Impact of physicochemical properties of DNA/PEI complexes on transient transfection of mammalian cells

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Abstract

Polyethyleneimine (PEI) has been used extensively for transient gene expression (TGE) in mammalian cell cultures. However, the relationship between DNA/PEI complex preparation and their biological activity has not been fully established. Here, a systematic study of DNA/PEI complexes, their physicochemical properties during formation and their transfection efficiency was performed on a virus-like particle (VLP) production platform. The same chemically defined cell culture medium for DNA/PEI complex formation was used as an alternative to simple ionic solutions to minimize changes in complex properties during transfection. Upon formation, an initial concentration of 1×10^{10} DNA/PEI complexes/mL underwent partial aggregation with an average size of 300 nm. The participation of NaCl ions in the evolution of complexes was analyzed by X-ray spectroscopy, stressing the relevance of complexing media composition in TGE strategies. After 15 minutes incubation, 250 complexes plus aggregates per cell were estimated at the time of transfection. Such heterogeneous preparations cannot be easily characterized; subsequently, nanoparticle tracking analysis (NTA) and cryo-electron microscopy were combined to achieve a complete picture of the preparation. Finally, the contribution of each DNA/PEI complex subpopulation was tested by drug inhibition endocytosis. Interestingly, all complexes delivered DNA efficiently and high size aggregates, which enter through macropinocytosis, when inhibited presented a major contribution to transfection efficiency. There is a need to understand the physicochemical factors that participate in DNA delivery protocols. Hence, this study provides new insights into the characterization of DNA/PEI complexes that will assist in more productive and reproducible TGE strategies.

Key Words: Transient Transfection, DNA/PEI complexes, Mammalian cells, Particle tracking analysis, Electron Microscopy

38 **Highlights**

39

- 40 • *DNA/PEI complexes formed immediately and underwent aggregation.*
- 41 • *1E+10 complexes/mL with 300-nm sizes were found after DNA/PEI complex formation.*
- 42 • *250 complexes per cell and aggregates were found at the time of transfection.*
- 43 • *Morphology and composition of complexes were analyzed by EM.*
- 44 • *Complex aggregates were required to enhance HEK 293 transient transfection.*

45 **Abbreviations**

46

47	Cl	Chloride
48	CPZ	Chlorpromazine
49	Cryo-TEM	Cryogenic Transmission Electron Microscopy
50	DLS	Dynamic Light Scattering
51	EDS	Energy Dispersive X-ray Spectroscopy
52	eGFP	enhanced Green Fluorescence Protein
53	EIPA	5(N-ethyl-N-isopropyl) amiloride
54	FreeStyle	FreeStyle™ cell culture medium
55	FU	Fluorescence Units
56	Hpt	Hours Post Transfection
57	Kcps	Kilo counts per second
58	Na	Sodium
59	Nys	Nystatin
60	NTA	Nanoparticle Tracking Analysis
61	Min	Minute
62	P	Phosphorus
63	PBS	Phosphate Buffer Saline
64	PDI	Polydispersity Index
65	PEI	Polyethyleneimine
66	RFU	Relative Fluorescence Units
67	RT	Room Temperature
68	SEM	Scanning Electron Microscopy
69	SD	Standard Deviation
70	TEM	Transmission Electron Microscopy
71	TGE	Transient Gene Expression
72	U	Uranium
73	VLP	Virus-like particle
74		

75 **Introduction**

76 Cationic polymers represent a very widely used means for the delivery into cells of large DNA plasmids and
77 small oligonucleotides [1,2]. Polyethyleneimine (PEI) has become a major reagent in this field [1,3]. PEI is
78 simple to use, efficient with suspension cells, compatible with serum free media and cost effective [4]. This
79 bulk chemical comprises amino groups with high cationic charge density capable of being protonated, that
80 were discovered to efficiently introduce DNA into cells [5]. Over the last 3 decades, it has been used in large-
81 scale production of recombinant proteins by transient gene expression (TGE) [4]. TGE enables the rapid
82 screening of potential therapeutic candidates, and the generation of sufficient quantities of biomaterial for
83 preclinical and early clinical studies [4]. Its use in vaccine production platforms to mitigate emerging epidemics
84 is of particular relevance, and huge efforts are being undertaken in this direction [6–8]. In previous studies,
85 intracellular tracking of a PEI-mediated TGE for the production of HIV-1 based virus-like particles (VLP) as
86 vaccine candidates was conducted [9,10]. It was found that DNA/PEI complex enter every cell, whereas only a
87 percentage produced VLPs. Hence, the assessment of the intracellular fate of those complexes might explain
88 to what extent protein production is achieved.

89

90 From a molecular point of view, cationic amines reduce the negative charges of DNA and thereby protect DNA
91 from nuclease degradation. The resulting positively charged complexes interact with the negatively charged
92 cell membranes [1]. Subsequently, their cellular uptake by several endocytic pathways has been widely
93 described [1,11]. While clathrin and caveolae-mediated endocytosis have been the most studied routes, there
94 are other mechanisms, such as macropinocytosis or non-endocytic pathways, that have also been reported
95 [1,11,12]. Indeed, when one pathway is inhibited, complexes may enter by alternative means [13]. Thereafter,
96 PEI molecules are thought to escape from endosomes through the known “proton sponge” effect and liberate
97 the complexes into the cytoplasm, from where they enter the nucleus and liberate the plasmid DNA [5].
98 However, this theory has not yet been confirmed [1]. For instance, the role of free polycations has also been
99 reported to participate actively in intracellular trafficking of the complexes [1].

