIC platform” at CBM. We thank Jéril Degrouard for his technical assistance with the electron microscope. The electron microscopy imaging was supported by “Investissements d’Avenir” LabEx PALM (ANR-10-LABX-0039-PALM). We are grateful to Frances Westall (CBM) for editing the manuscript. This work was supported by grants from ‘Association Française contre les Myopathies’ (Strategic project 2009, #15628AFM (AFM, Evry, France).

References

- Ahmed, M., Narain, R., 2013. Cell line dependent uptake and transfection efficiencies of PEI-anionic glycopolymer systems. *Biomaterials* 34, 4368-4376.
- Bar-Nur, O., Gerli, M.F.M., Di Stefano, B., Almada, A.E., Galvin, A., Coffey, A., Huebner, A.J., Feige, P., Verheul, C., Cheung, P., Payzin-Dogru, D., Paisant, S., Anselmo, A., Sadreyev, R.I., Ott, H.C., Tajbakhsh, S., Rudnicki, M.A., Wagers, A.J., Hochedlinger, K., 2018. Direct Reprogramming of Mouse Fibroblasts into Functional Skeletal Muscle Progenitors. *Stem Cell Reports* 10, 1505-1521.
- Bertrand, E., Goncalves, C., Billiet, L., Gomez, J.P., Pichon, C., Cheradame, H., Midoux, P., Guegan, P., 2011. Histidinylated linear PEI: a new efficient non-toxic polymer for gene transfer. *Chem Commun (Camb)* 47, 12547-12549.
- Billiet, L., Gomez, J.P., Berchel, M., Jaffres, P.A., Le Gall, T., Montier, T., Bertrand, E., Cheradame, H., Guegan, P., Mevel, M., Pitard, B., Benvegna, T., Lehn, P., Pichon, C., Midoux, P., 2012. Gene transfer by chemical vectors, and endocytosis routes of polyplexes, lipoplexes and lipopolyplexes in a myoblast cell line. *Biomaterials* 33, 2980-2990.
- Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 92, 7297-7301.
- Breuzard, G., Tertilt, M., Goncalves, C., Cheradame, H., Guegan, P., Pichon, C., Midoux, P., 2008. Nuclear delivery of NFkappaB-assisted DNA/polymer complexes: plasmid DNA quantitation by confocal laser scanning microscopy and evidence of nuclear polyplexes by FRET imaging. *Nucleic Acids Res* 36, e71.
- Brisson, M., He, Y., Li, S., Yang, J.P., Huang, L., 1999. A novel T7 RNA polymerase autogene for efficient cytoplasmic expression of target genes. *Gene Ther* 6, 263-270.

- 457 • Castan, L., Jose da Silva, C., Ferreira Molina, E., Alves Dos Santos, R., 2018.

458 Comparative study of cytotoxicity and genotoxicity of commercial Jeffamines(R) and

459 polyethylenimine in CHO-K1 cells. *J Biomed Mater Res B Appl Biomater* 106, 742-

460 750.
- 461 • Cozens, A.L., Yezzi, M.J., Kunzelmann, K., Ohnishi, T., Chin, L., Eng, K., Finkbeiner,

462 W.E., Widdicombe, J.H., Gruenert, D.C., 1994. CFTR expression and chloride

463 secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell*

464 *Mol Biol* 10, 38-47.
- 465 • Dalkara, D., Sahel, J.A., 2014. Gene therapy for inherited retinal degenerations. *C R*

466 *Biol* 337, 185-192.
- 467 • Demeneix, B., Behr, J.P., 2005. Polyethylenimine (PEI). *Adv Genet* 53, 217-230.
- 468 • Donkuru, M., Badea, I., Wettig, S., Verrall, R., Elsayahy, M., Foldvari, M., 2010.

469 Advancing nonviral gene delivery: lipid- and surfactant-based nanoparticle design

470 strategies. *Nanomedicine (Lond)* 5, 1103-1127.
- 471 • Gomez, J.P., Goncalves, C., Pichon, C., Midoux, P., 2017. Effect of IL-1beta, TNF-

472 alpha and IGF-1 on trans-endothelial passage of synthetic vectors through an in vitro

473 vascular endothelial barrier of striated muscle. *Gene Ther* 24, 416-424.
- 474 • Gomez, J.P., Pichon, C., Midoux, P., 2013. Ability of plasmid DNA complexed with

475 histidinylated IPEI and IPEI to cross in vitro lung and muscle vascular endothelial

476 barriers. *Gene* 525, 182-190.
- 477 • Goncalves, C., Akhter, S., Pichon, C., Midoux, P., 2016. Intracellular Availability of

478 pDNA and mRNA after Transfection: A Comparative Study among Polyplexes,

479 Lipoplexes, and Lipopolyplexes. *Mol Pharm* 13, 3153-3163.

- 480 • Goncalves, C., Ardourel, M.Y., Decoville, M., Breuzard, G., Midoux, P., Hartmann,

481 B., Pichon, C., 2009. An optimized extended DNA kappa B site that enhances plasmid

482 DNA nuclear import and gene expression. *J Gene Med* 11, 401-411.
- 483 • Gu, J., Chen, X., Xin, H., Fang, X., Sha, X., 2014. Serum-resistant complex

484 nanoparticles functionalized with imidazole-rich polypeptide for gene delivery to

485 pulmonary metastatic melanoma. *Int J Pharm* 461, 559-569.
- 486 • Gu, J., Wang, X., Jiang, X., Chen, Y., Chen, L., Fang, X., Sha, X., 2012. Self-

487 assembled carboxymethyl poly (L-histidine) coated poly (beta-amino ester)/DNA

488 complexes for gene transfection. *Biomaterials* 33, 644-658.
- 489 • He, Y., Cheng, G., Xie, L., Nie, Y., He, B., Gu, Z., 2013. Polyethyleneimine/DNA

490 polyplexes with reduction-sensitive hyaluronic acid derivatives shielding for targeted

491 gene delivery. *Biomaterials* 34, 1235-1245.
- 492 • Hobel, S., Aigner, A., 2013. Polyethylenimines for siRNA and miRNA delivery in

493 vivo. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 5, 484-501.
- 494 • Jackson, M.F., Hoversten, K.E., Powers, J.M., Trobridge, G.D., Rodgers, B.D., 2013.

495 Genetic manipulation of myoblasts and a novel primary myosatellite cell culture

496 system: comparing and optimizing approaches. *FEBS J* 280, 827-839.
- 497 • Jafari, M., Soltani, M., Naahidi, S., Karunaratne, D.N., Chen, P., 2012. Nonviral

498 approach for targeted nucleic acid delivery. *Curr Med Chem* 19, 197-208.
- 499 • Jiang, H.L., Kim, T.H., Kim, Y.K., Park, I.Y., Cho, M.H., Cho, C.S., 2008. Efficient

500 gene delivery using chitosan-polyethylenimine hybrid systems. *Biomed Mater* 3,

501 025013.
- 502 • Junquera, E., Aicart, E., 2016. Recent progress in gene therapy to deliver nucleic acids

503 with multivalent cationic vectors. *Adv Colloid Interface Sci* 233, 161-175.

- 504 • Ke, J.H., Young, T.H., 2010. Multilayered polyplexes with the endosomal buffering

505 polycation in the core and the cell uptake-favorable polycation in the outer layer for

506 enhanced gene delivery. *Biomaterials* 31, 9366-9372.
- 507 • Kichler, A., 2004. Gene transfer with modified polyethylenimines. *J Gene Med* 6

508 Suppl 1, S3-10.
- 509 • Kircheis, R., Wightman, L., Wagner, E., 2001. Design and gene delivery activity of

510 modified polyethylenimines. *Adv Drug Deliv Rev* 53, 341-358.
- 511 • Kodama, Y., 2016. Development of a Multi-functional Nano-device for Safe and

512 Effective Gene Delivery to Target Organs. *Yakugaku Zasshi* 136, 1533-1539.
- 513 • Kodama, Y., Yatsugi, Y., Kitahara, T., Kurosaki, T., Egashira, K., Nakashima, M.,

514 Muro, T., Nakagawa, H., Higuchi, N., Nakamura, T., Sasaki, H., 2015. Quaternary

515 complexes modified from pDNA and poly-l-lysine complexes to enhance pH-

516 buffering effect and suppress cytotoxicity. *J Pharm Sci* 104, 1470-1477.
- 517 • Kurosaki, T., Kitahara, T., Fumoto, S., Nishida, K., Nakamura, J., Niidome, T.,

518 Kodama, Y., Nakagawa, H., To, H., Sasaki, H., 2009. Ternary complexes of pDNA,

519 polyethylenimine, and gamma-polyglutamic acid for gene delivery systems.

