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Combining polyethylenimine and Fe(III) for mediating pDNA transfection

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ABSTRACT

Background: The potential use of Fe(III) ions in biomedical applications may predict the interest of its combination with pDNA–PEI polyplexes. The present work aims at assessing the impact of this metal on pDNA complex properties.
Methods: Variations in the formation of complexes were imposed by using two types of biological buffers at different salt conditions. The incorporation of pDNA in complexes was characterised by gel electrophoresis and dynamic light scattering. Transfection efficiency and cytotoxicity were evaluated in HeLa and HUH-7 cell lines, supported by flow cytometry assays.
Results: Fe(III) enhances pDNA incorporation in the complex, irrespective of the buffer used. Transfection studies reveal that the addition of Fe(III) to complexes at low ionic strength reduces gene transfection, while those prepared under high salt content do not affect or, in a specific case, increase gene transfection up to 5 times. This increase may be a consequence of a favoured interaction of polyplexes with cell membrane and uptake. At low salt conditions, results attained with chloroquine indicate that the metal may inhibit polyplex endosomal escape. A reduction on the amount of PEI (N/P 5) formed at intermediary ionic strength, complemented by Fe(III), reduces the size of complexes while maintaining a transfection efficiency similar to that obtained to N/P 6.
Conclusions: Fe(III) emerges as a good supporting condensing agent to modulate pDNA–PEI properties, including condensation, size and cytotoxicity, without a large penalty on gene transfection.
General significance: This study highlights important aspects that govern pDNA transfection and elucidates the benefits of incorporating the versatile Fe(III) in a gene delivery system.

1. Introduction

Gene therapy creates medical opportunities for the treatment of severe diseases, including cancer. In the past decades, attention has been directed to the development of nonviral vectors with the ability to overcome critical biological obstacles by presenting inherent properties that should comprise the following: stability in the extracellular environment, interaction with target cell surface and cell internalisation, release from endo/lysosomal vesicles and vector decompaction and translocation into the nucleus [1,2]. Such properties demand the development of an extremely efficient vector that is able to defeat adverse and sometimes opposite conditions along its pathway through the cell. The improvement and evaluation of different nonviral formulations such as polymer-based pDNA complexes (polyplexes) rely largely on transfection measurements. Hitherto, among the cationic polymers, polyethylenimine (PEI) is the most effective polycationic transfection agent, known as a golden standard for polymeric gene delivery [3–5]. The most important feature of this class of molecules is the possibility of attaining a high positive charge density and high buffering capacity due to partial protonation at physiological pH [6,7]. The latter facilitates the disruption of the endo/lysosomal membrane, for which a proton sponge property has been postulated [8,9]. The inherent toxicity of PEI is mainly related to the respective strong positive charge, which leads to a strong interaction with the mitochondria [10], and/or interaction with the cell membrane, resulting in a perturbation of the membrane structure and pore formation [11,12]. Hence, modifications of the polymeric backbone that reduce the positive charge of PEI might be beneficial in order to reduce the toxicity of the polycation. A number of modifications have been reported that convert PEI into a suitable vector and improve gene transfection [13,14].

Recently, we have used a ternary DNA-PEI-Fe(III) complex approach and have shown that the addition of Fe(III) to DNA-PEI complexes enhances DNA condensation and, in addition, allows an easier DNA release from the complexes, in the presence of a decondensing agent [15]. The replacement of PEI molecules by Fe(III) also results in polyplexes with similar sizes and zeta potential but reduced cytotoxicity [16]. Moreover, the ability of Fe(III) to improve DNA condensation is independent of the architecture (linear, branched) of the PEI molecules used and is

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governed by Fe(III)-amine chelation [17]. The protonation degree of the polycation has a determinant role in the PEI–metal chelation [18–20] as well as other parameters, such as metal concentration, ionic strength, structure of the polycation and nature of inorganic and organic ligands [18,21–27]. The versatility of the combination of the metal ions with organic compounds provides a variety of promising applications in diverse areas, including the biomedical field. For instance, metal pH-responsive systems are of special interest for controlling the "host–metal–drug" coordination bonding architecture on the mesopores releasing chemotherapy drugs by small pH variations [27]. In addition, the metal itself seems to possess the ability to reduce the tumour growth [28]. However, and specifically for Fe(III), controversial studies have shown that both a depletion or an excess of this metal in the cells results in an arrest of the growth of cancer cells [29].

The aim of this study was to evaluate the influence of the addition of Fe(III) on the formation of pDNA–lPEI22 complexes and on the diverse challenges the complexes face up to the final goal, gene expression. The complex formation was modulated by systematic changing buffer composition and ionic strength, and a collection of techniques was employed to elucidate the physicochemical properties and biological activity of pDNA–lPEI22 and pDNA–lPEI22–Fe(III) complexes over a range of N/P and Fe(III)/P ratios.

2. Materials and methods

2.1. Materials

Plasmid pCMVLuc (*Photinus pyralis* luciferase under control of the CMV enhancer/promoter) described in Plank et al. [30] was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). Linear PEI (22 kDa) was synthesised as described in Schaffert et al. [31]. Ferric chloride hexahydrate, sodium acetate and acetic acid were purchased from Sigma-Aldrich (Germany). Cell culture media, glutamine, antibiotics and foetal calf serum (FCS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Cy5-labeling kit for pDNA was obtained from Mirus Bio (Madison, WI, USA). GelRed was obtained from Biotium (Hayward, USA). Glucose was purchased from Merck (Darmstadt, Germany), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Biomol GmbH (Hamburg, Germany) and sodium chloride from Prolabo (Haasrode, Belgium). Dilutions were prepared as follows: (i) 20 mM HEPES-buffered 150 mM NaCl (HBS) pH 7.4, (ii) 20 mM HEPES-buffered 5% glucose (HBG) pH 7.4, (iii) 30 mM acetate buffer (1.6 mM CH_3COOH, 28.4 mM $NaCH_3COO.3H_2O$) (Ac30) pH 6.0, (iv) 50 mM acetate buffer (2.7 mM CH_3COOH, 47.3 mM $NaCH_3COO.3H_2O$) (Ac50) pH 6.0 and (v) 100 mM acetate buffer (5 mM CH_3COOH, 95 mM $NaCH_3COO.3H_2O$) (Ac100) pH 6.0. Luciferase for cell culture lysis reagent and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany).

2.2. Complex formation

For gel mobility, transfection and metabolic activity assays, pDNA–lPEI and pDNA–PEI–Fe(III) complexes were prepared as follows. A fixed volume of PEI solution (4.5 μL) with variable concentration was added to 200 ng of pCMVLuc solution (15 μL) and rapidly mixed by pipetting. The final concentration of pDNA in solution was 10 μg mL^{-1}. The trivalent salt solutions were always freshly prepared and added, at a fixed volume (0.5 μL), directly to the pDNA–PEI mixture after it equilibrated for 15 min and, again, mixed by pipetting. Polyplexes were allowed to equilibrate for 40 min at room temperature before use. In this work, specific N/P ratios were determined, considering the mass of 330 g mol^{-1} corresponding to one phosphate group on pDNA. In PEI, the mass of 43 g mol^{-1} corresponds to one amine group ($-CH_2CH_2NH$-). The chosen N/P ratios were 6 and 10, corresponding to 0.8 and 1.3 w/w, respectively. The same calculation was performed to determine the Fe(III)/P ratio considering the molar concentration of positive charges for the Fe(III) in solution. The ratio between the condensing agents, PEI:Fe(III), in these studies was 2:1 and 1:1. Note that both N/P and Fe(III)/P ratios are calculated on the basis of the amounts of pDNA, PEI and Fe(III) added to the solution.

In the case of polyplexes prepared for flow cytometry assays, the aforementioned procedure was used, but the final concentration of pDNA in solution was 50 μg mL^{-1} in a total volume of 100 μL. Further details are given in the corresponding section.