100

Overall, there is a high level of heterogeneity reported in PEI-mediated processes, which is likely caused by the variety of transfection protocols developed over the years [4,11,14]. Firstly, several physicochemical factors critically affect the DNA/PEI complexation process, such as incubation time, temperature, medium viscosity, salt ion concentration or pH [4,14–18]. Secondly, DNA concentrations and DNA:PEI ratios also determine the stability and charge density of the complexes formed, as well as PEI composition [3,19,20]. Moreover, cell culture conditions (i.e. medium, cell concentration at time of transfection), cell type and the product of interest also have a relevant impact on TGE yields [4,14]. In addition, essential parameters of nanocarriers have recently been described in the nanomedicine field, including particle concentration and shape which must also be considered in PEI-mediated processes [19,21,22].

110

Here, a comprehensive study of how preparation time affects physicochemical properties of DNA/PEI complexes and their correlation with transfection performance has been conducted. The work was performed using a previously characterized VLP production method with suspension-adapted HEK 293 cells cultured in a chemically defined and protein-free cell culture medium, which was also used for the incubation of complexes before transfection [9,10]. The use of the same cell culture medium to prepare complexes reduced the alterations of DNA/PEI complex properties between their formation and their addition to the cells. In addition, its use is beneficial for the cultured cells, being a more nutritional solution compared with standard saline preparations used in TGE strategies, hence not diluting the medium during transfection.

119

120 **Materials and Methods**

121 *HIV-1 Gag-GFP VLP expression plasmid*

Complexes were formed by using a pGag-eGFP plasmid, encoding a Rev independent HIV-1 Gag protein fused in frame to the enhanced GFP (eGFP) [23]. The plasmid from the NIH AIDS Reagent Program (Cat 11468) was constructed by cloning the Gag sequence from pCMV55M1-10 [24] into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). It was prepared and purified as previously described [25].

126 *Cell line, culture conditions and transient transfection*

127 The cell line used was a serum-free suspension-adapted HEK 293 cell line (HEK293SF-3F6) kindly provided by
128 Dr. Amine Kamen from McGill University (Montreal, Canada). Cells were cultured in FreeStyle™ 293 medium
129 (FreeStyle) (Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% Pluronic® (Invitrogen, Paisley, UK).
130 Medium for cell growth was also supplemented with 1.6 mg/L of r-transferrin (Merck Millipore, Kankakee, IL,
131 USA), 19.8 mg/L of r-insulin (Novo Nordisk Pharmatech, Køge, Denmark) and 0.9X of an in-house developed
132 lipid mixture to maximize cell growth, as previously described [9]. Cells were maintained routinely at 20 mL
133 final volume in exponential growth phase in 125 mL disposable polycarbonate erlenmeyer flasks (Corning,
134 New York, NY, USA), shaken at 120 rpm in a humidified incubator at 37°C and 5% CO₂ in air.

135

136 HEK 293 cells were transiently transfected using 25-kDa linear polyethylenimine (PEI) (PolySciences,
137 Warrington, PA, USA) at 2E+6 cells/mL after a medium exchange with fresh FreeStyle medium. Transfections
138 were performed using a final DNA concentration of 1 µg/mL. DNA/PEI complexes were formed by adding PEI
139 to plasmid DNA diluted in fresh culture medium (10% of the total culture volume to be transfected). Briefly,
140 pGag-eGFP plasmid was diluted with FreeStyle medium (Invitrogen, Carlsbad, CA, USA) and vortexed for 10
141 sec (10µg/mL). PEI was then added at a 1:2 (w/w) DNA:PEI ratio and vortexed 3 times for one sec. The mixture
142 was incubated for 15 minutes (min) at room temperature (RT) and added to the cell culture [9]. Sampling was
143 conducted every 24 h after transfection (hpt). Cell growth and viability were assessed with the
144 NucleoCounter® NC-3000 automatic cell counter (Chemometec, Allerød, Denmark).

145 Particle Size Measurement

146 DNA/PEI complexes were immediately added to the cell cuvette for analysis. Dynamic light scattering (DLS)
147 experiments were performed using a Zetasizer Nano ZS instrument (Malvern instruments, Malvern, UK) with
148 a He/Ne 633 nm laser at 173°. The hydrodynamic diameter and polydispersity index (PDI) were calculated with
149 cumulative fit correlation at 25°C and 0.8872 cP. Briefly, DNA/PEI complexes were prepared in 1 mL final
150 volume, as previously described, and placed in disposable plastic macro cuvettes (Scharlab S.L., Barcelona,
151 Spain) followed by automated experimental data collection. Complex formation was observed for 30 min; 11
152 scans were performed in each independent measurement.

153 Nanoparticle Tracking Analysis (NTA)

154 DNA/PEI complexes were immediately injected for sample analysis. Nanoparticle tracking analysis was
155 performed with a NanoSight® LM20 device (NanoSight Ltd., Amesbury, UK). The data were analyzed with
156 NanoSight® NTA 3.1 software. Samples were diluted in sterile PBS (HyClone, Little Chalfont, GE Healthcare,
157 UK) prior to injection into the device chamber to obtain a concentration of approximately 1E+08 particles/mL.
158 Samples were injected, and independent analyses were carried out. Video recordings of 60sec length were
159 made. Subsequently, particles were identified and tracked by their Brownian motion at RT. Capture settings
160 were recorded with an sCMOS camera (camera level of 5 for complexes, and 11 for controls, viscosity: 0.9 cP)
161 and analyzed with a detection threshold of 5.