520 *Biomaterials* 30, 2846-2853.
- 521 • Lachelt, U., Wagner, E., 2015. Nucleic Acid Therapeutics Using Polyplexes: A

522 Journey of 50 Years (and Beyond). *Chem Rev* 115, 11043-11078.
- 523 • Lai, W.F., Green, D.W., Jung, H.S., 2014. Linear poly(ethylenimine) cross-linked by

524 methyl-beta-cyclodextrin for gene delivery. *Curr Gene Ther* 14, 258-268.
- 525 • Li, G., Peng, H., Corsi, K., Usas, A., Olshanski, A., Huard, J., 2005. Differential effect

526 of BMP4 on NIH/3T3 and C2C12 cells: implications for endochondral bone

527 formation. *J Bone Miner Res* 20, 1611-1623.

- 528 • Lo, C.W., Liao, W.H., Wu, C.H., Lee, J.L., Sun, M.K., Yang, H.S., Tsai, W.B.,
529 Chang, Y., Chen, W.S., 2015. Synergistic Effect of PEI and PDMAEMA on
530 Transgene Expression in Vitro. *Langmuir* 31, 6130-6136.
- 531 • Lungwitz, U., Breunig, M., Blunk, T., Gopferich, A., 2005. Polyethylenimine-based
532 non-viral gene delivery systems. *Eur J Pharm Biopharm* 60, 247-266.
- 533 • Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and
534 cationic polymers in gene delivery. *J Control Release* 114, 100-109.
- 535 • Mamchaoui, K., Trollet, C., Bigot, A., Negroni, E., Chaouch, S., Wolff, A., Kandalla,
536 P.K., Marie, S., Di Santo, J., St Guily, J.L., Muntoni, F., Kim, J., Philippi, S., Spuler,
537 S., Levy, N., Blumen, S.C., Voit, T., Wright, W.E., Aamiri, A., Butler-Browne, G.,
538 Mouly, V., 2011. Immortalized pathological human myoblasts: towards a universal
539 tool for the study of neuromuscular disorders. *Skelet Muscle* 1, 34.
- 540 • Maury, B., Goncalves, C., Tresset, G., Zeghal, M., Cheradame, H., Guegan, P.,
541 Pichon, C., Midoux, P., 2014. Influence of pDNA availability on transfection
542 efficiency of polyplexes in non-proliferative cells. *Biomaterials* 35, 5977-5985.
- 543 • Min, S.H., Lee, D.C., Lim, M.J., Park, H.S., Kim, D.M., Cho, C.W., Yoon, D.Y.,
544 Yeom, Y.I., 2006. A composite gene delivery system consisting of polyethylenimine
545 and an amphipathic peptide KALA. *J Gene Med* 8, 1425-1434.
- 546 • Morgan, J.E., Beauchamp, J.R., Pagel, C.N., Peckham, M., Ataliotis, P., Jat, P.S.,
547 Noble, M.D., Farmer, K., Partridge, T.A., 1994. Myogenic cell lines derived from
548 transgenic mice carrying a thermolabile T antigen: a model system for the derivation
549 of tissue-specific and mutation-specific cell lines. *Dev Biol* 162, 486-498.
- 550 • Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival:
551 application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63.

- 552 • Muses, S., Morgan, J.E., Wells, D.J., 2011. A new extensively characterised

553 conditionally immortal muscle cell-line for investigating therapeutic strategies in

554 muscular dystrophies. PLoS One 6, e24826.
- 555 • Neuberger, P., Kichler, A., 2014. Recent developments in nucleic acid delivery with

556 polyethylenimines. Adv Genet 88, 263-288.
- 557 • Nienhuis, A.W., Nathwani, A.C., Davidoff, A.M., 2017. Gene Therapy for

558 Hemophilia. Mol Ther 25, 1163-1167.
- 559 • Pandey, A.P., Sawant, K.K., 2016. Polyethylenimine: A versatile, multifunctional non-

560 viral vector for nucleic acid delivery. Mater Sci Eng C Mater Biol Appl 68, 904-918.
- 561 • Qu, X., Li, P., Liu, D., Liu, C., Zhang, N., 2012. Enhanced gene transfer with

562 multilayered polyplexes assembled with layer-by-layer technique. IET

563 Nanobiotechnol 6, 122-128.
- 564 • Rezaee, M., Oskuee, R.K., Nassirli, H., Malaekheh-Nikouei, B., 2016. Progress in the

565 development of lipopolyplexes as efficient non-viral gene delivery systems. J Control

566 Release 236, 1-14.
- 567 • Sanjoh, M., Hiki, S., Lee, Y., Oba, M., Miyata, K., Ishii, T., Kataoka, K., 2010.

568 pDNA/poly(L-lysine) Polyplexes Functionalized with a pH-Sensitive Charge-

569 Conversional Poly(aspartamide) Derivative for Controlled Gene Delivery to Human

570 Umbilical Vein Endothelial Cells. Macromol Rapid Commun 31, 1181-1186.
- 571 • Sethuraman, V.A., Na, K., Bae, Y.H., 2006. pH-responsive sulfonamide/PEI system

572 for tumor specific gene delivery: an in vitro study. Biomacromolecules 7, 64-70.
- 573 • Sun, C., Tang, T., Uludag, H., 2011a. Molecular dynamics simulations of PEI

574 mediated DNA aggregation. Biomacromolecules 12, 3698-3707.

- 575 • Sun, C., Tang, T., Uludag, H., Cuervo, J.E., 2011b. Molecular dynamics simulations

576 of DNA/PEI complexes: effect of PEI branching and protonation state. *Biophys J* 100,

577 2754-2763.
- 578 • Thorne, B., Takeya, R., Vitelli, F., Swanson, X., 2017. Gene Therapy. *Adv Biochem*

579 *Eng Biotechnol*, 1-49.
- 580 • Tian, H., Guo, Z., Lin, L., Jiao, Z., Chen, J., Gao, S., Zhu, X., Chen, X., 2014. pH-

581 responsive zwitterionic copolypeptides as charge conversional shielding system for

582 gene carriers. *J Control Release* 174, 117-125.
- 583 • Tros de Ilarduya, C., Sun, Y., Duzgunes, N., 2010. Gene delivery by lipoplexes and

584 polyplexes. *Eur J Pharm Sci* 40, 159-170.
- 585 • Wang, M., Tucker, J.D., Lu, P., Wu, B., Cloer, C., Lu, Q., 2012. Tris[2-

586 (acryloyloxy)ethyl]isocyanurate cross-linked low-molecular-weight polyethylenimine

587 as gene delivery carriers in cell culture and dystrophic mdx mice. *Bioconjug Chem* 23,

588 837-845.
- 589 • Wang, Y., Xu, Z., Zhang, R., Li, W., Yang, L., Hu, Q., 2011. A facile approach to

590 construct hyaluronic acid shielding polyplexes with improved stability and reduced

591 cytotoxicity. *Colloids Surf B Biointerfaces* 84, 259-266.
- 592 • Widom, J., Baldwin, R.L., 1980. Cation-induced toroidal condensation of DNA

593 studies with $\text{Co}^{3+}(\text{NH}_3)_6$. *J Mol Biol* 144, 431-453.
- 594 • Zhao, Q.Q., Chen, J.L., Han, M., Liang, W.Q., Tabata, Y., Gao, J.Q., 2008.

595 Combination of poly(ethylenimine) and chitosan induces high gene transfection

596 efficiency and low cytotoxicity. *J Biosci Bioeng* 105, 65-68.

LEGENDS OF FIGURES

Figure 1: Agarose gel electrophoresis shift assay of pDNA complexed with His-IPEI, IPEI alone or IPEI/HisIPEI at different molar ratios. N/P stands for the pDNA/polymer charge (negative to positive) ratios assuming that at pH 7.4, IPEI and His-IPEI exhibited 102 and 85 cationic charges per polymer molecule, respectively (Bertrand et al., 2011). pDNA free or complexed with the polymers was analyzed on a 0.6% agarose gel. Gel running buffer was TAE (40 mM Tris-acetate, 1 mM EDTA) and pDNA gel was stained with ethidium bromide (EtBr) at a concentration of 0.5 µg/ml.