For size and zeta potential measurements, the concentration of plasmid DNA was the same, 10 μg mL^{-1}, but a larger volume sample of the polyplex mixture (1000 μL) was prepared according to the proportions and procedure described previously.

2.3. Gel shift assay

pDNA complexes (200 ng of pCMVLuc in 20 μL) with loading buffer (prepared from 6 mL of glycerine, 1.2 mL of 0.5 M EDTA, 2.8 mL of H_2O, 0.02 g bromophenol blue) were loaded into the wells of a 1% agarose gel in TBE buffer (Trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g, in 1 L of water) containing GelRed for pDNA detection. The gel was run at 120 V for 80 min and then photographed under UV light.

2.4. Particle size and zeta potential

Hydrodynamic diameter and zeta potential of pDNA–PEI and pDNA–PEI–Fe(III) complexes were measured by dynamic light scattering using a Delsa Nano C Submicron (Beckman Coulter, Krefeld, Germany). After equilibration, samples were measured in triplicate at 25 °C with a detection angle of 160°. In the case of size measurements, the correlation functions were fitted using the instrumental software to obtain the averaged hydrodynamic diameter (CONTIN) and the size distribution (non-negative least squares, NNLS). All the measurements of size in the DNA-PEI-Fe(III) system are compared with the standard DNA-PEI complexes.

2.5. Cell culture

HUH-7 hepatocellular carcinoma cells (JCRB 0403; Tokyo, Japan) were cultured in a 50:50 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium. HeLa cells (human cervical carcinoma cells, ATCC CCL-2) were grown in DMEM. Both media were supplemented with 10% foetal calf serum (FCS), 4 mM stable glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Both cell lines were grown at 37 °C in 5% CO_2 humidified atmosphere.

2.6. Cell transfection

In vitro pCMVLuc transfection efficiency was assessed in HUH-7 and HeLa cells. Each cell line was seeded 24 h prior to the transfection assay at a density of 1×10^5 cells/well in 96-well plates (TPP, Trasadingen, Switzerland). Before transfection, medium was replaced with 80 μL of fresh growth medium; thereafter, 20 μL of complexes was added to each well and incubated at 37 °C in 5% CO_2 for 24 h. All samples were analysed in at least triplicate. In assays performed in the presence of chloroquine, this chemical was added to the buffer solutions (final concentration of 100 μM) in which the complexes were prepared. After 4 h in post-transfection, the medium was replaced by fresh growth medium to avoid cell damage. At 24 h after transfection, the remaining portion of medium in each well was removed by suction and cells were treated with 100 μL cell lysis buffer (25 mM Tris pH 7.8, 2 mM dithiothreitol (DTT), 10% glycerol, 1% Triton® X-100). Luciferase activity in 35 μL cell lysate was assessed in white 96-well plates using a luciferase assay kit (100 μL Luciferase assay buffer, Promega, Mannheim, Germany) and measured on a Lumat LB9507 luminometer for 10 s (Centro LB 960 instrument, Berthold, Bad Wildbad, Germany).

2.7. Flow cytometry

To assess the level of internalisation of pDNA–PEI and pDNA–PEI–Fe(III) complexes, the following procedure was conducted in HUH-7 cell line. Cells were seeded in 24-well plates at a density of 5×10^5 cells/well in 500 μL medium 24 h prior to transfection and incubated at 37 °C and 5% CO_2. Before the addition of polyplexes, medium was replaced with 400 μL of fresh growth medium. An amount of 100 μL polyplex solution with 1 μg of pCMVLuc containing Cy5-labeled pDNA (20%) was added to the cells. Four hours after polyplexes addition, the cells were washed once with phosphate buffer saline (PBS) and treated with 500 IU mL^{-1} heparin to remove extracellularly bound complexes. Afterwards, the cells were harvested by treatment with trypsin/EDTA solution (Invitrogen GmbH, Karlsruhe, Germany), taken up in cell culture medium and centrifuged at 2000 rpm for 5 min. The remaining supernatant was removed and the pellet resuspended in 500 μL PBS with 10% FCS and analysed using a Cyan™ ADP flow cytometer (Dako, Hamburg, Germany). Samples were analysed using Flow Jo software (Treestar, Inc., San Carlos, USA). Experiments were performed in duplicate.

2.8. Metabolic activity assay

Metabolic activity was analysed 48 h after transfection using the CellTiter-Glo™ luminescent cell viability assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. The assay is based on the quantitative measurement of the cellular ATP content. A volume of 50 μl was removed from each well of the transfection plate and replaced with 50 μl of CellTiter-Glo™ reagent (Promega, USA). The plate was shaken for 2 min to induce cell lysis and allowed to incubate at room temperature for 15 min. The relative metabolic activity was determined as the ratio of measured luminescent signal proportional to the amount of ATP present over the signal of untreated cells. For this purpose, the Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used.

3. Results

3.1. Gel shift assay

The spontaneous association of DNA with polycations and subsequent DNA condensation in aqueous media is a result of the strong electrostatic interactions and, therefore, the use of different biological buffers is likely to influence their association [32]. In the literature, condensation frequently encompasses the concept of reduction in size, at the single chain level, and multi-chain complex formation [33]. In these initial sections we study, the influence of Fe(III) on pDNA electrophoretic mobility and size.

First, the magnitude of electrophoretic mobility of polyplexes prepared in different buffers with various ionic strengths was examined. In more detail, we have used HEPES with no extra addition of salt (HBG) and in the presence of salt (150 mM, HBS) and acetate buffer (Ac) with salt concentrations from 30 to 100 mM. The HEPES buffer is generally considered to have an excellent buffering capacity over the required pH range and, additionally, to possess poor ability for metal complexation [34]. Previously, the efficient incorporation of DNA in DNA-PEI-Fe(III) polyplexes was reported using acetate buffer at 150 mM [15]. However, in the present study, acetate buffers over the range 30–100 mM were used instead.

Fig. 1 displays the electrophoretic mobility of pDNA in polyplexes composed of lPEI22 alone or lPEI22 and Fe(III), prepared in four different media. As expected, the addition of lPEI22 results in the decrease of the migration of pDNA through the gel, indicating pDNA complex formation. The addition of the second condensing agent, Fe(III), induces pDNA neutralisation at even lower N/P ratios, which is consistent with our earlier findings [7,16]. These observations are independent of the buffers used. However, comparing panels a and b reveals a lower retention of pDNA in the wells, that is, pDNA neutralisation is lower when the solutions are prepared in a buffer with high ionic strength (HBS). This fact is observed for both pDNA–PEI and pDNA–PEI–Fe(III) complexes. The same trend is observed for pDNA–PEI and pDNA–PEI–Fe(III) complexes prepared in acetate buffers, Ac30 and Ac100, see panels c and d. The delay in pDNA neutralisation obtained in high ionic strength media is a strong indication of the predominant electrostatic nature of polyplex formation. Furthermore, differences in polyplex mobility are visible between the two different buffers used. Thus, neutral pDNA–PEI complexes are formed at low N/P ratio in acetate buffer, panel c, similarly to those found in HBG, panel a, whereas in the presence of Fe(III), a more marked pDNA retention is observed for complexes prepared in acetate buffer. For high ionic strength buffers, panels b and d, the inhibition of pDNA neutralisation is more evident in the case of complexes prepared with HBS than with Ac100. These divergences detected between HEPES and acetate buffers might be explained by the difference in the pH of the media since a lower pH increases the protonation of PEI [7,35]. Despite the differences in the media, all systems are able to incorporate pDNA and the synergy of PEI-Fe(III) is clearly observed.