162 Cryo-Transmission Electron Microscopy (Cryo-TEM)

163 DNA/PEI complexes incubated for the desired time, were immediately plunged into liquid ethane. Their
164 morphology was studied by Cryo-TEM. A 2-3 µL amount of sample was blotted onto holey carbon grids
165 (Quantifoil Micro Tools, Großlobichau, Germany and Micro to Nano, Haarlem, Netherlands) previously glow
166 discharged in a PELCO easiGlow glow discharger unit. The samples were subsequently plunged into liquid
167 ethane at -180°C using a Leica EM GP cryo workstation and observed in a JEM-2011 electron microscope (JEOL
168 Ltd., Tokyo, Japan) operating at 200 kV. During imaging, samples were maintained at -181°C, and pictures
169 were taken using a CCD-multiscan camera (Gatan Inc., Pleasanton, CA, USA).

170 Electron Microscopy and X-ray spectroscopy

171 DNA/PEI complexes incubated for the desired time were immediately deposited onto the grid for preparation
172 by air-dried negative staining. Briefly, 8 µL of the mixture was placed onto grids and incubated at RT for 1 min.
173 Excess sample was carefully drained off the grid with the aid of Whatman filter paper. Sample was negatively
174 stained with 8 µL uranyl acetate (2%) by incubation for 1 min at RT. Excess stain was drained off, and the grids
175 were dried. Transmission electron microscopy (TEM) examination was performed with a JEM-1400 (JEOL USA
176 Inc., Pleasanton, CA, USA) transmission electron microscope equipped with an ES1000W Erlangshen charge-
177 coupled device camera (Model No. 785; Gatan, Pleasanton, CA, USA). Scanning electron microscopy (SEM)
178 images were assessed with a FE-SEM Merlin (Zeiss, Jena, Germany) scanning electron microscope. Images
179 were taken with in-lens mode with an EHT of 2-5 eV at 3 or 5.5 mm working distance and 100 pa spot size.
180 Samples prepared for SEM analysis were further characterized by X-ray spectroscopy. Samples coated with

181 uranyl acetate were scanned and analyzed with a coupled energy dispersive X-ray spectroscopy (EDS) unit at
182 a resolution of 138 eV. Samples were scanned in X-ray mode with an EHT of 5 EV, a 5.5 mm working distance
183 and a size spot of 500 pa in 20.000-30.000X. Phosphorous, chloride, sodium and uranyl elements were tracked
184 and quantified with Inca software (Oxford Instruments, Abingdon, UK).

185 Covalent labeling of PEI

186 Linear 25 kDa PEI (25 kDa, Polysciences, Warrington, PA, USA) was covalently labeled with Cy5 monoreactive
187 dye (Amersham, GE Healthcare, Little Chalfont, UK) by suspending one vial with 2 mg of PEI dissolved in 1 mL
188 PBS at pH 8.0, adapted from [16]. Reaction was performed for 4 h at RT under continuous mixing. To eliminate
189 Cy5 excess, the resultant Cy5-labeled PEI solution was dialyzed through a 5 kDa-cutoff cellulose membrane
190 (Sigma Aldrich, San Luis, MO, USA) in a 2 L PBS solution and pH 7.4 at RT for 7 d. Dialysis solutions were changed
191 every 24 h. PEI labeling was confirmed by absorbance measurement with Nanodrop 1000 (Thermo Fisher
192 Scientific, Waltham, MA, USA). Maintenance of PEI activity was confirmed by its consistency with the standard
193 transient transfection protocol. Cy5-labeled PEI was stored at -20 °C protected from light.

194 Flow cytometry

195 The percentage of GFP-positive cells and of cells interacting with DNA/PEI complexes was assessed using a BD
196 FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) with a two-laser configuration (488 nm and
197 635 nm). Laser 488 was used for GFP measurement and detected with GFP FITC-A detector, and laser 635 and
198 detector APC-A were used for Cy5 measurement. A total of 20,000 events were analyzed in every sample.
199 First, SSC-H vs FSC-A and FSC-A vs. FSC-H density plots were used to gate individual HEK 293 cell populations.
200 Secondly, positive Cy5 and GFP populations were assessed with GFP FITC-A vs. APC-A density plot and the
201 individual histogram for each fluorochrome. Gating was adjusted manually for each channel with an intensity
202 between 5E+2 and 1E+3 multiple fluorescence units. The results were analyzed with FACS DIVA software (BD
203 Biosciences, San Jose, CA, USA).

204 HIV-1 Gag VLP quantitation

205 The concentrations of HIV-1 Gag VLPs were assessed by fluorimetry using an in-house developed and validated
206 quantification assay [26]. VLP containing supernatants were recovered by cell culture centrifugation at 1000×g

for 5 min. Relative fluorescence unit (RFU) values were calculated by subtraction of fluorescence unit (FU) values of non-transfected negative control samples.

Inhibition of cell DNA/PEI complex uptake

To selectively inhibit endocytosis pathways, the following compounds were used: 10-30 μ M chlorpromazine (Sigma Aldrich, San Luis, MO, USA), 20-60 μ M nystatin (Sigma Aldrich, San Luis, MO, USA), and 10-50 μ M 5(N-ethyl-N-isopropyl) amiloride (EIPA) (Sigma Aldrich, San Luis, MO, USA). Passive transport was evaluated by maintaining the cells at 4°C for 4 h after transfection. Specific inhibitors were incubated for 1 h at 37°C at 120 rpm after a medium exchange process. Transient transfections were then performed with Cy5-labeled PEI, and at 4 h post transfection (hpt), a second medium exchange was performed. Sampling was conducted every 24 h until 96 hpt.

Prior to the definitive experiment, different concentrations of each inhibitor were tested to select the one showing transfection effects with minimal side effects on cell viability. To explore these conditions, preliminary experiments were conducted in 6-well culture plates (Thermo Fisher Scientific, Waltham, MA, USA), whereas the validation experiment was performed using standard cell culture conditions described previously.