Figure 2: Size of pDNA complexed with His-IPEI, IPEI or IPEI/HisIPEI at different molar ratios. Dashed line is the fit curve between size measurements values and IPEI/His-IPEI molar ratio. (▲) is N/P values as a function of IPEI/HisIPEI molar ratio. Values are means ± SD.

Figure 3: ζ potential of pDNA complexed with His-IPEI, IPEI or IPEI/HisIPEI at different molar ratios. (▲) is N/P values as a function of IPEI/HisIPEI molar ratio. Values are means ± SD.

Figure 4: Dye exclusion assay. The fluorescence of 2 µg pDNA in the presence of SYBR Green in HEPES buffer was measured in the presence of various quantities of His-IPEI, IPEI or IPEI/HisIPEI. Fluorescence intensity is expressed as the percent of fluorescence in the absence of any polymer. (▲) is N/P values as a function of IPEI/HisIPEI molar ratio. Values are means ± SD.

Figure 5: Polymers composition of polyplexes. A series of FITC-IPEI/IPEI polyplexes and FITC-IPEI/His-IPEI polyplexes was prepared as a function of the polymers molar ratio in the mixture. The mean fluorescence intensity (MFI) of polyplexes was measured by flow cytometry (488 nm excitation; 520 nm emission). (A) Fluorescence histograms of FITC-IPEI/IPEI polyplexes containing (red) 70%, (yellow) 50%, (blue) 30% and (green) 0% non-fluorescent IPEI. (B) Variation of the MFI of (●) FITC-IPEI/IPEI and (▲) FITC-IPEI/His-IPEI polyplexes expressed as MFI/MFI_{100} where MFI_{100} corresponds to polyplexes containing 100% of FITC-IPEI. Dashed line corresponds to the linear regression fit of MFI of FITC-IPEI/IPEI polyplexes.

Figure 6: Titration of polymer solutions with 0.1 N HCl. Solutions of 3 mg/ml (□) IPEI, (●) His-IPEI or (▲) the HisIPEI/IPEI mixture (molar ratio of 72/28; %/%) were adjusted to pH = 12 with 0.1 N NaOH, then 20 ml aliquots of 0.1 N HCl were progressively added and the pH measured.

Figure 7: Polyplexes stability. pDNA in the presence of SYBR Green (1 : 10000) in 10 mM HEPES buffer, pH 7.4 was complexed at DNA/polymer molar ratio of 1/6 either with (□) IPEI, (●) His-IPEI or IPEI/His-IPEI mixture containing (Δ) 30% or (▲) 57% IPEI. Then, the fluorescence intensity was measured in the absence and in the presence of various amounts of Dextran Sulphate (DS) of 500 kDa molecular weight. The fluorescence intensity was expressed as the ratio between fluorescence of polyplexes in the presence to that in the absence of DS. Values are means \pm SD.

Figure 8: Cryo-TEM micrographs of (a and b) His-IPEI/IPEI, (c) IPEI and (d) His-IPEI polyplexes. Scale bar is 50 nm.

648

649 **Figure 9: Transfection efficiency of NIH3T3 cells.** The cells were transfected with lPEI-,
650 HislPEI- or lPEI/HislPEI polyplexes containing pDNA encoding the luciferase gene (5
651 $\mu\text{g/ml}$). The luciferase activity in cell lysates was measured 48 h post-transfection and
652 expressed as relative light units (RLU) *per* mg of protein. (\blacktriangle) is N/P values as a function of
653 lPEI/His-lPEI molar ratio. Values are means \pm SD. (*: $p < 0.05$ *versus* lPEI; \diamond : $p < 0.05$ *versus*
654 His-lPEI).

655

656 **Figure 10: Transfection efficiency of H2K mdx cells.** The cells were transfected with lPEI-
657 , His-lPEI- or lPEI/HislPEI polyplexes containing pDNA encoding the luciferase gene (5
658 $\mu\text{g/ml}$). The luciferase activity in cell lysates was measured 48 h post-transfection and
659 expressed as relative light units (RLU) *per* mg of protein. (\blacktriangle) is N/P values as a function of
660 lPEI/His-lPEI molar ratio. Values are means \pm SD. (*: $p < 0.05$ *versus* lPEI).

661

662 **Figure 11: Cytotoxicity in H2K mdx cells.** The cells were transfected with lPEI-, HislPEI-
663 or lPEI/His-lPEI polyplexes as in Fig. 10. The cytotoxicity was measured 48 h post-
664 transfection using MTT assay and the percentage of cytotoxicity is expressed relatively to
665 non-transfected cells. (\blacktriangle) is N/P values as a function of lPEI/His-lPEI molar ratio. Values are
666 means \pm SD. (**: $p < 0.01$ *versus* lPEI; $\diamond\diamond$: $p < 0.01$ *versus* His-lPEI).

667

668

669

670

671

672

673

674

675

Table 1: Molar quantity of His-IPEI and IPEI associated to polyplexes (%)

in the mixture		within polyplexes	
His-IPEI	IPEI	His-IPEI	IPEI
100	0	100	0
85	15	70 ± 4	30 ± 2
72	28	43 ± 2	57 ± 3
59	41	33 ± 2	67 ± 4
49	51	27 ± 2	73 ± 3
39	61	15 ± 1	85 ± 5
30	70	3 ± 0.5	97 ± 3
22	78	0	100
14	86	0	100
7	93	0	100
0	100	0	100

676

677

678

679

680

681

682

683

Table 2 : His-IPEI/PEI polyplexes improvement on transfection of mouse cell lines							
Polyplexes			Cell lines				
His-IPEI	IPEI	RLU His-IPEI/PEI polyplexes /RLU His-IPEI polyplexes					
(%)	(%)	N/P	MLE-12	NiH3T3	C2C12	H2K 2B4	H2K mdx
100	0	4.88	1	1	1	1	1
70	30	5.31	0.6	5.5	6.5	6.7	11.5
43	57	5.74	0.1	51	7.0	6.8	7.6
33	67	6.32	0.4	130	4.3	6.4	4.2
27	73	6.6	0.2	212	3.1	8.5	1.9
15	85	7.08	0.15	208	3.2	5.1	0.8
3	97	7.45	0.1	249	4	4.4	0.5
0	100	9.18	0.01	114	3.1	1.0	0.03

Data shown corresponds to representative experiment performed in triplicate and reproduced at least three times. RLU is the relative light unit per mg of protein

Table 3 : His-IPEI/PEI polyplexes improvement on transfection of human cell lines

Polyplexes		Cell lines					
His-IPEI	IPEI	RLU His-IPEI/PEI polyplexes /RLU His-IPEI polyplexes					
(%)	(%)	N/P	16HBE14o-	293T7	HeLa	SKMH	SKM DMD
100	0	4.88	1	1	1	1	1
70	30	5.31	1.2	0.9	0.6	3.2	2.9
43	57	5.74	0.70	0.6	0.15	0.7	0.1
33	67	6.32	0.5	0.8	0.25	0.15	0.02
27	73	6.6	0.5	0.5	0.40	0.1	0.1
15	85	7.08	0.6	0.3	0.46	0.2	0.04
3	97	7.45	0.5	0.2	0.71	0.1	0.01
0	100	9.18	0.2	0.1	0.42	0.001	-

Data shown corresponds to representative experiment performed in triplicate and reproduced at least three times. . RLU is the relative light unit per mg of protein

0	0	1107	996	885	775	664	553	443	350	221	111	His-IPEI/DNA (mol)
0	1735	0	174	347	549	694	868	1041	1215	1388	1562	IPEI/DNA (mol)
	0/100	100/0	85/15	72/28	59/41	49/51	39/61	30/70	22/78	14/86	7/93	His-IPEI/IPEI molar ratio (%)
	9.18	4.88	5.31	5.74	6.32	6.6	7.08	7.45	8.00	8.31	8.75	N/P