3.2. DLS measurements

In order to monitoring the effect of the addition of Fe(III) in the size of pDNA–PEI polyplexes in aqueous buffer solutions, dynamic light scattering (DLS) was used. Fig. 2 shows the intensity distribution graphs of pDNA–PEI and pDNA–PEI–Fe(III) complexes formed in different buffer solutions, at N/P ratios similar to those later used in the transfection assays. pDNA–PEI complexes at N/P 6 in HBG, Fig. 2a, were found to have a bimodal distribution with the strongest intensity belonging to complexes with roughly 100 nm, and a second population at larger sizes (ca. 1316 nm) and a lower intensity. Increasing the N/P ratio leads to a reduction of the particle size, now with ca. 70 nm, in agreement with data reported in the literature [36,37]. The addition of Fe(III) ions, on the other hand, leads to an increase in the size of the complexes from approximately 70 to 100 nm, for a Fe(III)/P ratio of 5. At N/P:Fe(III)/P = 10:10, the intensity distribution graph shows the appearance of a third peak at intermediate values around 220 nm arising possibly from complexes aggregation, and/or from the expansion of the pDNA–PEI complexes. Note that the phenomena of aggregation are related with the neutralisation of complexes, and under these conditions, pDNA is fully neutralised, as observed in Fig. 1. In the case of complexes prepared in HBS, Fig. 2b, larger particles are observed, as expected. It has been shown that, at physiological conditions, polyplexes with lPEI22 possess an inherent tendency to aggregate that evolves with time, conversely to what occurs with branched PEI chains with 25 and 800 kDa [36–38]. At higher ionic strength, the effective charge of the free polymer segments is reduced and, consequently, also that of the complexes. This decreases the electrostatic stabilisation of the particles, leading to high levels of aggregation [39]. Therefore, at N/P 10, three different populations are observed, displaying a large range of sizes. The presence of Fe(III) influences the populations differently: the peak corresponding to the population of smaller sizes becomes more intense, but no significant differences are found for the larger populations when compared with the analogous polyplex in the absence of Fe(III).

The distribution of sizes for pDNA–PEI and pDNA–PEI–Fe(III) complexes prepared in Ac50 was further assessed at N/P values typically used in transfection, see Fig. 2c. Complexes at N/P 5 present a distribution shifted to higher values when compared to N/P 6, with two populations, one with an average value of ca. 757.2 nm and the other corresponding to larger sizes ca. 1119.7 nm. In the case of complexes prepared at N/P 6, again two peaks are clearly distinguishable, corresponding to one population of small complexes of about 180 nm and the other with larger sizes, ca. 720 nm. Fe(III) contributes by reducing the size of pDNA–PEI complexes, similarly to what was observed using HBS (Fig. 2b). The complexes prepared with N/P:Fe(III)/P = 5:10

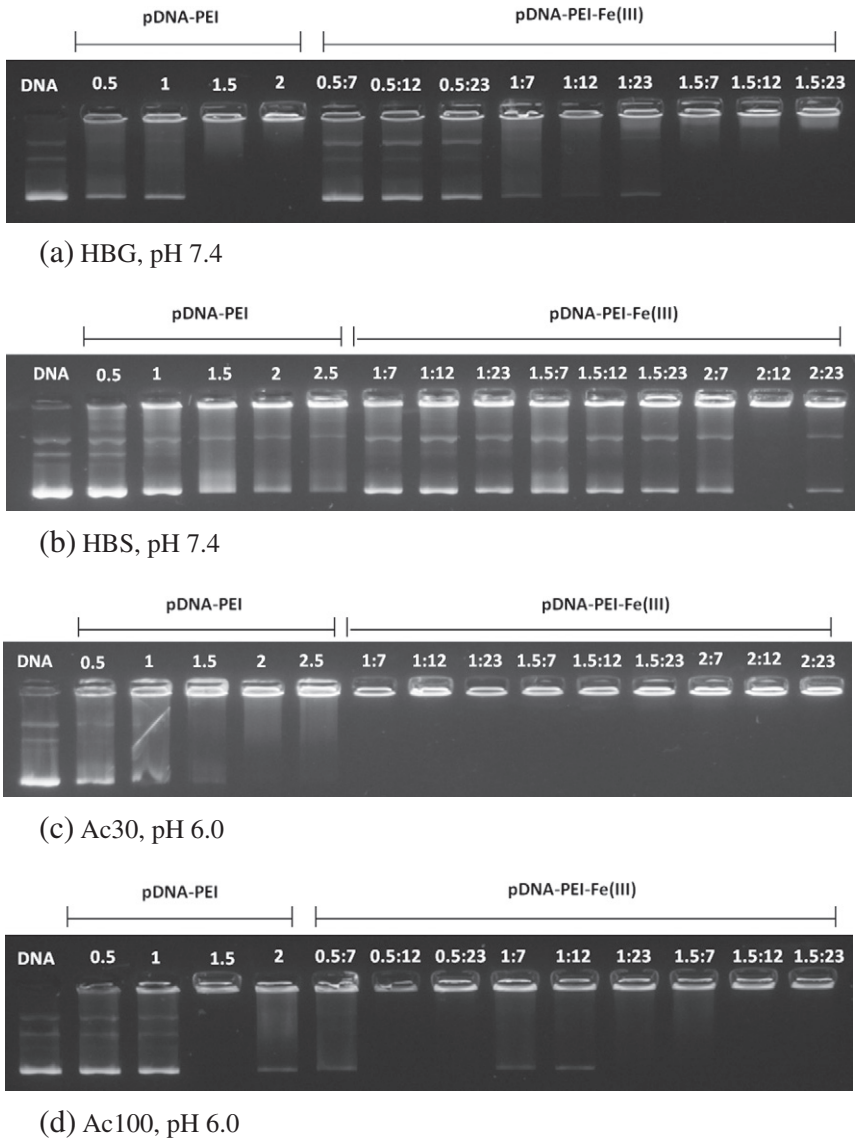


Fig. 1. pDNA–lPEI22 and pDNA–lPEI22–Fe(III) binding assays. The values of N/P and N/P:Fe(III)/P ratios are depicted for each lane. Lane 1 corresponds to pDNA in the absence of condensing agents. pDNA concentration is 10 μg mL^{-1} in each well. The polyplexes were prepared in HEPES and acetate buffers at different ionic strengths as indicated.

show roughly the same distribution as those of N/P 6, with one population with about 225 nm and a second one with larger sizes (ca. 964.5 nm). Considering these data, it is concluded that Fe(III) reduces the size of polyplexes prepared at high or moderate ionic strength. This contrasts with the results obtained for the complexes prepared in HBG. As it is observed in pDNA incorporation assays, Fig. 1, panels a and c, a low concentration of PEI is able to efficiently condense pDNA when in conjunction with Fe(III) at low salt conditions. However, at high N/P ratio, the addition of the extra condensing agent seems to destabilise the complexes. For high salt conditions (HBS and Ac50), the presence of the extra condensing agent offers both stabilisation and condensation of the complex.

In an attempt to understand the behaviour of ternary polyplexes as a function of the ionic strength of the buffer, four acetate buffer solutions at different ionic strength ranging from 30 to 100 mM were used as the media for polyplex preparation. The apparent average size and zeta potential obtained for the ternary complexes with low N/P ratio are gathered in Fig. 2d. The reduction of salt concentration in the media leads to the formation of smaller and more positively charged polyplexes. This correlates the trend observed in electrophoresis results (Fig. 1), but by this technique, normally positive charged complexes appear to higher N/P ratios, and they should be small enough to be mobile in electrophoresis gel [16]. At low N/P ratios using Ac30, it is possible to attain small complexes with 261 ± 4 nm that simultaneously possess a high surface charge.

It should be noted that, in general, complexes display a high polydispersity, especially when aggregation takes place. In these size and zeta-potential measurements, the main objective is to compare and establish trends, which are believed to be sufficiently robust to withstand the underlying assumptions of the techniques.