221

Results and Discussion

Size determination of DNA/PEI complexes

DNA/PEI complexes used in a standard transient transfection protocol of HEK 293 cells were examined with particle tracking techniques. The evaluation of DNA/PEI complexes over time was followed by DLS and NTA. In Figure 1A, the mean hydrodynamic diameter and the relative kilo counts per second (kcps) determined by DLS are depicted, while in Figure 1B, the mean hydrodynamic diameter and the concentration of particles analyzed by NTA are shown.

229

DLS revealed a rapid increase in size of DNA/PEI complexes. A mean hydrodynamic diameter of approximately 344 nm was recorded as the initial point, which increased up to 690 nm at 30 min of incubation. Interestingly, the kcps decreased during this period. The kcps value is proportional to the total light scattered intensity,

233 which in turn, is proportional to the concentration of particles and size. The decreased kcps was likely to be
234 due to aggregation or precipitation phenomena. Because no precipitation was observed, and the
235 hydrodynamic diameter increased with time, an aggregation process was the probable cause of this
236 observation.

237

238 NTA measurements presented a slight growth in DNA/PEI complex size starting from 220 nm and reaching a
239 plateau at 30 min (300 nm). Interestingly, while the mean hydrodynamic diameter was nearly constant during
240 the entire process, the concentration of particles quickly decreased by an order of magnitude within the first
241 5 min. In the entire process analyzed, DNA/PEI complexes began at a concentration of 1.08×10^{10} particles/mL,
242 which was reduced to 1.80×10^9 particles/mL by 30 min of incubation. Three kinetic rates could be identified
243 within this period: a rapid fall in the first 5 min, a second trend until the 15 min and a third period with a slow
244 rate from that time on. The background levels detected in the controls (FreeStyle medium, free DNA and free
245 PEI) are included in Supplementary materials A1 (APPENDIX A1). Furthermore, the presence of particles in the
246 controls was only detected using high laser intensities (Camera level 11), whereas in DNA/PEI complexes the
247 laser intensity was much lower (Camera level 5).

248

249 Overall, a DNA/PEI complex aggregation process is proposed. DLS is highly affected by large aggregates
250 because the scattered light is proportional to the hydrodynamic diameter, which occludes or prevents the
251 detection of small particles. NTA is unable to analyze large aggregates; consequently, only smaller particles
252 are observed. When both analytical methods are combined, insights into DNA/PEI complexes can be obtained.
253 First, DNA/PEI complexes are formed immediately. They then evolve during incubation in cell culture medium
254 and some small complexes aggregate with time. After 30 min, the samples contain small complexes with sizes
255 of approximately 300 nm (detected by NTA) and large aggregates of micron-size (detected by DLS).

256

257 Due to the intrinsic limitation of each methodology, such heterogeneous systems cannot be characterized by
258 one technique alone, to avoid misleading results. A similar approach has been taken by van Gaal *et al.* [27],

259 where flow cytometry was assessed to analyze DNA/PEI complex populations. The heterogeneous results
260 found in TGE protocols could be better explained by these combined characterizations [21].

261

262 Morphology study of DNA/PEI complexes

263 Cryo-TEM, TEM and SEM have been used to evaluate DNA/PEI complex morphology. This feature has been
264 recently described as highly relevant in DNA and drug delivery strategies [28,29]. Cryo-TEM enables the study
265 of nanoscopic molecular assemblies in their aqueous form; Cryo-TEM analysis of DNA/PEI complexes after 1
266 min incubation (Figure 2A, 2D), 15 min (Figure 2B, 2E) and 30 min (Figure 2C, 2F) are shown in Figure 2. DNA/PEI
267 complexes showed electrodense structures with ellipsoid-like shapes (Figure 2, white arrows) with sizes of
268 approximately 150-200 nm (Figure 2A). The observed density was elevated, in agreement with the previous
269 NTA measurements (Figure 1B). After 15 min incubation, the heterogeneity of DNA/PEI complex population
270 and the aggregation process was evident: micrometric aggregates with irregular shapes (Figure 2, black
271 arrows) were present and the apparent concentration of particles was reduced. At 30 min, aggregates with
272 diameters greater than 3 μm could be observed. However, this technique cannot identify objects with
273 thicknesses larger than 1 μm [30], suggesting that the positively charged complexes may deform in the grid
274 since it is negatively charged after glow discharge.

275 SEM and TEM are simpler characterization techniques; nonetheless, these measurements are carried out on
276 negatively stained and dried samples which may result in artifacts. To improve image resolution and reduce
277 background, samples were deposited on holey nickel carbon grids (Copper-carbon grid preparations of
278 DNA/PEI complexes are presented in APPENDIX A2, for comparison). Changing the nature of the support
279 improved the resolution: DNA/PEI complexes of ~300 nm (Figure 3, white arrows) and micron-sized aggregates
280 were clearly distinguishable (Figure 3, black arrows) in both TEM (Figure 3A and C) and SEM (Figure 3B and D).
281 Background noise caused by the precipitation of the salts present in the FreeStyle medium was also reduced
282 (higher levels are detected in the control and in the copper-carbon grid, APPENDIX A1 and A2, respectively. Of
283 note, DNA/PEI complexes maintained their 3-dimensional structure under SEM analysis. In addition, the
284 heterogeneity of the complex population was evidenced by all the analyses. The presence of a DNA/PEI
285 complex subpopulation was suggested by Choosakoonkriang et al. more than 10 years ago [21]. In this work,

286 the novel application of NTA and Cryo-TEM have confirmed this hypothesis in the system tested (Figures 1 and
287 2).

