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Control of the transfection efficiency of human dermal fibroblasts by adjusting the characteristics of jetPEI®/plasmid complexes/polyplexes through the cation/anion ratio

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Abstract

Primary cells are in general more difficult to transfect than most animal cells. Here we report on the efficient transfection of human dermal fibroblasts with jetPEI®/DNA complexes (polyplexes). The polycation/DNA ratio was varied with respect to both transfection efficiency and cell viability and the different DNA-polyplexes were characterized by different physicochemical methods, namely ζ -potential measurement, light scattering and scanning electron microscopy. Interestingly these data point to the fact that our DNA-polyplexes possess a core-shell structure in the wet state. We find that the range of jetPEI®/DNA ratios, expressed as N/P (nitrogen-to-phosphate) ratio, at which high transfection and high cell viability is observed is quite narrow. Transfection efficiency is high for N/P-ratios below 15 and cell viability is high at high N/P-ratios, the optimum of both occurs in the range of 10 to 15. The transfection maximum is observed at over 80% of transfected cells in the range of N/P-ratio of 7.5 to 10, whereas the generally recommended N/P-ratio for jetPEI®/DNA transfection experiments is 5. At a N/P-ratio of 15, transfection is still as high as at a N/P-ratio of 5, but viability is almost twice as high. We found that the cytotoxicity of the polyplexes is related to their size, and is very small for particles of small size. Our data suggest that the morphologies of the polyplex-particles discussed here are better described by a core-shell structure rather than a conventional homogeneous “scrambled egg” structure.

Introduction

Gene therapy has attracted much interest as an alternative approach for the treatment of severe human diseases. The therapeutic effect is achieved by delivering exogenous nucleic acids into the target cells that induce the production of therapeutic proteins or modulate damaged cellular functions [1–3]. Due to large size, negative charge, and hydrophilicity, nucleic acids cannot easily cross the cellular membrane and need to be incorporated into a delivering carrier [4]. This carrier condenses DNA into small particles and allows DNA transfer through the cell membrane and protects genes from degradation in the cellular environment [1–4]. Depending on the carrier, gene delivery can be achieved using viral or non-viral methods. The viral techniques are based on the use of viruses which genetic material has been replaced with therapeutic genes [5]. Although these systems were found highly effective, their therapeutic applications are however limited due to the possible side effects such as immunogenicity and oncogenic effects [6,7]. In this context, non-viral carriers such as positively charged polymers have attracted much interest due to their high accessibility and the low adverse effects level. In this case, gene delivery performed by polyplexes, soluble interpolyelectrolyte complexes of the nucleic acid and the positively charged polyelectrolyte also called transfection agent [8–12]. The transfection yield and the cytotoxicity depend on the chemistry transfection agent and polyplex properties such as size, charge, and composition. These characteristics are controlled by the parameters of the flocculation process such as the concentration, the molecular weight and the chemical nature of the reagents, their stoichiometric balance (N/P ratio), the mixing order, the ionic strength, pH and the temperature of the reaction mixture [13]. In addition, the transfection ability of the complexes prepared under similar conditions may vary for different cell lines. Thus, for the fabrication of an efficient carrier with low toxicity, it is indispensable to optimize its preparation for a specific cell line.

A general approach that allows adjusting the transfection rate of a particular type of cells is to control the stoichiometric balance between the number of positive and negative charges by employing an increased concentration of positively charged macromolecules in the reaction mixture (increasing N at constant P in the N/P ratio). Typically, this leads to the formation of the smaller size complexes with an increased ζ -potential. The latter is assumed to play an important role in the floc stability in a buffer by preventing particle fouling and sedimentation and their interactions with biological media such as blood [13,14]. The positively charged vectors are known to enhance vector cell binding and their internalization [15–20] through the ionic interactions with proteoglycans present on the cell surface [21–23]. The main drawback of this approach is that with an increase of N/P ratio the polyplexes with a high charge density induce undesirable cytotoxic effects due to the aggregation of the complexes on the cell surface leading to membrane rupture [24,25]. In this context, particularly challenging remains the transfection of primary cells, where a low efficiency of the complexes is typically coupled with high cytotoxicity [26–29].