3.3. Gene expression of pDNA–PEI–Fe(III)

3.3.1. Complexes prepared in HBS and HBG

The effect of adding Fe(III) to pDNA–PEI complexes on the reporter gene expression was determined using two different cell lines, HUH-7 and HeLa. Transfection efficiency was analysed for polyplexes formed under low salt conditions, HBG, and under higher salt conditions, HBS (Fig. 3). The trends attained are comparable for both cell lines, but polyplexes prepared in HBG show lower *in vitro* transfection efficiencies when compared to HBS, in agreement with the literature [36,38]. Generally, it is observed that for complexes formed in HBG the addition of

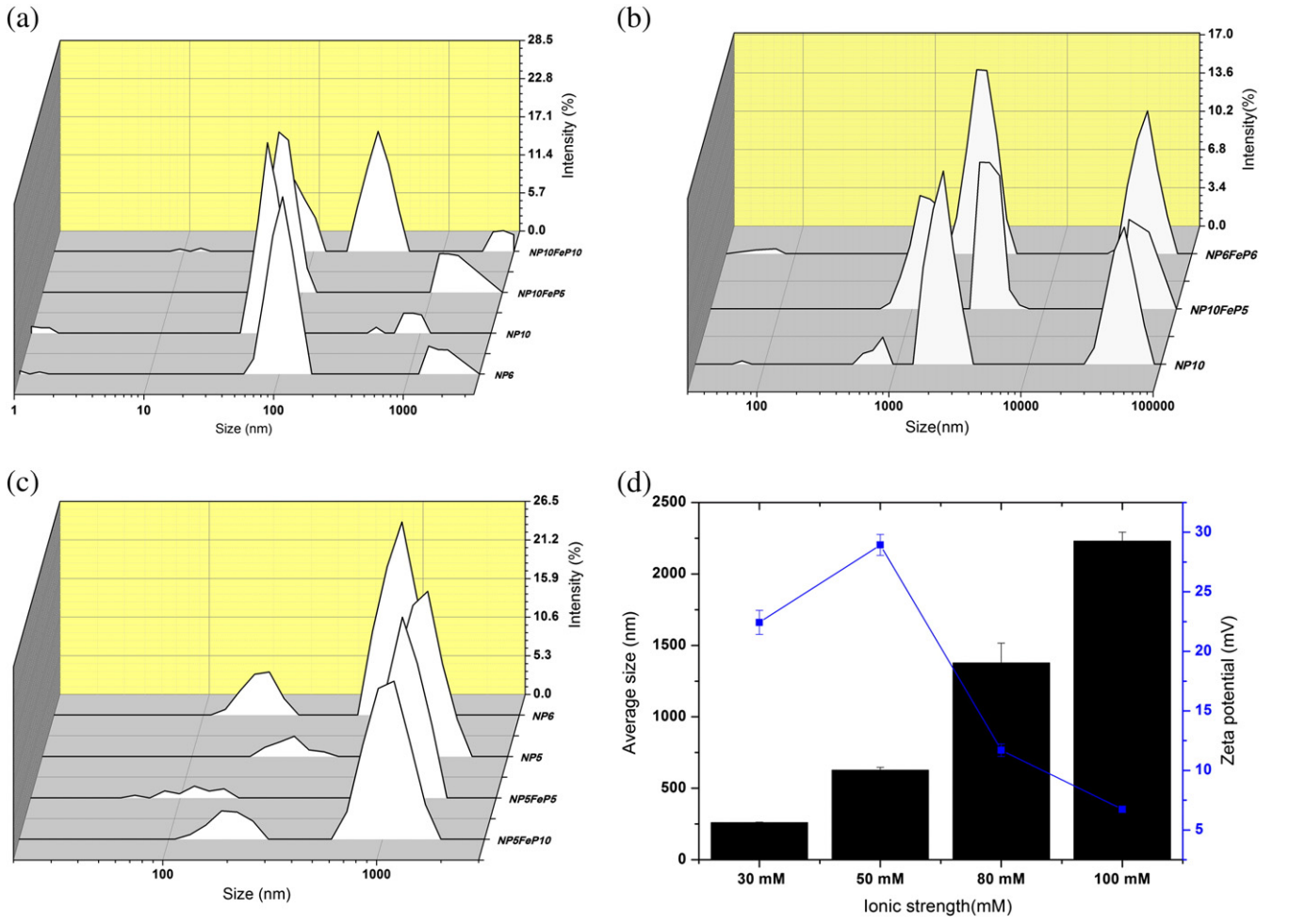


Fig. 2. DLS curves of intensity size distribution for complexes prepared in biological buffers, (a) HBG, (b) HBS and (c) Ac50. In panel d, average size and zeta potential versus acetate buffer ionic strength of complexes at N/P:Fe(III)/P = 3:12 are represented.

Fe(III) reduces their transfection efficiency, panels a and d, in a direct correlation to the Fe(III)/P ratio, i.e., the larger the amount of Fe(III) added, the larger is the reduction on transfection efficiency. In the case of complexes with N/P 6 prepared in HBS, the higher concentration of Fe(III) did not affect the transfection efficiency, in HeLa cell line, panel b. In the case of the HUH-7 cell line, an increase in transfection efficiency of approximately 5.3-fold, is found for the higher studied Fe(III)/P ratio, panel a. When N/P 10 complexes in HBS are considered instead, and for both cell lines, the gene expression is not significantly affected at the lowest Fe(III)/P 5 but is reduced for higher Fe(III) concentrations, panels c and d.

3.3.2. Complexes prepared in acetate buffers

The same analyses were conducted in systems prepared in acetate buffer, with increasing ionic strength, as shown in Fig. 4. Here, the reference samples prepared in identical acetate buffer are represented as well, for an easier comparison. Also represented are reference samples prepared in HBG or HBS, according to the similarity of the sizes of complexes. Results confirm the effect of the ionic strength of the medium on the transfection efficiency, as observed previously, yielding an increase in transfection efficiency in both cell lines when high ionic strength buffers are used to prepare the polyplexes. The influence of the addition of Fe(III) on the transfection efficiency seems to be also correlated with this property. Complexes prepared in low concentrated buffer (Ac30), panels a and b, in the presence of the metal ions show a decrease in gene expression in HUH-7 cells, whereas in HeLa, a sharp reduction is only observed for complexes at N/P:Fe(III)/P = 6:6. In the case of complexes at N/P:Fe(III)/P = 6:3, there is a slight increase of the transfection efficiency when compared with the analogous N/P 6 in Ac30.

As the ionic strength is increased (Ac50), panels c and d, higher values of gene transfer are attained. The differences observed between pDNA–PEI and pDNA–PEI–Fe(III) complexes follow exactly the same trend as before, in panels a and b, but now the values are approximately equal to those of the reference samples. Recalling that the ionic strength of the medium influences considerably the size of the polyplexes (Fig. 2), this suggests that larger particles are more efficient in gene transfection *in vitro*, as has been reported [38]. For the highest concentrated buffer (Ac100), panels e and f, the addition of Fe(III) is less noticeable. The ternary complexes prepared in (Ac100) present values of reporter gene transfer higher than those in HBS but quite similar to their analogues in the absence of metal and in the same buffer. Note that at this stage, aggregation is very pronounced which consequently leads to high transfection efficiency *in vitro*.