288

289 Elemental analysis of DNA/PEI complexes

290 Aggregation of DNA/PEI complexes has been evidenced by particle tracking (Figure 1) and EM (Figures 2 and
291 3). This process has been widely reported in simpler hypertonic solutions [13,15,18]. In this work, the use of
292 cell culture medium to form DNA/PEI complexes was used: FreeStyle medium is protein-free and chemically
293 defined but its composition is proprietary. In this regard, cell culture media have been reported to change the
294 properties of the formed complexes after its addition [15,18]. Moreover, since some cell culture media contain
295 proteins, such as albumin, they would interact with the complexes, complicating the interpretation of
296 physicochemical properties in TGE processes [27,31]. Since FreeStyle has no protein, the problems presented
297 in the literature should not affect the present approach, although unknown compounds could be participating
298 in the complexation process.

299

300 To go further into DNA/PEI complex formation in the present methodology, SEM was coupled with X-ray
301 spectroscopy to determine the distribution of molecules within complexes. Figure 4 presents an example of
302 an elemental analysis obtained. Figure 4A presents the SEM image coupled to the profile line analyzed in a
303 200 nm size DNA/PEI complex. Four other elements were detected, other than carbon and oxygen from the
304 grid (Figure 4B and 4C): phosphorus (P), chloride (Cl), sodium (Na) and uranium (U). All 4 pooled within the
305 DNA/PEI complex structure. For additional information, APPENDIX A3 shows an X-ray spectroscopic elemental
306 analyses of a series of DNA/PEI particles observed by SEM.

307

308 Remarkable levels of Na and Cl were detected in the particle, which may come from the FreeStyle medium.
309 Consequently, NaCl salt is indicated as the driving force for DNA/PEI complex evolution in the cell culture
310 medium. It is possible to infer that the presence of counter ions in the DNA/PEI complex accounted for salt
311 precipitates in the SEM and TEM images in all DNA/PEI particles observed (Figure 3). This was more evident

312 on the carbon-coated grids where all salt precipitates were retained on the grid due to the lack of holes
313 (APPENDIX A2).

314

315 To confirm this hypothesis, transfections were performed in simpler PBS buffer and Milli Q water following
316 the same DNA/PEI complex preparation. In those cases, complexes prepared in salt buffer produced almost
317 the same protein yield upon transfection (96hpt); otherwise, complexes prepared in water showed inefficient
318 TGE yields in terms of protein production, despite having a similar percentage of producing cells (APPENDIX
319 A3). In addition, DNA/PEI complex formation was analyzed in both media, and aggregation was found only in
320 the salt buffer (PBS) (data not shown), thus corroborating the hypothesis of direct salt intervention in PEI-
321 mediated TGE processes (as also reported in the literature)[15,18].

322

323 Apart from salt ions, the presence of phosphorous (P) might derive from the phosphate group of the DNA,
324 whereas the presence of uranium was due to the negative staining pretreatment required for the vacuum
325 conditions. Although phosphorous was clearly identified, it did not appear in the whole spectrum due to its
326 low concentration compared with the other elements (Figure 4B). The surrounding counter ions might hinder
327 the detection of DNA/PEI atomic composition. To assess that possibility, an extra wash step with MiliQ water
328 was performed before negative staining addition. After sample drying, MiliQ water was added to the grid and
329 dried with filter paper to decrease salt concentration (APPENDIX A4). At this point, a nitrogen peak,
330 characteristic of DNA and PEI molecules, was clearly detected.

331 In short, NaCl was indicated as being the main cause of the DNA/PEI complex aggregation shown. Raup et al
332 [18] recently analyzed the behavior of DNA/PEI complexes in different concentrations of NaCl and observed
333 aggregation in all, despite showing different aggregation rates.

334

335 Average of DNA/PEI load in cell culture

336 Prior to any biological assay, the number of complexes in the cell culture was determined to calculate
337 transfection efficiency. DNA complexation by PEI was confirmed by gel retardation assay (data not shown).
338 Preliminary theoretical calculations of the number of pGag-eGFP plasmid copies per complex based on the

339 data obtained were then performed (Table I). Based on the NTA results (number of DNA/PEI complexes of
340 220-nm size recorded at the beginning of the incubation) and the concentration of pGag-eGFP plasmids, 144
341 plasmid copies per DNA/PEI complex were obtained. Moreover, at the time of transfection (after 15 min
342 incubation; 4.90E+9 particles/mL from NTA data), approximately 250 complexes of 279-nm average size per
343 cell plus aggregates were expected after incubation. The number of complexes per cell should be sufficient to
344 guarantee efficient transfection. In previous confocal microscopy studies, similar estimations were performed
345 by considering only large DNA/PEI aggregates visualized in mammalian cells, leading to results of fewer than
346 10 aggregates per cell [10,32]. The presence of small complexes of ~300-nm sizes could not be visualized due
347 to optical limitations.

348

349 Effect of DNA/PEI physicochemical heterogeneity in transient transfection

350 The influence of the physicochemical properties of DNA/PEI complexes aggregation in transient transfection
351 was investigated. A previously optimized TGE protocol for the production of HIV-1 based VLPs was used [9].
352 These VLP vaccine candidates are based on the self-assembly capacity of the Gag-polyprotein of the HIV-1.
353 Upon expression, Gag is able to accumulate underneath the plasma membrane and, through a budding
354 process, VLPs are released to the cell culture medium. Gag polyprotein was labeled with eGFP; thus, its
355 production could be monitored by fluorescence-based techniques.