Here, we report on how the DNA concentration controls the cytotoxicity and the ability of the polyplexes to transfect CPRE2 cell line, human adult dermal fibroblasts, established from the abdominoplasty of a 38-year-old woman suffering from obesity [30]. Linear poly(ethylene imine) (jetPEI®, Polyplus Transfection™) was selected as a transfection agent. Due to its high positive charge density jetPEI® effectively condenses DNA into small particles and protects them from the degradation in endosomes [16,31]. The buffering capacity of amine groups or “proton sponge” effect results in the osmotic swelling of endosomes leading to the endosomal escape of the particles into the cytosol [32]. Although PEI-based vectors were proved to be highly efficient in the transfection of various cell lines [7,9,11,12,33], the possible toxic side effects of PEI limit its application in systematic transfection experiments [34–36]. In present work, polyplexes with

different N/P ratios were prepared using jetPEI® and plasmid DNA coding green fluorescent protein (GFP) and were characterized by ζ -potential measurement, light scattering, and scanning electron microscopy. The transfection yield was calculated based on GFP expression detected by flow cytometry and fluorescent microscopy. We found that the cytotoxicity and the transfection properties of the polyplexes are related to their size (N/P ratio) and not to their ζ -potential. Our data suggest also that the morphologies of the studied polyplex-particles are better described by a core-shell structure rather than a conventional homogeneous “scrambled egg” structure.

Experimental Section

Materials

Linear poly(ethylene imine) (jetPEI®, $\overline{Mw} \approx 22\ 000$ g/mol, Polyplus Transfection™, Illkirch-Graffenstaden, France) was used as received. Plasmid DNA (gWiz GFP, 5757 bp) was obtained from Aldevron (Fargo, ND). Sodium chloride ($\geq 99.5\%$ pure, suitable for cell culture) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). All chemicals were used without further purification. Water used for the preparation of all solutions was purified with a Milli-Q Gradient water purification system (Millipore, Molsheim, France). The solution of sodium chloride was filtered through a 0.22 μm filter before use. All experiments were performed with freshly prepared solutions.

Cell culture

CPRE2 cell line, primary adult human dermal fibroblasts, was established from an abdominoplasty of a 38-year-old woman suffering from obesity. Briefly, the tissue was treated mechanically to

remove hypoderm (especially adipose tissue) and then treated with enzymes to remove the epidermal part. Then, dermal fibroblasts were selected upon culture.

The cells were maintained at 37 °C under 5% CO₂ atmosphere in Opti-MEM® medium supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO). The complete procedure for establishing the cell line is described elsewhere [30].

Complex preparation

The solutions of PEI/DNA complexes were prepared by mixing jetPEI® and DNA solutions in 0.15M NaCl at corresponding concentrations (Table 1). Briefly, 50 µL of PEI solution in 0.15M NaCl was added to 50 µL of DNA solution in 0.15M NaCl, and the resulting mixture (100 µL) was gently vortexed. After 20 min of incubation time at room temperature, the polyplexes were added to the CPRE2 cells.

N/P ratio	Concentration of initial DNA solution, µM	Concentration of initial jetPEI® solution, µM
5	60	300
7.5	50	300
10	30	300
15	20	300
30	10	300

Table 1. Concentrations of jetPEI® and DNA solutions in 0.15M NaCl applied for polyplex preparation at different N/P ratios.

Transfection experiments

CPRE2 cells were seeded in 12-well cell culture plate (Costar, Corning, NY) at a density of 10 000 cells/well in Opti-MEM® medium supplemented with 10% (v/v) FBS. After 24 h the culture medium was changed with a fresh portion, and 100 µL of polyplexes were added to each well containing cells in 1 mL of the serum-containing medium. The GFP expression was analyzed 48 h after transfection by flow cytometry. All experiments were performed in triplicate at cell passage 6.

Flow cytometry assay

Transfected cells were analyzed for GFP expression on a FACSCalibur using CellQuest software (BD Biosciences). To discriminate between viable and dead cells, cells were stained using TOPRO®-3 assay (dead cell indicator) before cytometry analysis. Experiments were performed in triplicate.