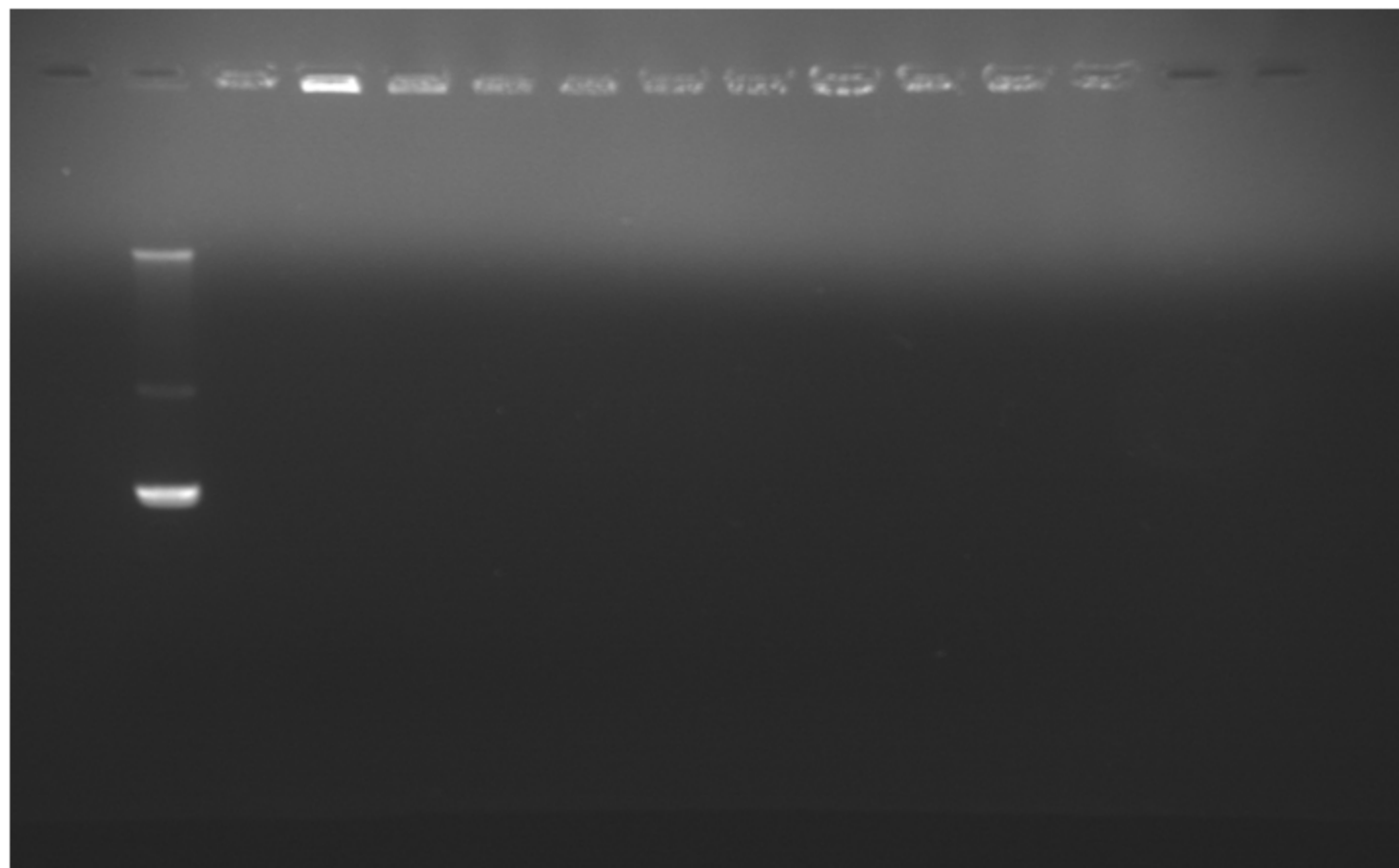


Fig 1

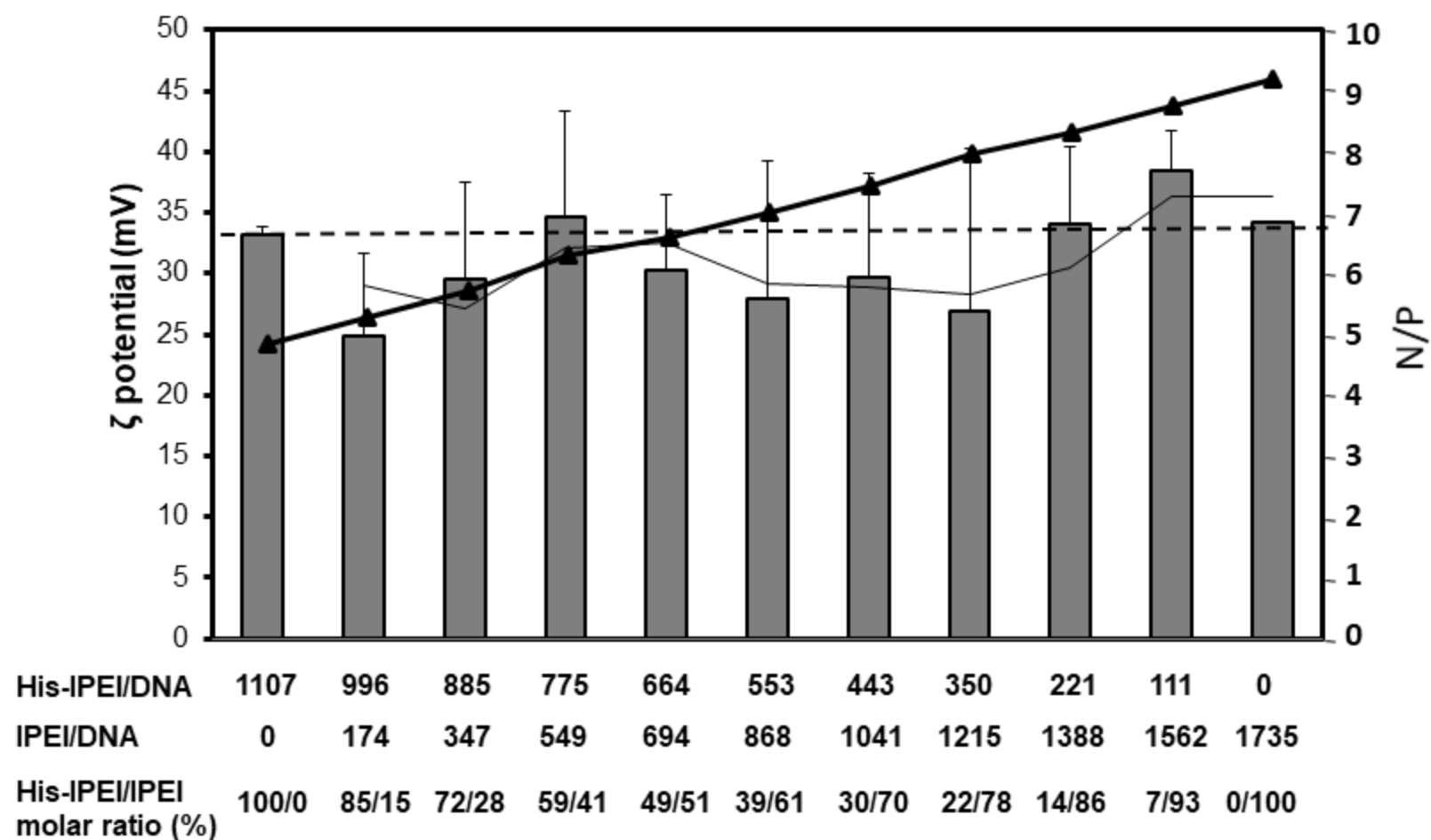


Fig 3