356

357 DNA/PEI complexes formed under the same DNA:PEI (w:w) ratio, but with different incubation times prior to
358 transfection, were prepared (Preparations 1-2-3 with 0-15-30 min of incubation, respectively; Figure 5A).
359 Figure 5B shows that HEK 293 cell growth and viability were not affected by the presence of small complexes,
360 aggregates or mixtures of the two. Hence, size and particle concentration did not show cytotoxic effects.
361 However, complexation time affected the production of HIV-1 Gag VLPs, determined in terms of GFP-positive
362 cells (Figure 5C) and relative fluorescence units (RFU) in the supernatant corresponding to the final HIV-1 Gag
363 VLP production (based on an in-house developed quantification assay) (Figure 5D) [26]. Preparation 1 with
364 non-incubated complexes had no aggregates and showed the lowest production. No significant differences
365 were observed between preparations 2 and 3 (incubation times of 15 and 30 min, respectively). Thus, even

366 though complexation kinetics are rapid, a minimum complexation time is necessary to allow for aggregation
367 before cell transfection. These data suggested that aggregation critically contributed to the transient
368 transfection process and 15 min was sufficient to produce the highest yield.

369

370 Effect of endocytosis inhibition in transient transfection

371 To determine which type of complex had a higher impact on transient transfection, specific inhibitors of
372 endocytosis were used to block entry of the different DNA/PEI complexes subpopulations (complexes were
373 formed with DNA:PEI ratio 1:2 w:w and 15 min incubation, corresponding to Preparation 2 in Figure 5). A
374 scheme of the experimental approach is shown in Figure 6A. Four main pathways in DNA/PEI complex entry
375 into HEK 293 cells have been reported: clathrin-mediated endocytosis (typically identified by entry of particles
376 with sizes between 100-150 nm), caveolae-mediated endocytosis (characterized by entry of particles of 50-
377 100 nm), macropinocytosis (typically identified for particles between 1-5 μ M) and passive transport.
378 Phagocytosis was not considered in this work because it is a special endocytosis pathway specific in phagocyte
379 cells (the HEK 293 cells used in this work are derived from human kidney cells lacking this ability).

380

381 The results of endocytosis inhibition are found in Figure 6. First, as shown in Figure 6B, cell growth and viability
382 were only slightly affected by the addition of inhibitors. Secondly, when the transfection efficiency was
383 evaluated in terms of GFP-positive cells (Figure 6C), at 24 hpt, different effects on transfection were observed.
384 These effects were attenuated during the production phase (48-96hpt). The same results were observed when
385 the final HIV-1 Gag VLP production was quantified in the supernatant. For all conditions, cells were successfully
386 transfected and HIV-1 Gag VLPs were produced, suggesting that DNA/PEI complex transfection occurred
387 through all tested pathways. However, when cell-DNA/PEI complex interactions in the presence of inhibitors
388 were evaluated using Cy5-labeled PEI, no significant changes in the percentage of cells interacting with a
389 complex were observed (APPENDIX A5). This indicated that DNA/PEI complex trafficking to the cell membrane
390 was not affected by endocytosis inhibition and suggests that the recovery of GFP-positive cells at 48 hpt in
391 nearly all endocytosis inhibition tests could have been due to the decreased effectiveness of inhibitors, i.e.,
392 complexes entered through their natural pathways. This may also account for the levels of transfection

393 achieved by passive transport, where a fast recovery in the percentage of GFP-positive cells was observed
394 between 24 and 48 hpt despite decreased overall production.

395

396 Different concentrations of product were obtained depending on the pathway inhibited. In the inhibition of
397 clathrin-mediated endocytosis (CPZ), a slightly negative effect was observed in terms of GFP-positive cells and
398 RFU, indicating that a percentage of DNA/PEI complexes may enter via the clathrin pathway. Caveolae-
399 mediated inhibition (Nys) showed a slight but not significantly positive effect in transfection, suggesting that
400 DNA/PEI complexes might not use this pathway, which agrees with previous DNA/PEI complex
401 characterizations, where small populations had hydrodynamic mean diameters of approximately 300 nm.
402 Finally, when the macropinocytosis pathway was inhibited, a significant reduction in HIV-1 Gag VLP production
403 was observed, achieving levels like those observed with passive transport. These results suggested that, from
404 the two previously characterized populations (Complexes 2 in Figure 5), the large aggregates contributed to a
405 greater extent to the final transfection efficiency. This was probably because large aggregates carried more
406 DNA and likely acted as reservoirs. Cervera et al. [10] showed the presence and efficient endocytosis of
407 DNA/PEI complex aggregates in all transfected cells after 1 h incubation, despite only partial production of
408 HIV-1 Gag VLPs by the cells. Note that the drug inhibitor endocytosis approach is an indirect method since the
409 drugs used could have other effects in the cell metabolism hindering the experimental objective [11,33]. In
410 this sense, the characterization of complexes and the previous confocal microscopy analyses contribute to the
411 hypothesis; nevertheless, the effectiveness of ~300-nm complexes have also been largely reported in other
412 systems as well as the present work [1,11,13].

413

414 On the whole, PEI-mediated TGE will still be a reference methodology in industrial biotechnology. There is a
415 high heterogeneity in PEI-mediated processes for both intracellular fate and TGE yields. Other from the cell
416 line, this could be caused by (1) the large number of variables which change the physicochemical properties
417 of DNA/PEI complexes and/or (2) the polydispersity of the DNA/PEI complexes formed [1,14,21,27]. Here, the
418 use of a chemically defined and protein-free cell culture media for all complex formation and cell culture has
419 enabled the comprehensive correlation between its formation and its efficiency, since no chemical alteration

is made when complexes are added to the cell culture. Furthermore, the improvements in the nanotechnology field have enabled a better description of the present HIV-1 Gag VLP production platform. The aggregation of DNA/PEI complexes has been directly visualized by Cryo-TEM and quantified by NTA. The present study will allow one to decipher the key aspects of PEI-mediated process and enhance TGE yields.