Dynamic Light Scattering (DLS) measurements

The estimated diameter and size distribution of polyplexes were determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, Ltd, UK). We stress that in order to reliably measure the hydrodynamic diameter of the floc particles (polyelectrolyte complex particles), the sample volume must be at least 1 mL. For DLS measurements, 200 µL of corresponding jetPEI® (300 µM, 0.15M NaCl) were added to 200 µL of DNA solutions (various concentrations, please refer to Table 1) and the mixture was vortexed gently. After 20 min of incubation at room temperature, the polyplex solution was placed in a quartz cuvette and diluted with 600 µL of 0.15M NaCl in

order to reach the desired sample volume and the particle concentration. The size distribution was obtained at 25 °C. Time for sample equilibration was kept at 60 s.

ζ-potential measurements

The ζ-potential of the polyplexes was determined at 25 °C (Zetasizer Nano ZS ZEN3600, Malvern Instruments, Ltd, UK). The polyplexes were prepared according to the protocol used for transfection. After 20 min of incubation at room temperature, the sample was diluted with Milli-Q water up to 1 mL and transferred into the measuring cell.

Transmission electron microscopy (TEM)

The samples were analyzed with a transmission electron microscope operating at 120 kV (Philips CM12) equipped with a Megaview III Soft Imaging System camera. jetPEI®/DNA complexes or polyplexes were prepared according to the protocol used for transfection. For TEM grid preparation, 5 μL of polyplex solution were placed on 400 mesh carbon-coated copper grids. After 2 min, the solution was removed with a filter paper and the sample was treated with a staining solution of uranyl acetate (2 wt % in water) for 1 min and then dried with a filter paper.

Results and Discussion

In this study, polyplexes were prepared by adding the solution of transfection agent to the solution containing DNA. We used a plasmid DNA (gwiz GFP) coding for GFP for studying transfection activity of the complexes by the methods of flow cytometry and fluorescent microscopy. The cell viability after transfection was evaluated using TOPRO®-3 staining assay.

In the present experimental sequence, the jetPEI® concentration was kept constant (15 nmol), while the concentration of DNA was altered (Table 1). The cells transfected according to the standard protocol provided by Polyplus transfection™ with polyplexes prepared at N/P=5 using the solution of DNA at concentration 60 μM (3 nmol of DNA in 50 μL of 0.15M NaCl) [37] was used as a reference. The transfection rate was determined as a percentage of fluorescent cells over the total cell population 48 h after transfection. The bright field and fluorescence microscopy images of the transfected cells are presented in Fig. 1. The highest fluorescent rate was found for samples treated with polyplexes prepared at N/P=7.5, whereas cell viability increased when cells were incubated with polyplexes formed at N/P=15 and 30.