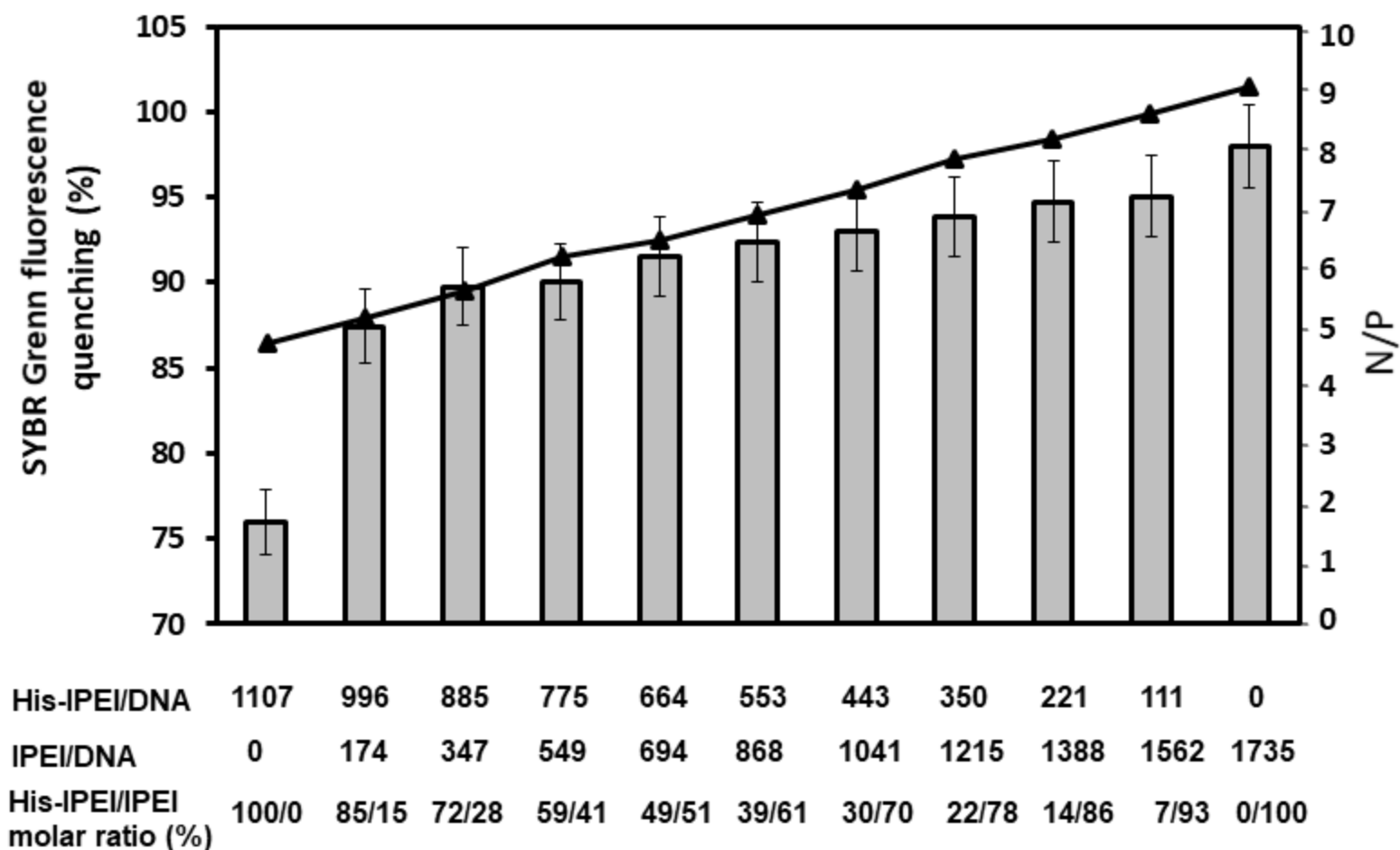


Fig 4

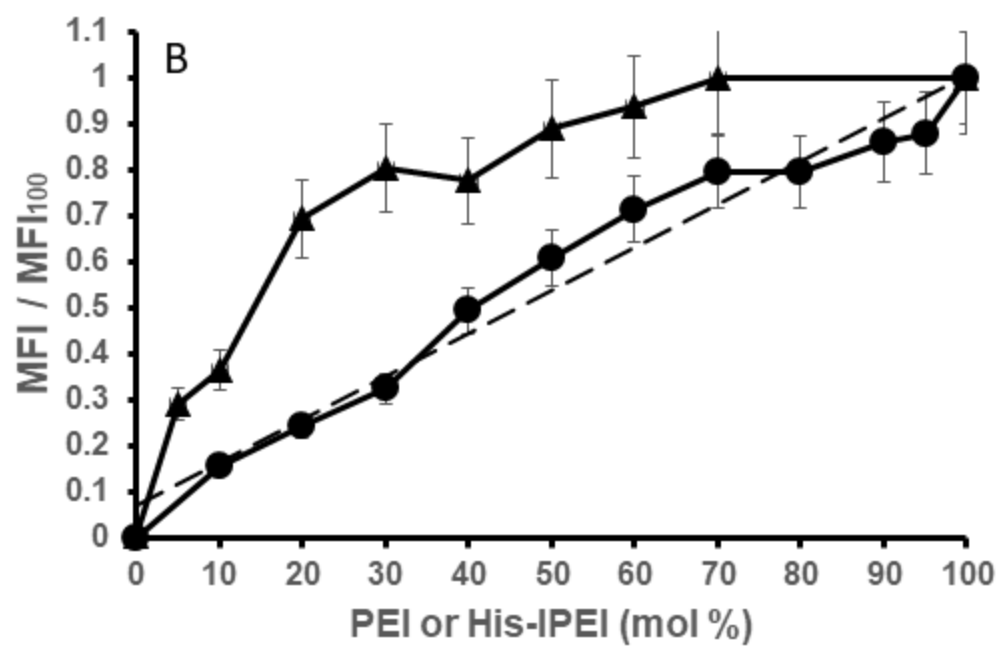
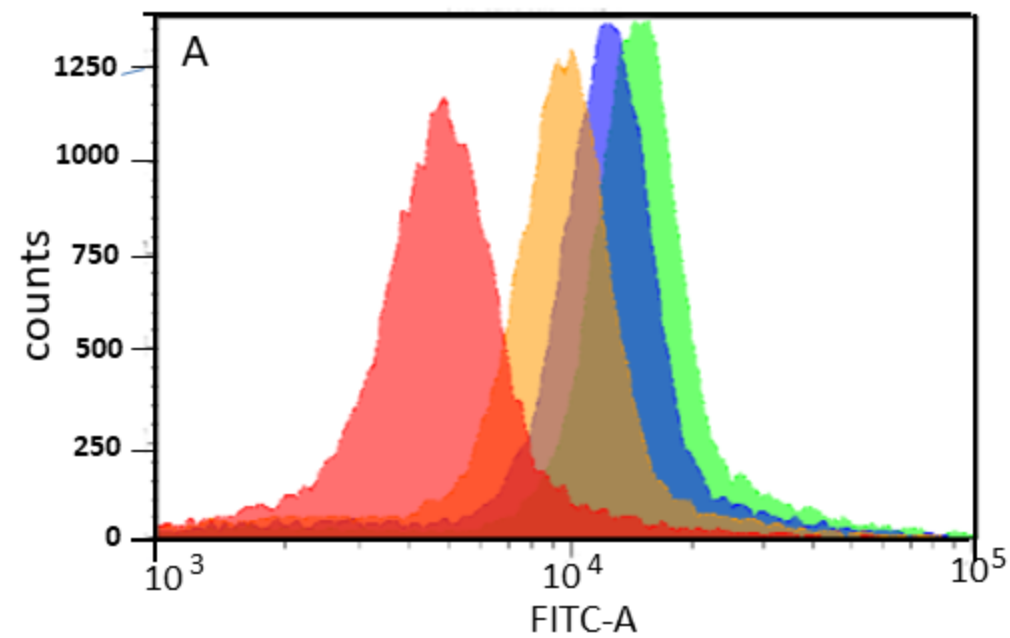