Conclusion

A strong relationship between DNA/PEI complex aggregation level and transient transfection efficiency was observed. Aggregation did not affect the entire population evenly, resulting in heterogeneous complexes. The combination of particle tracking and electron microscopy techniques enabled the comprehensive characterization of DNA/PEI complexes.

The extent of aggregation was correlated with incubation time, which is a relevant parameter to be considered at the moment of complex preparation. Moreover, the concentrations of Na and Cl ions in culture media played important roles in DNA/PEI complex formation. Interestingly, DNA/PEI complex aggregates seemed to significantly contribute to transient transfection. This was likely to be because these aggregates carried more DNA and acted as reservoirs.

This work provides highlights for a better understanding of DNA/PEI complexes to improve TGE processes, reducing transfection variability and increasing production yields by adjusting simple parameters, such as incubation time. This approach can aid characterization of similar nanocarriers used in biomedicine for non-viral DNA delivery strategies.

Author Contributions

I.G-D. designed and performed the experiments. N.G. supervised the experiments. I.G-D, N.G., L.C. and F.G. analyzed the results. IG-D., N.G., L.C., N.V. and F.G. wrote the manuscript.

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552

553 **Figure and Table Legends**

554

555 Figure 1: Particle tracking analysis of DNA/PEI complex kinetics in FreeStyle medium. DNA/PEI complexes were
556 formed instantaneously after the mixing of DNA and PEI; a partial aggregation of the population was observed
557 by (A) DLS measurement of mean size and kilo count per second (kcps), respectively, and (B) NTA analysis with
558 Nanosight® of mean size and particle concentration, respectively, in the range from 0 to 1000 nm; standard
559 deviation (SD) in every mean size measurement is also shown. Samples were diluted 1:10 with PBS to achieve
560 a concentration between 10^8 - 10^9 particles/mL.

561

562 Figure 2: Cryo-electron microscopy analysis of DNA/PEI complexes in FreeStyle medium. DNA/PEI complexes
 563 were observed as electrodense sphere-like structures just after mixing DNA and PEI. Heterogenous population
 564 size was observed after incubation: large DNA/PEI aggregates appeared at 15 min incubation and increased in
 565 size at 30 min with diameters up to 3μm. (A, D) Cryo-TEM images at 0 min incubation; (B,E) 15 min incubation;
 566 (C,F) 30 min incubation. White arrows indicate ~300nm-size DNA/PEI complexes, and black arrows indicate ≥
 567 1 μm-size DNA/PEI complex aggregates.

568

569 Figure 3: Electron microscopy analysis of DNA/PEI complexes at 15 min incubation in holey-nickel grids in
 570 FreeStyle medium. Identification of DNA/PEI complexes with negative staining was achieved using holey grids.
 571 (A, C) TEM analysis and (B, D) SEM analysis. White arrows indicate ~300nm-size DNA/PEI complexes, and black
 572 arrows indicate $\geq 1 \mu\text{m}$ -size DNA/PEI complex aggregates.

573

574 Figure 4: X-ray spectroscopy mapping elemental analysis of a DNA/PEI complex. Presence of Na and Cl ions

575 surrounding DNA/PEI complexes were identified as the driving force in complex formation and aggregation.

576 (A) SEM image of the particle analyzed, histogram analyzed is presented as a yellow line. (B) Sum spectrum of

577 X-ray analysis. (C) Specific X-ray spectra composition for the elements found in more abundance: phosphorous

578 (P) represented (red), chlorine (Cl) (green), sodium (Na) (blue) and uranium (U) (purple).

A **B** **C** **D**

580 Figure 5: DNA/PEI complex formation effect on the production of HIV-1 Gag VLPs by transient transfection of
581 HEK 293 cells. Aggregation of DNA/PEI complexes before transfection was required to obtain higher yields. (A)
582 Scheme of the different DNA/PEI complexes aggregation levels 1, 2 and 3 prepared by non-incubation, 15 and
583 30 min incubation in FreeStyle medium, respectively. *NTA data **DLS data. (B) Viable cell concentration and
584 cell viability. (C) Percentage of GFP-positive cells. (D) Relative fluorescence units (RFUs) in the supernatant.
585 Experiments were performed in triplicate. Mean values $\pm$ SD are presented. Student t-test analysis for every
586 condition with the control of each time is also included: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

588 Figure 6: Effect of endocytosis inhibition on transient transfection efficiency in the production of HIV-1 Gag
589 VLPs in HEK 293 cells. DNA/PEI complexes may enter through several endocytic pathways and DNA/PEI
590 aggregates are required to obtain the highest TGE yields. (A) Endocytosis inhibition experimental design. Four
591 pathways were analyzed through drug inhibition endocytosis experiment. Each pathway was specifically
592 inhibited with a drug inhibitor or with a temperature treatment: chlorpromazine 25 μ M (CPZ), nystatin 30 μ M
593 (Nys), N-ethyl-N-isopropyl 30 μ M (EIPA) for evaluation of clathrin-mediated endocytosis, caveolae-mediated
594 endocytosis and macropinocytosis, respectively. Finally, another condition for testing the passive transport
595 was evaluated by maintaining the cells at 4°C after transfection until 4 hpt. (B) Cell growth and viability of HEK
596 293 cells. (C) Percentage of GFP-positive cells. (D) Relative fluorescence units (RFUs) in the supernatant. After
597 medium replacement, HEK 293 cells were pretreated with inhibitors 1 hpt; a second medium replacement was
598 performed at 4hpt. Experiments were performed in triplicate. Mean values $\pm$ SD are shown. Student t-test
599 analysis for every condition with the control of each time is also included: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
600