3.3.3. Complexes prepared at low N/P ratios

A factor of paramount importance in the use of PEI is the control of its intrinsic cytotoxicity. A strategy proposed to improve the biocompatibility of pDNA–PEI complexes consists in reducing the concentration of PEI by replacement with Fe(III) ions, attaining stable and positively charged complexes, according to the trends shown in previous work [16]. Typically, the values of the N/P ratios used to form the complexes for transfection assays are equal or higher than 5 and normally at N/P 5 present higher values of transfection efficiency than those prepared at N/P 6 [36,40]. However, in high salt conditions, the complexes formed

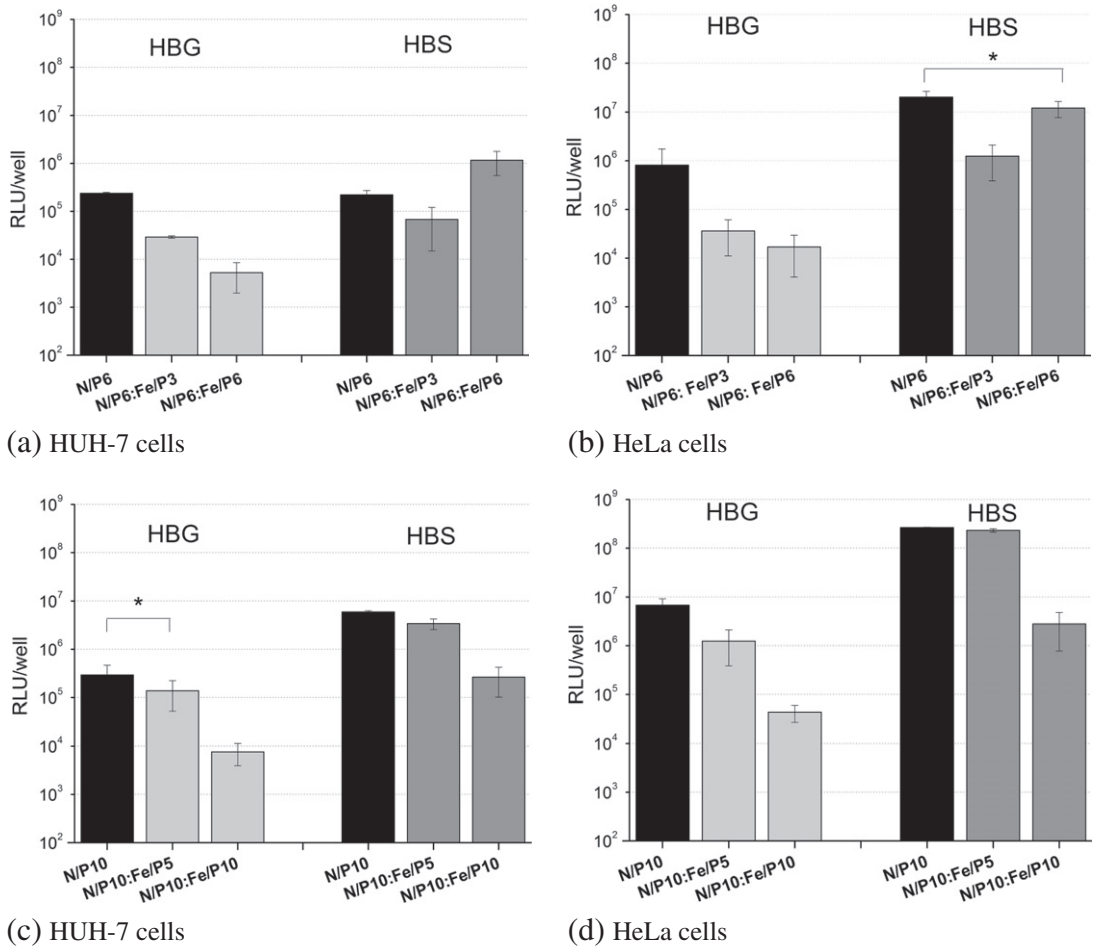


Fig. 3. Reporter gene transfection of pDNA–PEI and pDNA–PEI–Fe(III) polyplexes on HUH-7 and HeLa cell lines, as indicated. pDNA–PEI–Fe(III) complexes were prepared in HBG and HBS at N/P ratios of 6 (a, b) and 10 (c, d) and compared to standard PEI complexes with the same N/P ratios. Luciferase activity is presented as mean values ± SD of at least triplicates. pDNA concentration is 10 μg mL^{-1} in each sample. A Student's *t*-test was performed to assess statistical significance between each sample and the reference N/P 6 and N/P 10 (HBG or HBS) (*$p > 0.05$: samples are not significantly different compared to reference).

at lower N/P tend to present larger sizes. Herein, the replacement of PEI by Fe(III) in complexes prepared at low N/P ratios is evaluated. The values of the N/P ratios were decreased towards N/P 4 and 5, while the previously studied Fe(III)/P ratios were maintained. Acetate buffer solution with intermediate ionic strengths (Ac50) was chosen for these studies. The resulting gene transfer efficiency of these ternary complexes is depicted in Fig. 5.

Comparing the transfection efficiencies obtained in both cell lines, a marked dependence on cell type is clearly observed panels a and b. The addition of Fe(III) reduces the transfection efficiency when compared with related complexes in the absence of metal ions, in both cell lines, in agreement with previous observations. Nevertheless, the transfection of complexes pDNA–PEI–Fe(III) at low N/P is approximately the same as complexes prepared at N/P 6 in HBS, for HUH-7 cells (panel a). In the case of HeLa cells, complexes prepared from low concentrations of PEI combined with Fe(III) show a steep decrease in the transfection efficiency, panel b. This fact may be a consequence of the different mechanisms used by different cell lines to internalise the polyplexes [41]. Panel c gathers the results of the transfection experiments of the complexes with lower concentration of PEI in the presence and absence of Fe(III), including the reference complexes, as well as the respective average sizes. The first point that should be remarked is that the transfection efficiency of N/P 6 complexes in HBG and HBS are very similar, although the sizes of these polyplexes are very different. Moreover, for complexes prepared in Ac50 for a low N/P ratio of 5, the size of the polyplexes is very large, ca. 1123 nm, and so is the value of transfection attained. As the concentration of condensing agent is increased to N/P 6, a higher condensation of pDNA is visible with the complexes attaining values of roughly 544.2 nm, as well as the consequent reduction in transfection efficiency. The primary effect of the addition of Fe(III) to complexes at N/P 5 seems to be the narrowing of polyplex size distribution, now with an average size of 890.7 nm. This happens due to an increase in the percentage of small polyplexes, and the overall shift of the larger complexes to lower values, see Fig. 2c. As observed, the addition of metal ions leads to a reduction of the size of the complexes with a concomitant penalty in transfection efficiency, when compared to the equivalent system without Fe(III) (N/P 5 and in Ac50), but close to that obtained for the reference systems N/P 6 in HBG and HBS.

3.4. Effect of Fe(III) on complexes cytotoxicity

To investigate the influence of the presence of Fe(III) in complexes formed in HBG and HBS on the cytotoxicity in HUH-7 cells, the level of adenosine triphosphate (ATP) was determined by the bioluminescent assay. ATP is just present in metabolically active cells. HeLa cells were also analysed using this assay, but no cytotoxicity was found within the tested conditions (data not shown). The Fe(III)/P ratios used in these assays were chosen according to their transfection efficiency