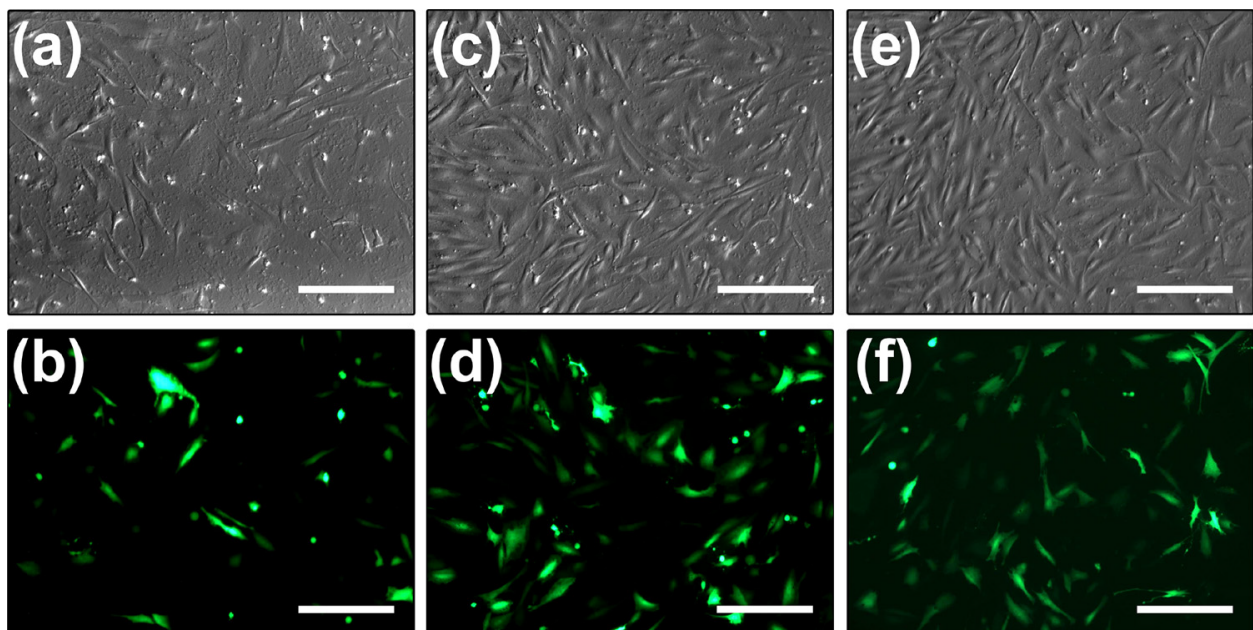


Fig. 1. Optical and fluorescence micrographs of CPRE2 cells treated with polyplexes prepared at N/P=5 (a and b), N/P=7.5 (c and d) and N/P=15 (e and f) 48 h after transfection. The polyplexes were prepared at a constant concentration of jetPEI® (15 nmol). Scale bars are 250 μm. For the observation of fluorescence micrographs in colour in this figure, the reader is referred to the web version of this article.

The flow cytometry data (Fig. 2a) confirmed that the highest transfection efficiency (about 89%) was observed for the polyplexes prepared at N/P=10. The transfection rates for complexes formed at N/P=5 (P=3 nmol), N/P=10 (P=1.5 nmol) and N/P=15 (P=1 nmol) were about 72%, 89% and 73%, respectively. We observed that the viability increases for cells exposed to complexes formed at higher N/P ratio, i.e. at lower DNA concentration. The lowest transfection rate (15%) and the highest cell viability (87%) were obtained with the cells transfected with polyplexes prepared at N/P=30 (P =0.5 nmol). Those results might be explained by the low DNA concentration (0.5 nmol) applied for the cell transfection. The best compromise between the polyplex toxicity and efficiency lies in the range of N/P ratios of 10 to 15.

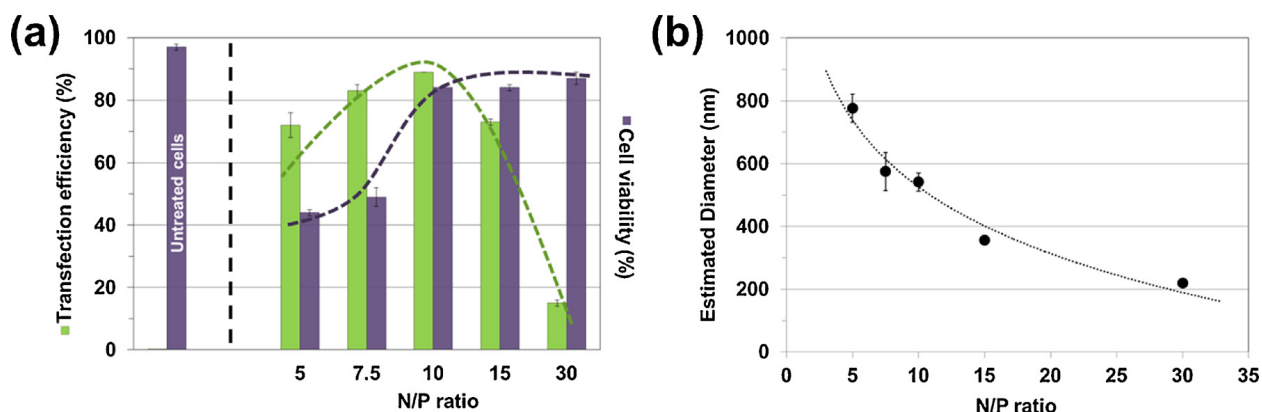


Fig. 2. (a) Variation of the percentage of transfection efficiency (green) and viability (violet) of CPRE2 cells treated with polyplexes formed at different N/P ratios determined from flow cytometry data. (b) Variation of the hydrodynamic diameter of polyplexes as a function of the N/P ratio. The viability is higher for the cells transfected with the particles of a smaller size. The best results (transfection rate about 89% and a cell viability of about 84%) were obtained for the polyplexes prepared at N/P=10. The dotted lines outlining the shape of the bar charts for transfection efficiency and for cell viability have no concrete physical meaning, they are only intended to guide the eye. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

To identify the parameters influencing the transfection rate, we primarily studied polyplex size and ζ -potential as these parameters are considered to play an essential role in the gene delivery process [9]. We found that the decrease of DNA concentration in the complex yielded the formation of particles with rather constant ζ -potential (Table 2). Since the ζ -potential of the particles underwent only slight changes, it was concluded that neither transfection efficiency nor cell viability was dependent on this parameter.