601 Table I. Theoretical calculation of pGag-eGFP plasmid copies in DNA/PEI complexes with a hydrodynamic
 602 diameter of 220 nm. * DNA/PEI complexes concentration has been adjusted to the final transfected cell culture
 603 volume.

pGag-eGFP concentration	1 (µg/mL) / 6.022+14(kDa/mL)	604 605
pGag-eGFP	6274 (bp) / 3880 (kDa)*	606
Cell Concentration at transfection time	2.00E+6 (cells /mL)	607
pGag-eGFP copies	1.55E+11 (copies/mL)	608
pGag-eGFP copies/cell	7.75E+4 (copies/cell)	609 610
DNA/PEI complex concentration at t= 0 of	1.08E+9 (complex /mL)**	611
incubation. with a mean hydrodynamic diameter of		612
220 nm at the transfected cell culture		613
pGag-eGFP copies/ DNA/PEI complex of 200nm at	1.44E+2 (copies/complex)	614
t:0 of incubation		615
DNA/PEI complexes/cell	5.40E+2 (complex/cell)	616
		617

618

619 **Molecular Weight calculated with Snap Gene Viewer®(GSL Biotech LLC)** DNA/PEI complexes concentration has been*
 620 *adjusted to the final transfected cell culture volume.*

621

622

624

625 APPENDIX A1. FreeStyle medium, free DNA and free PEI controls for electron microscopy analysis and particle

626 tracking analysis. CryoTEM, TEM and SEM images samples were prepared immediately after DNA and PEI

627 addition. DLS and NTA analyses were performed in 3 independent measurements. DLS analyses of FreeStyle

628 medium had a mean hydrodynamic diameter of 6 nm ± 3 nm with stable and monodisperse population

629 (Atn:11; kcps:60); free DNA had a mean hydrodynamic diameter of 485 nm ± 1 nm with unstable and

630 polydisperse population (Atn:11; Kcps:90); and free PEI had a mean hydrodynamic diameter of 8 nm ± 0 stable

631 and polydisperse population (Atn:11; Kcps:90). NTA analyses of FreeStyle medium had a mean hydrodynamic
632 diameter of $174 \text{ nm} \pm 4 \text{ nm}$ with a concentration of $6.35\text{E}+8 \pm 5.47\text{E}+7$ particles/ml; free DNA had a mean
633 hydrodynamic diameter of $150 \pm 12 \text{ nm}$ with a concentration of $1.87\text{E}+9 \pm 2.03\text{E}+8$ particles/ml; and free PEI
634 had a mean hydrodynamic diameter of $130 \pm 2 \text{ nm}$ with a concentration of $4.25\text{E}+8 \pm 2.89\text{E}+7$ particles/ml. All
635 NTA measurements for the control samples were performed with a Camera Level of 11 and detection threshold
636 of 5 for medium and PEI and 3 for DNA.
637

639 APPENDIX A2. DNA/PEI complex analysis by TEM and SEM at 0 and 15 min incubation in FreeStyle medium in
640 carbon-coated grids. White arrows indicate ~300nm-size DNA/PEI complexes, black arrows indicate $\geq 1 \mu\text{m}$ -
641 size DNA/PEI complex aggregates, and grey arrows indicate NaCl precipitates surrounding DNA/PEI complexes.

A **B** **C**

643 APPENDIX A3. Evaluation of other complex incubation media in transient transfection of HEK 293 cells. HEK
644 293 cells were incubated with the standard transfection process of 15 min of incubation prior to transfection
645 previously described but changing the media complexation: Standard incubation in FreeStyle (CN), incubation
646 PBS (PBS), MilliQ filtered water (MilliQ) and direct addition of DNA and PEI to the cell culture (DIRECT) were
647 tested. (A) Viable cell concentration and cell viability. (B) Percentage of GFP-positive cells. (C) Relative
648 fluorescence units (RFUs) in the supernatant. Experiments were performed in triplicate. Mean values $\pm$ SD are
649 presented. Student t-test analysis for every condition with the control of each time is also included: * $P<0.05$,
650 ** $P<0.01$, *** $P<0.001$

A

C	Na	P	Cl	O	Total
25.87	0.97	1.09	1.4	70.67	100
26.39	0.73	0.5	1.15	71.22	100
25.26	1.44	1.26	2.62	69.43	100

B

C	N	Na	P	Cl	Cu	O	Total
19.76	4.21	0.47	1.88	-0.03	5.14	68.56	100

C

D

651
652 APPENDIX A4. X-ray spectroscopy results of different DNA/PEI complexes from SEM analysis. (A) Table of
653 DNA/PEI histogram final composition and its (C) Summarized spectrum from an example histogram (B)
654 DNA/PEI complex analysis by X-ray spectroscopy after its wash with MiliQ water and its (D) Summarized
655 spectrum from an example histogram.

656
657

658

659 APPENDIX A5. Endocytosis inhibition preliminary studies in 6-well plates of HEK 293 cells transfection
660 efficiency. DNA/PEI complexes were formed with Cy5-labeled PEI and the transfection was performed
661 according to the standard methodology. Transfection efficiency was analyzed in terms of GFP (grey bars) and
662 Cy5 (orange line) positive cells and cell concentration and viability associated at 10 hpt are also present,
663 respectively: Chlorpromazine (CPZ) (A,B), nystatin (Nys) (C,D) and N-ethyl-N-isopropyl (EIPA) (E,F).
664 Experiments were performed in triplicate. Mean values $\pm$ SD are presented.