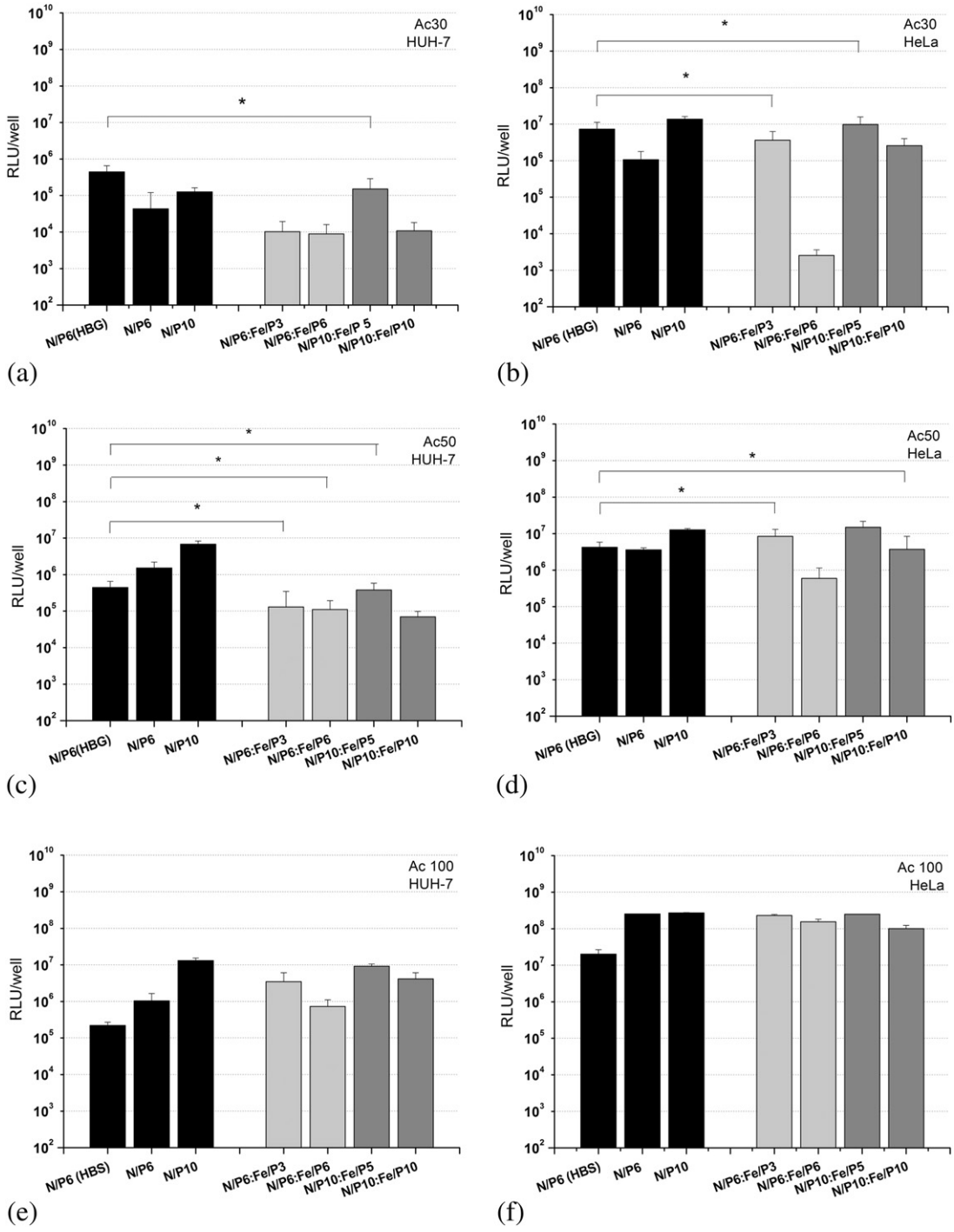
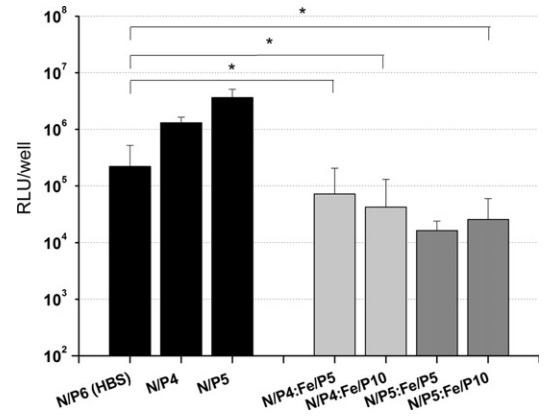


Fig. 4. Reporter gene transfection of pDNA–PEI and pDNA–PEI–Fe(III) polyplexes in HUH-7 and HeLa cell lines. pDNA–PEI and pDNA–PEI–Fe(III) complexes were prepared in acetate buffers with different ionic strength, (a) and (b) 30 mM (Ac30), (c) and (d) 50 mM (Ac50) and (e) and (f) 100 mM (Ac100), and then compared to standard pDNA–PEI complexes at the same N/P ratios in the absence of Fe(III), and with analogous polyplexes in terms of size) prepared in HBG or HBS. Luciferase activity is presented as mean values ± SD of at least triplicates. pDNA concentration is 10 μg mL^{-1} in each sample. A Student's *t*-test was performed to assess statistical significance between each sample and the reference N/P 6 (HBG or HBS) (* 0.05: samples are not significantly different compared to reference).

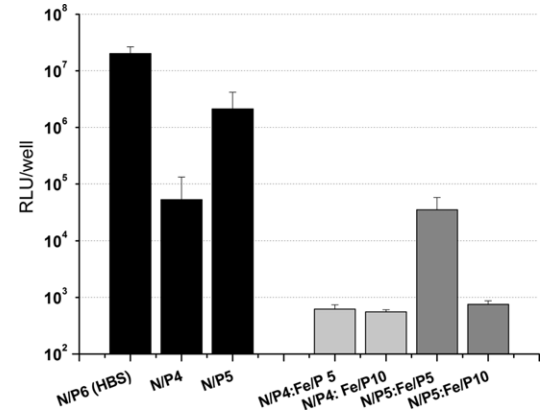
(see Fig. 3). The complexes formed at N/P 6 in the absence and presence of Fe(III) show minimal cytotoxicity (>80%) under the conditions used, for both buffers (Fig. 6). Increasing the concentration of PEI at N/P 10 leads to higher levels of cell damage, as expected. However, the addition of Fe(III) to the complex N/P 10 in HBG shows a slight increase in the biocompatibility of the complex (Fig. 6a), whereas, in the case of complexes formed in HBS, the ternary complexes are found to be equally cytotoxic as pDNA–PEI at N/P 6, Fig. 6b. It is again observed that Fe(III) mitigates the cytotoxicity of PEI, but this effect is also dependent on buffer type [16].

3.5. Effect of Fe(III) on endosome release and cellular internalisation

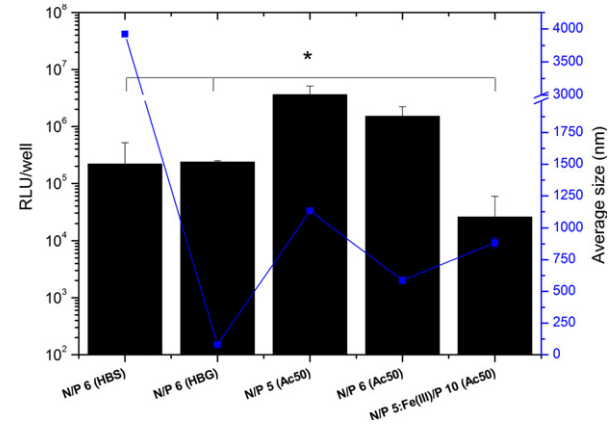
The influence of chloroquine, a known promoter of endo/lysosomal release, was tested in complexes prepared under low and high ionic strength, HBG and HBS (Fig. 7). The presence of this promoter has



(a) Ac50, in HUH-7 cells

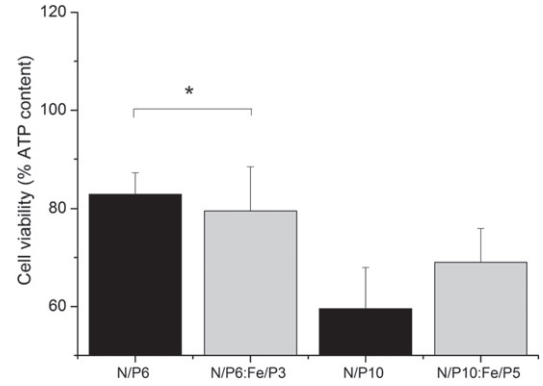


(b) Ac50, in HeLa cells

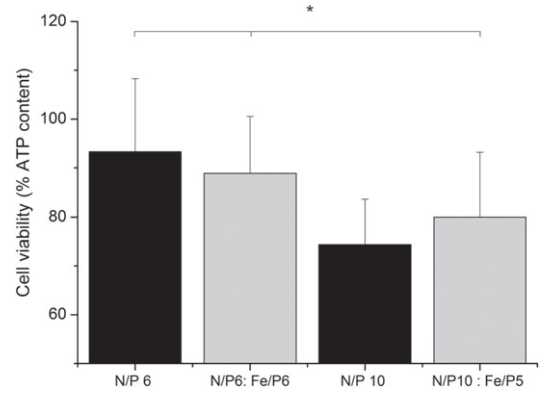


(c)

Fig. 5. Reporter gene transfection of pDNA–PEI and pDNA–PEI–Fe(III) polyplexes applied on (a) HUH-7 and (b) HeLa cells. Polyplexes prepared in Ac50, at low values of N/P ratios are depicted and compared with reference systems. Panel c shows the size and transfection efficiency of selected pDNA–PEI and pDNA–PEI–Fe(III) complexes in HUH-7. pDNA concentration is 10 μg mL^{-1} in each sample. A Student's *t*-test was performed to assess statistical significance (* 0.05: samples are not significantly different compared to reference).