Fig 5

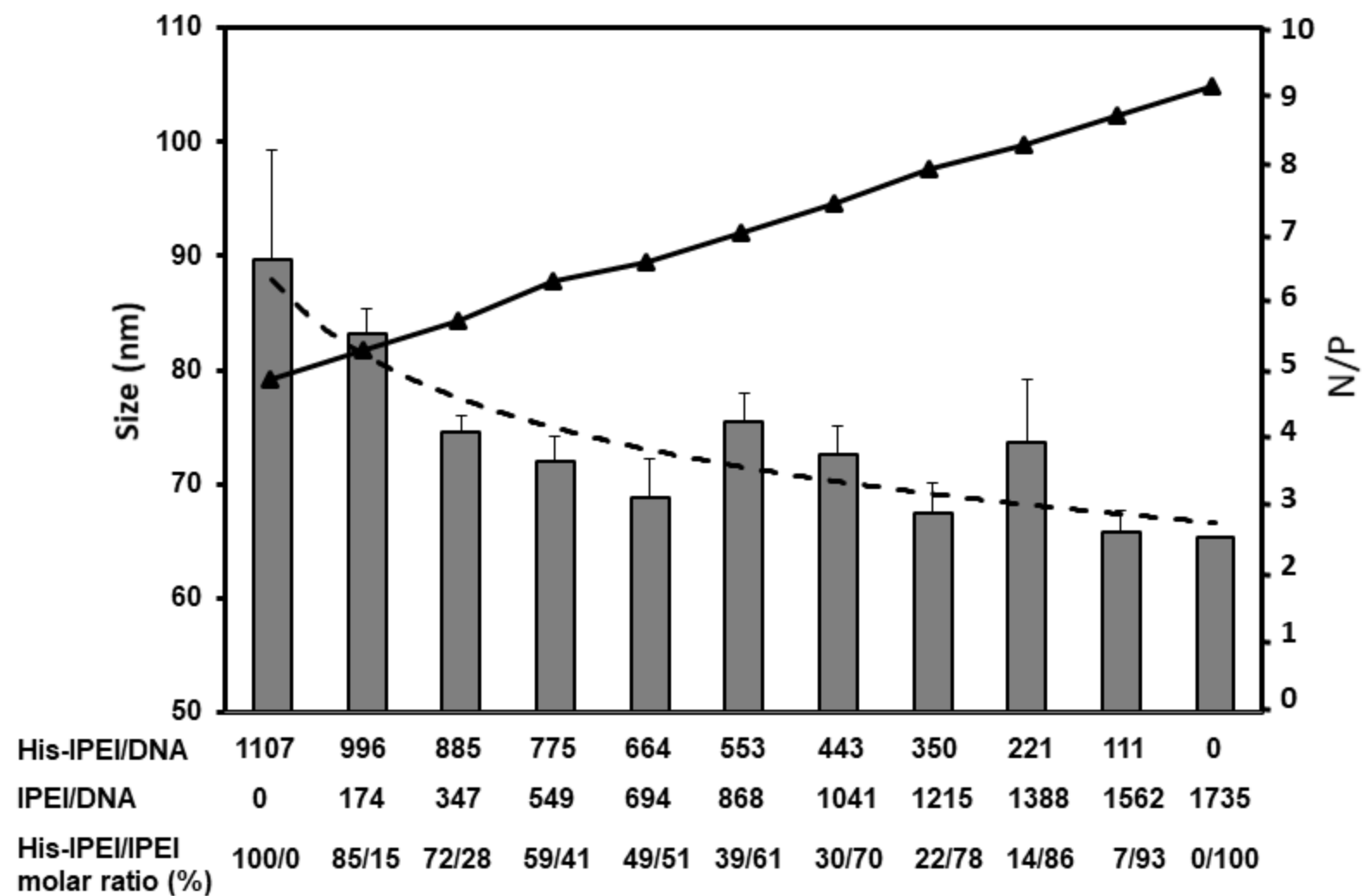


Fig 2

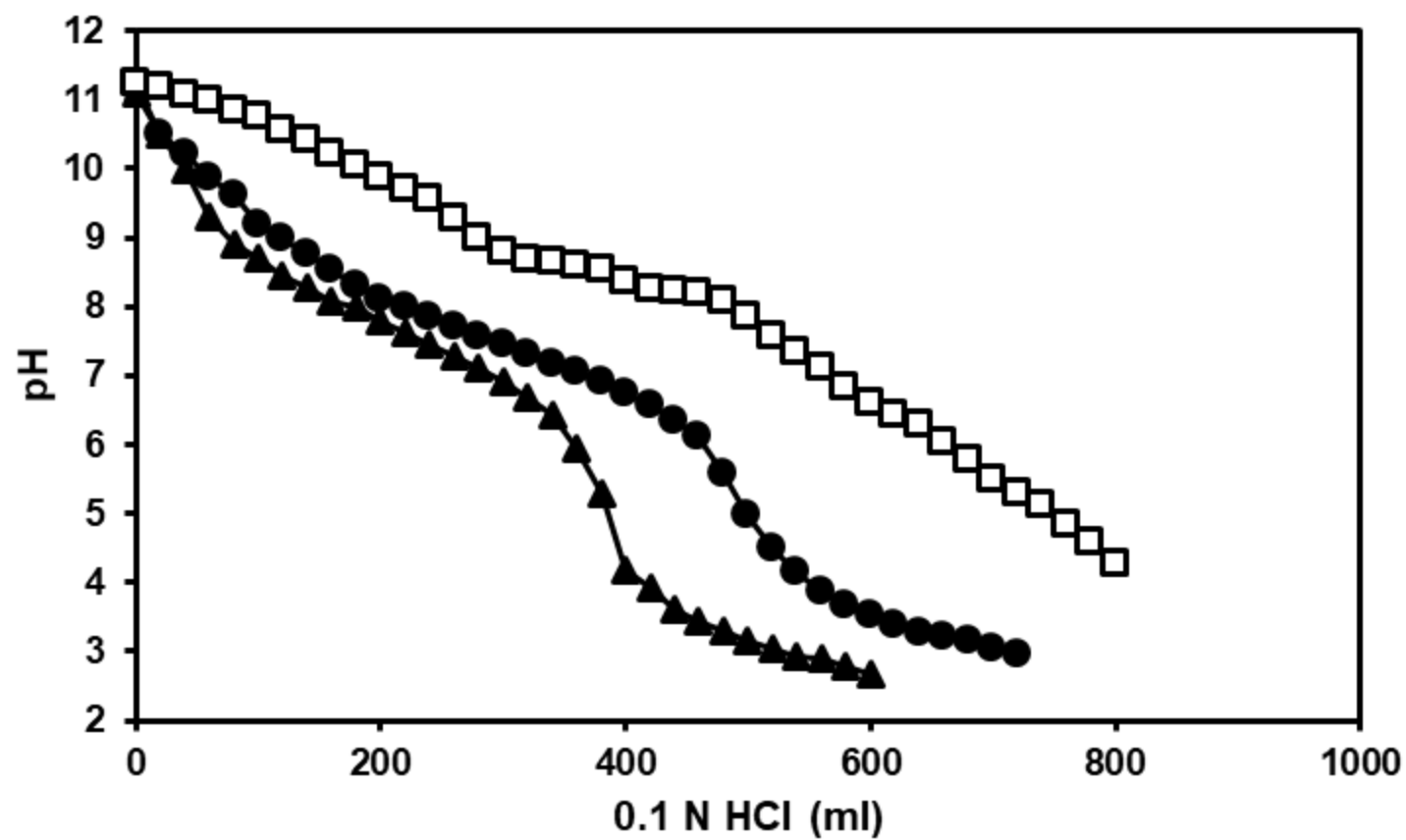


Fig 6

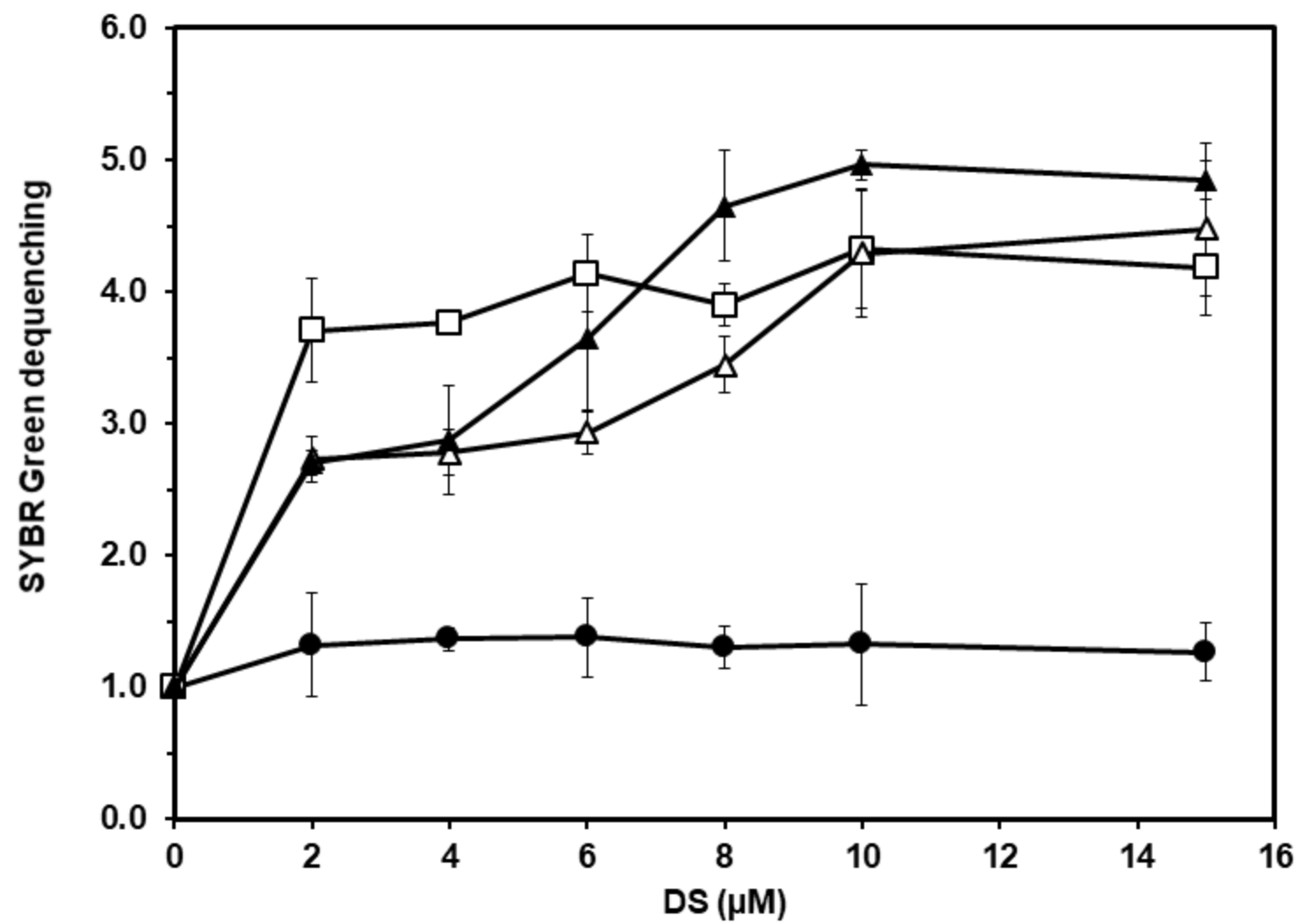