Then, the size of polyplexes was analyzed by dynamic light scattering (DLS) (Fig. 2b and Table 2). The data were obtained with freshly prepared samples in 0.15M NaCl at 25 °C. The DLS results showed that the decrease in the DNA concentration leads to the formation of smaller particles. The combined results from DLS and flow cytometry measurements suggest a strong correlation between the particle size and cell viability: smaller particles formed at lower P (at higher N/P ratio) were less toxic in the transfection of primary fibroblasts (Fig. 2). A maximum in the transfection yield was achieved for the particles with diameter 542 nm. A further decrease in the floc size led to a lower transfection efficiency and a higher cell viability.

The size distribution and the floc morphology were further studied by Transmission Electron Microscopy (TEM). For this purpose, the polyplexes were prepared according to the protocol used for transfection experiments. The samples were negatively stained with 2% uranyl acetate on the carbon-coated copper grids and then were dried with filter paper. As shown in Fig. 3, the jetPEI®/DNA complexes formed spherical particles with homogeneous structure. The faint dark rings visible around the polyplexes in the TEM images (Fig. 3) are common staining artifacts arising from the uranyl acetate treatment. Interestingly, the diameter of the dry particles is about 180 ± 70 nm and does not depend much on the composition of the complex. Although the

drying procedure may cause artefacts in TEM imaging, but the fact that we cannot distinguish any size differences in the different dry polyplexes has interesting consequences. First of all, if the dried objects have the same size, their dry mass per complex particle is very similar.

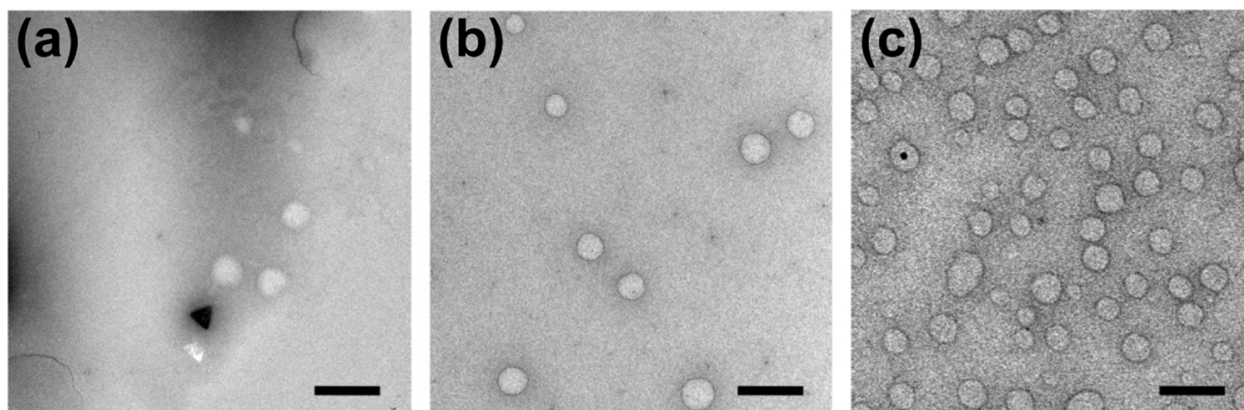


Fig. 3. Transmission electron micrographs of polyplexes prepared at (a) $N/P=10$ ($P = 1.5$ nmol), (b) $N/P=7.5$ ($P = 2$ nmol) and (c) $N/P=5$ ($P = 3$ nmol). Scale bars are 500 nm.