(a) HBG



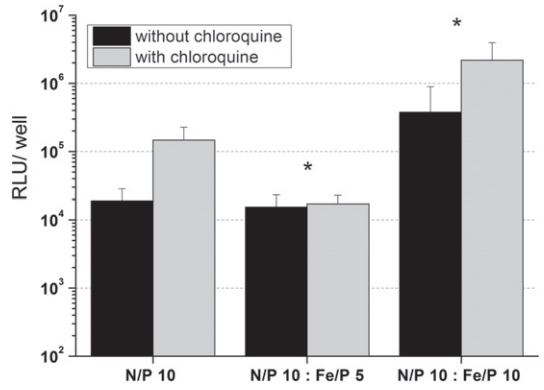
(b) HBS

Fig. 6. Cell viability was determined by a CellTiter Glo assay. Experiments were done in triplicates. A Student's *t*-test was performed to assess statistical significance (* 0.05: samples are not significantly different compared to reference). pDNA concentration is 10 μg mL^{-1} in each sample.

been showed to increase the transfection efficiency by roughly 10-fold when prepared in HBG and presenting small sizes [36]. In contrast, the transfection ability of polyplexes prepared in HBS shows no significant changes in the presence of chloroquine [36]. Our results corroborate these findings, showing an increase in the transfection efficiency when polyplexes are prepared in HBG, panel a, and no variation of those prepared in HBS, panel b. This is again related with the size of the complexes, showing that endosome escape is not a critical point for larger polyplexes. In the presence of Fe(III) the promoter effect of chloroquine is no longer observed when comparing with related complexes in its absence. The increase in polyplex size may explain the reduction of the positive action of chloroquine in gene expression, but for polyplexes at N/P:Fe(III)/P = 10:5, this explanation is not valid. This suggests that the presence of Fe(III) may directly inhibit the effect of chloroquine.

Moreover, a flow cytometry analysis was performed on HUH-7 transfected cells to inspect whether differences detected between pDNA–PEI and pDNA–PEI–Fe(III) complexes arise from the internalisation step. The uptake of pDNA complexes into the cells was quantified by measuring the emission of Cy5 dye attached to the plasmid. According to the experimental procedure, only the Cy5-pDNA internalised by the cells should be detected. The distribution of fluorescence intensities for the polyplex compositions that were tested are depicted in Fig. 8.

First, the internalisation of reference complexes prepared at N/P 6 and 10, in HBG and HBS, panel a, was analysed. In both buffers, a higher degree of internalisation is observed for complexes formed at N/P 6 than for those at N/P 10. This observation must be addressed taking into account the higher amount of free PEI molecules in the latter, which has been considered responsible for reducing the cellular association of complexes [42]. Furthermore, complexes formed in HBS reveal a moderate degree of internalisation, which is somewhat surprising considering their larger size. However, it should be noted that complexes formed in a medium with high ionic strength have a tendency to exhibit a lower surface charge than those prepared at low ionic strength, see Fig. 2d.



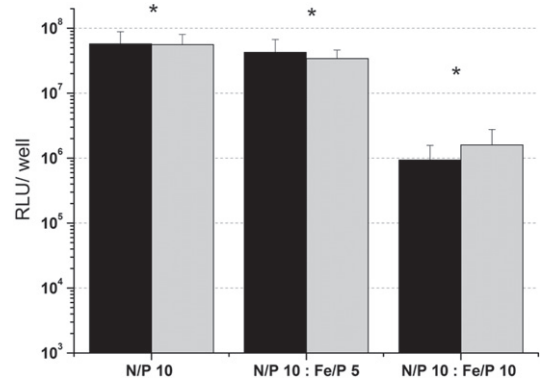
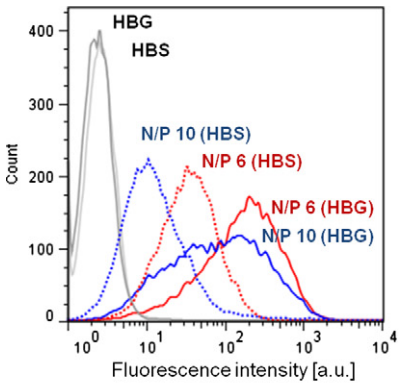
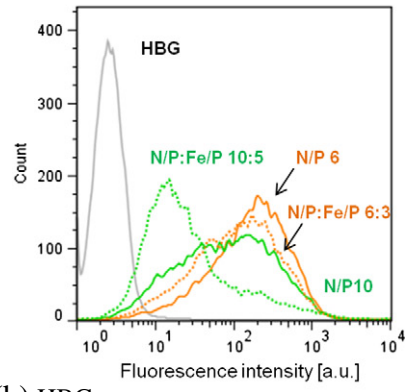


Fig. 7. The effect of the addition of chloroquine to the medium during transfection assays in HeLa cells. Luciferase activity is presented as mean values ± SD of triplicates. pDNA concentration is 10 μg mL-1 in each sample. A Student's *t*-test was performed to assess statistical significance between samples with and without chloroquine (* 0.05: samples are not significantly different compared to reference). Note that the experimental conditions are different from those of Fig. 3, now with the medium being changed 4 h post-transfection.





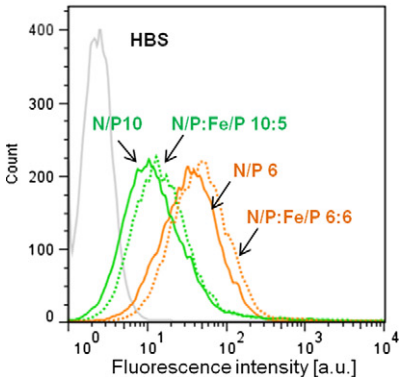


Fig. 8. Cellular uptake of pDNA complexes in HUH-7 cells represented in flow cytometry histograms. (a) pDNA–PEI complexes at N/P 6 and N/P 10 in both HBG and HBS; (b) pDNA–PEI and pDNA–PEI–Fe(III) complexes in HBG; and (c) pDNA–PEI and pDNA–PEI–Fe(III) complexes HBS. The charge ratios are indicated for each curve. Negative controls of unstained cells in HBS or HBG are also represented in all histograms. pDNA concentration is 50 μg mL^{-1} in each sample.

Figs. 8b and c gather the results obtained for complexes prepared in each of the buffers with and without Fe(III) for a clearer comparison.