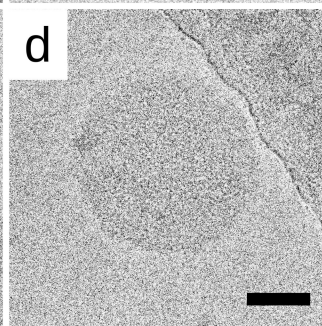
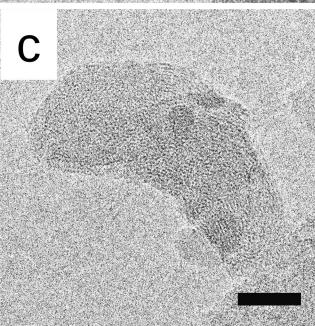
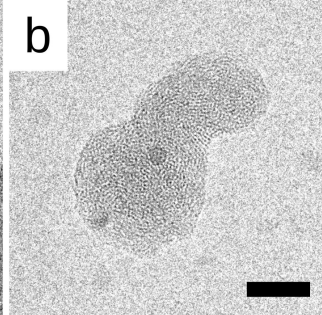
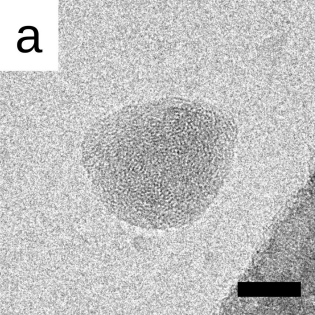