Together with the fact that their diameter in the wet state decreases from about 780 nm at $N/P=5$ to about 220 nm at $N/P=30$ while their ζ -potential stays more or less constant at about +33 mV, one is lead to conclude that the particles do not possess a homogeneous chemical composition (“scrambled egg”), but rather a core-shell structure in which the corona is rich in polycation whereas the core is rich in plasmid. As expected, the electron densities of the PEI and the plasmid are too close to corroborate any core-shell morphology via the TEM images. Since the volume per charge and the charge-to-charge distance are much smaller for PEI (molar mass=43 g/mol per charged unit) than for DNA (molar mass=304 g/mol per charged unit) it is difficult for both partners to form a complex with 1:1 stoichiometry (see also polycation/polyanion mass ratio in Table 2). For polyelectrolytes of similar length and similar charge density, one would expect that the polycation/polyanion complex is compact and little hydrated at 1:1 stoichiometry and less

compact and more hydrated as one moves away from stoichiometry. In the case discussed here, we not only have different charge densities but also different lengths of the partner polyelectrolytes which favours non-classical polyelectrolyte complexation.

N/P ratio	Polycation/ Polyanion mass ratio	<i>P</i>, nmol	ζ-potential, mV	Estimated Diameter, nm
5	0.4	3	34 ± 4	777 ± 45
7.5	0.5	2.5	36 ± 4	575 ± 61
10	0.8	1.5	37 ± 1	542 ± 29
15	1.0	1	30 ± 1	357 ± 10
30	2.3	0.5	27 ± 6	220 ± 12

Table 2. Summary of the particles hydrodynamic size and ζ -potential measured by DLS and polycation/polyanion mass ratio for given N/P ratios and DNA concentration (P).

In our case, we see large and strongly hydrated particles at N/P=5 and small and little hydrated particles at N/P=30. Overall this points to a structure in which the DNA is more hydrated when less PEI is present and in which the corona is always rich in PEI (because the surface potential is always positive), but with a composition of the complex in the shell which is independent of the N/P ratio (because the ζ -potential is constant). At N/P=5 there is not enough PEI for all the DNA which requires the DNA to form large and strongly hydrated particles for achieving a positively charged complex. As the N/P is increased the surface-to-volume ratio becomes favorable for forming smaller and smaller particles with a similarly thick shell rich in PEI. Note that coreshell

structures have already been discussed for polyplexes [38], but here we see evidence for such structures also in the wet state and not only after drying which in our case leads to a deswelling by a factor of 4–5 and such to major structural and/or morphological rearrangements.

Thus, while the dry size of the polyplex particles between $N/P=5$ and $N/P=10$ is basically constant, there is a clear correlation between the size of the hydrated polyplex particles and their transfection properties (Fig. 2). For an increasing N/P ratio from 5 to 30 the size of the hydrated complex particles is decreasing from about 780 nm to about 220 nm, while the cell viability and transfection efficiency show opposing trends in the same interval of N/P ratios. More specifically, the cell viability drops from above 80% to below 50% at $N/P=7.5$ and below whereas the transfection efficiency drops from values above 70% to below 20% at a N/P ratio of 30 (see the lines guiding the eye in Fig. 2a). Thus the optimum transfection conditions occur between $N/P=10$ and $N/P=15$, where the hydrated particle size is in the range of 360 to 540 nm. The present approach underlines the importance of DNA concentration for engineering polyplexes (Table 2) and demonstrates how advantageous polyplex properties can be attained by balancing between poor transfection efficiency and poor cell viability (Fig. 2).

In summary, we showed that primary adult human fibroblasts can be readily and efficiently transfected with the jetPEI®-based polyplexes. The transfection efficiency, cytotoxicity and the size of these vectors can be finely tuned by changing the concentration of DNA involved in the complex preparation. We found that the vector efficiency and cell viability depended on the particle size and not on the ζ -potential. The highest transfection efficiency and the highest cell viability occur in the range of $N/P=10$ to $N/P=15$.

Interestingly, our data suggest that the morphologies of the polyplex-particles discussed above are better described by a core-shell structure rather than a conventional homogeneous “scrambled egg”

structure. While this is a somewhat surprising result, core-shell structures have been suggested before in polyplex preparation and their occurrence may help to better understand transfection efficiencies in this group of transfection agents. We believe that the present work contributes to the development of a safe and simple approach to enhance the transfection of the “difficult-to-transfect” primary cells and in establishing a robust protocol for systematic transfection experiments.

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ABBREVIATIONS

DLS, dynamic light scattering; TEM, transmission electron microscopy; GFP, green fluorescent protein; PEI, poly(ethylene imine); DNA, Deoxyribonucleic acid.

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