The uptake efficiency of pDNA–PEI–Fe(III) complexes prepared in HBG is lower than that attained for pDNA–PEI complexes. This difference is more evident in system N/P:Fe(III)/P = 10:5 than N/P:Fe(III)/P = 6:3. However, this detrimental effect did not affect the transfection efficiency of the complexes for N/P:Fe(III)/P = 10:5 (Fig. 3c), contrarily to what is observed in complexes prepared in N/P:Fe(III)/P = 6:3 (Fig. 3a). In the case of polyplexes prepared in HBS, panel c, the curves obtained for the ternary complexes show a slightly beneficial effect in the cell entry stage, which can be ascribed to the presence of Fe(III). This happened to the same extent for both N/P ratios tested. As observed in Fig. 3a, pDNA–PEI–Fe(III) at N/P:Fe(III)/P = 6:6 showed a higher transfection efficiency. At N/P:Fe(III)/P = 10:5, in Fig. 3c, the transfection efficiency is nearly the same as that obtained for pDNA–PEI complexes.

4. Discussion

The condensation of DNA with Fe(III) has been observed experimentally, but the degree of condensation and stabilisation of the complex is limited [43,44]. On the other hand, PEI is known as one of the most efficient non-viral vector but possesses a main drawback, its high cytotoxicity. Fe(III) on its own is also harmful to the cells, but this property suggests the opportunity of an iron-mediated oxidative attack to vulnerable cancer cells, that are commonly deficient in antioxidant enzymes, as a mechanism to recede tumours [29,45,46]. It has been observed that Fe(III) acts as a promoter of DNA condensation in DNA-PEI complexes [15]. Furthermore, the stable polyplex prepared with lower concentration of PEI resulted in more efficient polyplex decondensation [15], as well as in a higher biocompatibility [16].

The functional effect that Fe(III) ions have on the properties of pDNA–PEI complexes generated in different buffer conditions and how, in turn, this affects their biological activity was investigated. It is well known that the formation of polyplexes is highly influenced by the chosen solution properties, including the nature of buffer used and the ionic strength of the solution [32,47]. This modulates the physical

properties of the resulting complexes, such as the extent of condensation, size and charge [36,48]. In the present work, the formation of pDNA–PEI and pDNA–PEI–Fe(III) complexes was investigated under different ionic strengths, ranging from the physiologically relevant (100–150 mM) to low (30–50 mM) and more indicated ionic strength to obtain suitable pDNA complexes for gene delivery. The addition of Fe(III) to pDNA–PEI polyplexes showed an enhancement on the condensation of pDNA, independently of the buffer used (Fig. 1). As expected, larger complexes were obtained at high N/P ratios and under high ionic strength (HBS), when compared to the observations at low salt conditions (HBG) (Figs. 2a and b). Two distinct behaviours were discerned after metal addition to highly charged complexes: (i) at low salt conditions (HBG), the presence of Fe(III) favours an increase in the size of the complexes, whereas (ii) at high and intermediate salt conditions (HBS and Ac50), a reduction occurs (Figs. 2b and c). According to the core-shell model [39], the excess of charge in overcharged complexes is accommodated in the form of positively charged polymer "tails" protruding from the complex, which produces an electrostatically stabilising shell around the particle avoiding inter-complex interactions and, thus, aggregation. At high ionic strength, the repulsion between the shells of two neighbour particles is screened, and aggregation may takes place. A possibility is that the presence of Fe(III) acts as destabiliser of this shell in low salt concentrations, and as a stabiliser in the opposite conditions. Some evidences of this effect has been observed in previous work using different mixing orders for polyplex formation, suggesting an interesting approach for the control of particle size [17]. These results deserve attention in future work. The transfection efficiency of pDNA–PEI complexes was generally found to increase with the size of the particles [36]. It should be noted that polyplexes with large sizes are generally less successful for gene delivery *in vivo* [49]. It is therefore highly recommended to prepare complexes in low salt conditions to attain small size particles. However, it has also been reported that, to reach efficient gene delivery, complex aggregation should occur before their entry into the cell [38,50]. Thus, it is still of great interest to study the aggregation properties of polyplexes and its influence on the process of transfection.

In general, the addition of Fe(III) to pDNA–PEI complexes leads to an equal or lower gene transfer efficiency, with the polyplexes prepared at low ionic strength buffers showing the least efficiency (Figs. 3 and 4, panels a and b). At high salt conditions, the inhibition of the transfection efficiency is less evident and, particularly in the case of polyplexes prepared at N/P:Fe(III)/P = 6:6 in HBS, there is an increase in transfection efficiency of 5.3 times compared to the analogous pDNA–PEI complex, in the HUH-7 cell line (see Fig. 3a). Since the average size of the complexes is smaller in the presence of Fe(III), size cannot account for this increase. The effect of the metal ions in complexes formed in acetate buffers at intermediate (Ac50) and high ionic strength (Ac100) is lower and not noticeable, respectively, Figs. 4b and c. This may suggest that the main hurdle to overcome by pDNA–PEI–Fe(III) complexes is the endosomal release. In previous work [17], we have concluded that chelation of Fe(III) with the amine groups of PEI is of paramount importance to the final complex conformation. This property is fundamental to produce a high condensation of pDNA and reduce the size of the pDNA–PEI–Fe(III) complexes. However, the complexation of the amine groups by the metal ions may represent a problem to transfection efficiency, due to the possible decrease in the buffering capacity of PEI. Note that Fe(III) is a very versatile metal, extremely efficient in acid–base reactions, and one which is able to chelate to Lewis bases. Upon chelation, it is difficult to reverse the process, considering that this interaction is very strong even at low pH [19]. This correlates well with the fact that chloroquine itself could not promote the release of pDNA–PEI–Fe(III) complexes from the endosome, as it did with the pDNA–PEI analogue, probably due to some interference of Fe(III). Some studies indicate that chelation between amine groups and metal species is dependent on the salt content of the medium [21,51], and this fact may be an important feature in some systems, but further studies are needed.

The reinforcement of the positive charge of the PEI chains by the chelation of Fe(III) and the increase of the weight of complex is likely to improve the interaction between the polyplex and the negatively charged cell membrane, improving the uptake of the cells. However, this is observed solely in the case of complexes prepared at high ionic strength media, HBS (Fig. 8c). The existence of PEI-Fe(III) chelation may indicate a cause to reduction of the harmful effect of PEI in the cells (Fig. 6), corroborating prior results [16]. An additional attempt to reduce the cytotoxicity of pDNA–PEI polyplexes resides in the reduction of the number of PEI by replacement with Fe(III). It can be seen, from analysis of the results, that the decrease of the N/P ratio is not relevant to the pDNA–PEI efficacy *in vitro*, but this may be related with the increase in the size of complexes, Fig. 2c. Systems in which Fe(III) is added maintain the same levels of transfection efficiency as the reference (N/P 6 in HBG and HBS), using N/P 4 and 5, and at the same time allowed to control, to some extent, the size of the complexes, Fig. 5c. In previous results, it was shown that the final conformation of the ternary complex is not dependent on PEI molecular weight and architecture [17], which may predict the possible advantage that may arise by the use of PEI with more biocompatible backbones. The use of Fe(III) proved to be a valuable strategy to regulate the properties of the complex, including complex size, and also polyplex biocompatibility.

5. Conclusions

This study presents a comparative investigation of the physicochemical properties and transfection efficiencies of pDNA–PEI and pDNA–PEI–Fe(III) complexes generated in different biological buffers, ranging from low to high salt concentration regimes. Results have shown that the effect of Fe(III) ions on the properties and transfection activity of polyplexes is strongly dependent on the ionic strength of the buffer. At high ionic strength, Fe(III) enhances pDNA–PEI condensation, reduces the complex size, increases the cellular uptake and shows less perturbation of the transfection efficiency when compared with reference systems. On the other hand, at low salt content, the metal also enhances the pDNA condensation but leads to some increase in the size of the complexes and to a reduction of their cellular uptake and transfection activity. Moreover, the interaction of Fe(III) with the amines of PEI is accompanied by a mitigation of polycation cytotoxicity. In this context, the decrease in the number of PEI molecules in the complexes leads to a small decrease in the transfection efficiency but still keeps values compared with those of reference complexes, while simultaneously allowing the regulation of the complex size.

Transparency document

The Transparency document associated with this article can be found, in online version.

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