Fig 7



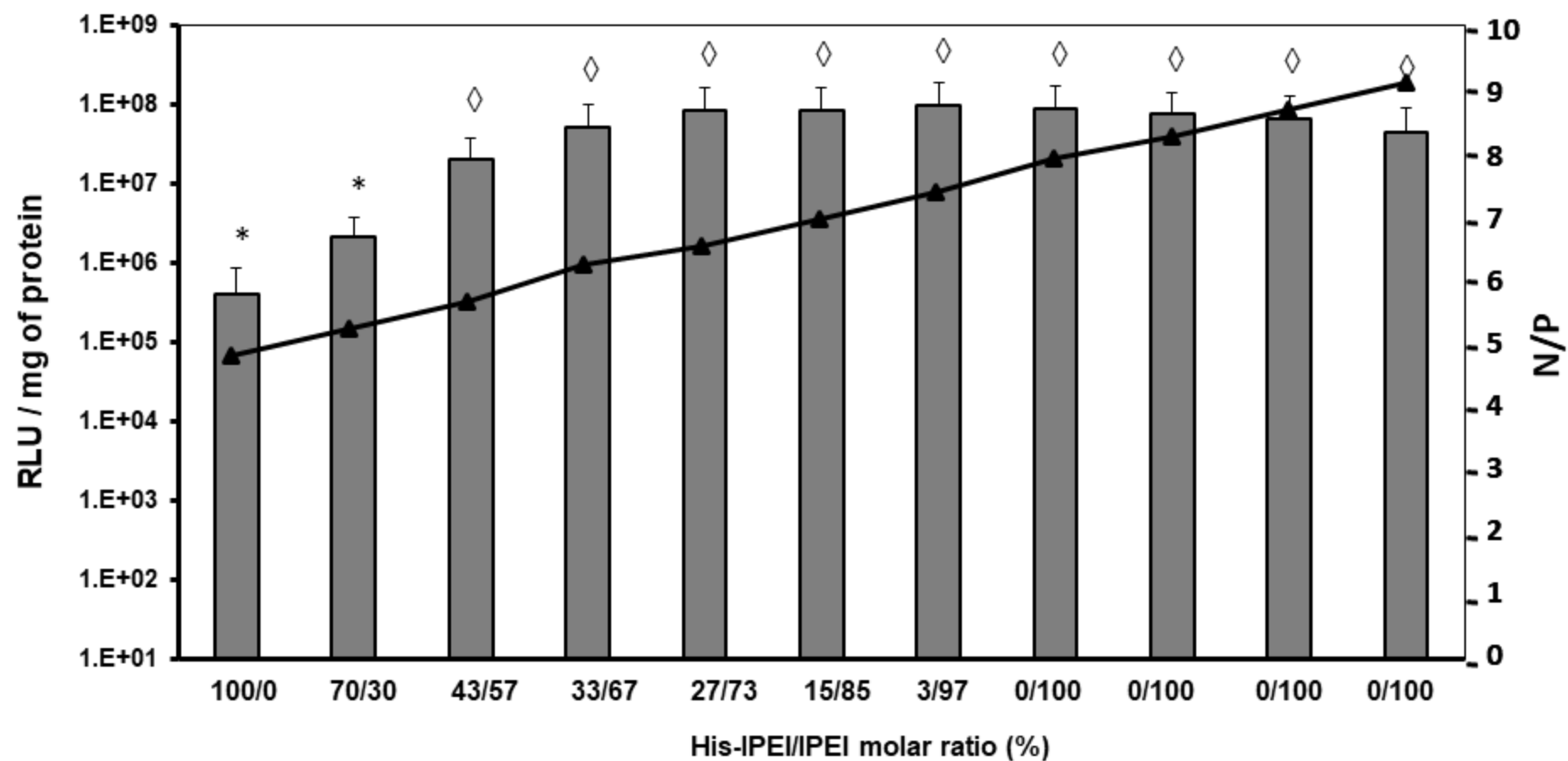


Fig 9

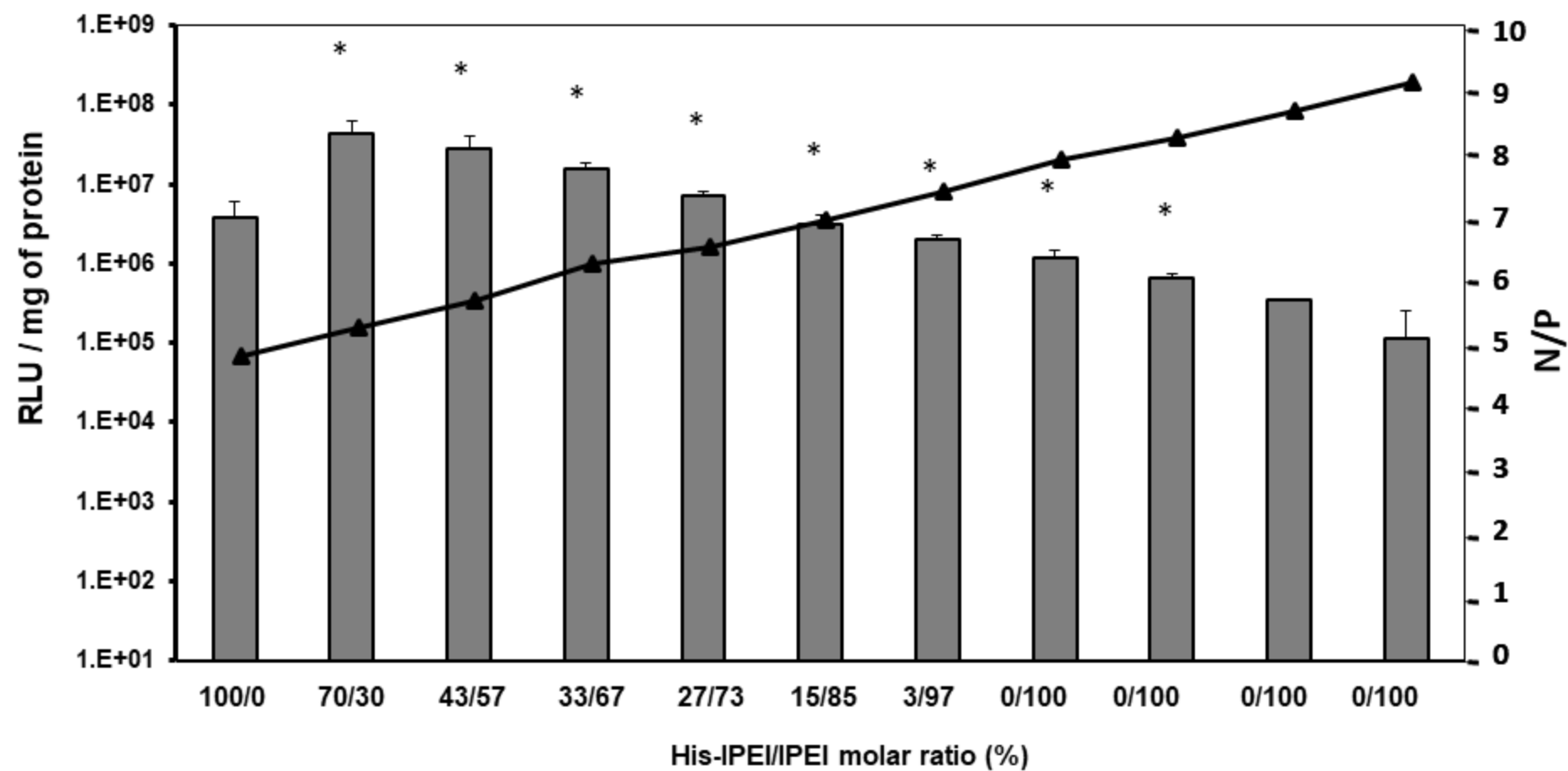


Fig 10

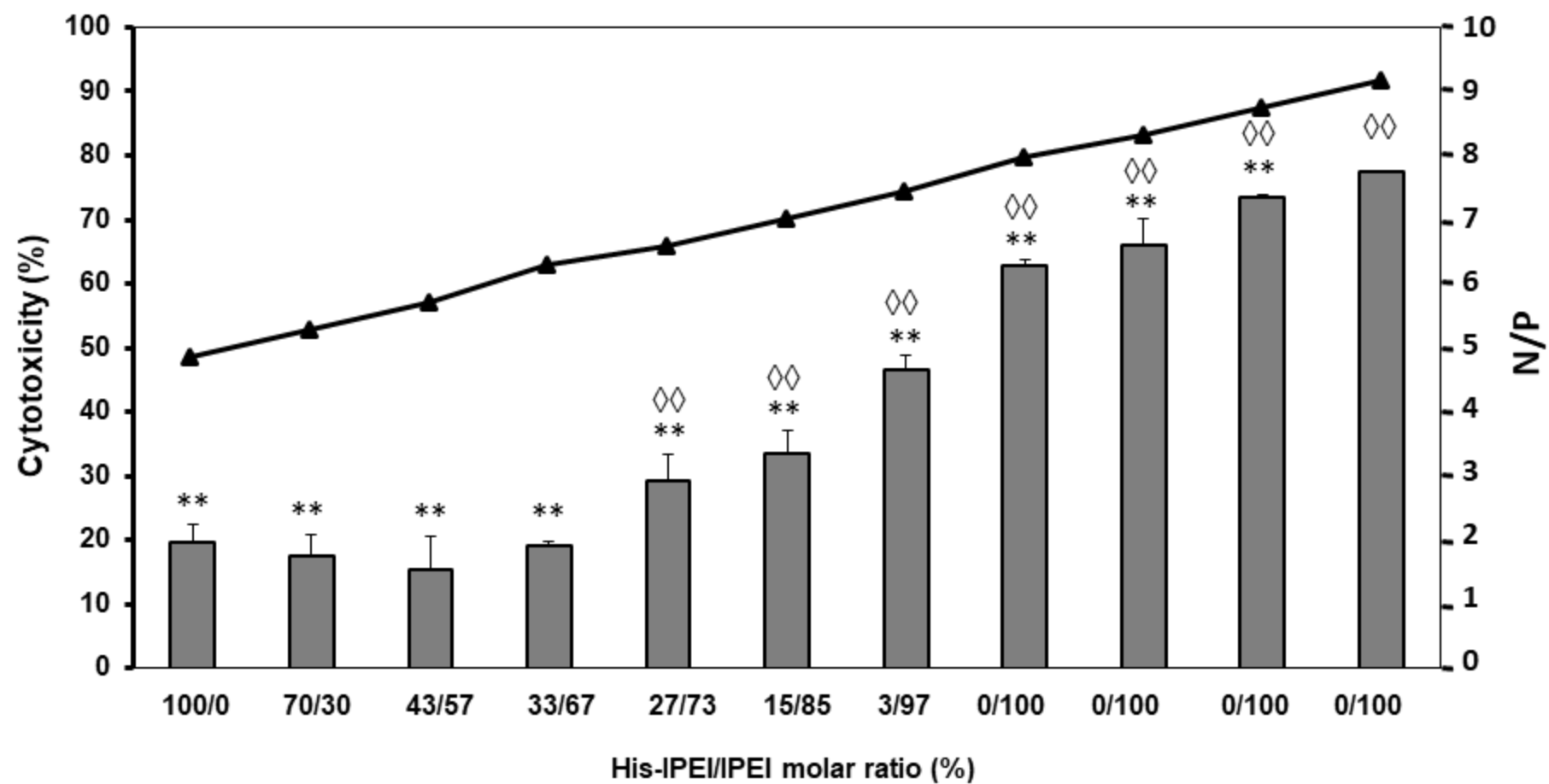
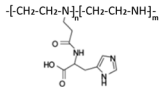


Fig 11

(1)+(2) polyplexes



His-IPEI (1)



DNA

+



(1)

+



(2)

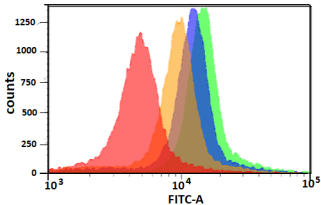
→



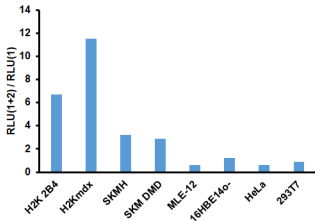
polyplex



IPEI (2)



Polyplex composition as function of the (1)/(2) ratio



Improved skeletal muscle cell transfection