**Single-particle electrophoresis to study the adsorption of polyplexes onto anionic particles: a model system for investigating polyplex-cell interactions**

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**Abstract**

Gene therapy is being used in many applications, including pest control and the treatment of diseases. However, protection during transport and delivery of the genetic material to the desired location remain important challenges. Cationic polymers have certain key advantages as delivery vectors, but the toxicity and transfection efficiency of new polymers are unpredictable and the polyplex-cell interactions remain largely underinvestigated. In this work, we aimed to study the electrostatic interactions of polyplexes with anionic particles, which serve as a simple model for a cell. To this end, we used single-particle electrophoresis to measure the electrophoretic mobility of the particles upon interacting with the polyplexes. We found that the dominant factor influencing the adsorption kinetics is the polymer concentration, while the N/P ratio has a smaller effect, at least in the investigated range. This research establishes single-particle electrophoresis as a new tool to study one of the processes involved in cytotoxicity and transfection and could have strong implications regarding the unraveling of the key parameters involved.

**Keywords**

Single-particle electrophoresis, cationic polymers, polyplexes, adsorption kinetics, N/P ratio

1. Introduction

Gene therapy is a very promising concept for many different applications, ranging from the treatment of various diseases to pest control.1–5 It involves the use of genetic material (DNA or RNA) to induce or modulate the expression of a certain gene, thereby obtaining a desired effect. Depending on which polynucleotide is used, a different pathway is activated, which in turn influences the final result.6 As an example, when small interfering RNA (siRNA) is introduced in the cell, it can be processed by the RNA interference (RNAi) machinery in the cells, leading to post-transcriptional knockdown of a certain (e.g. disease-causing) gene.7,8 When, on the other hand, DNA (e.g. plasmid DNA) is delivered to the nucleus of the cells, it is transcribed and consecutively translated, leading to the production of a certain protein (depending on the DNA sequence).6 These are only two examples of how genetic material can be used, while many other options exist (e.g. gene editing).6,9,10

Evidently, there are a lot of opportunities to use this technology in various applications. One of the most obvious applications is to cure diseases which are linked to or can be treated by the expression of proteins or the lack thereof (e.g. cancer, cardiovascular diseases, infectious diseases, etc.).9 However, to protect the polynucleotide from degradation by nucleases, a vector is necessary. Therefore, the success of gene therapy is largely dependent on the choice of vector for its delivery.11,12 Originally, in medicine, mainly viral vectors were investigated to deliver genetic material because of their inherent ability to infect cells.4,5 However, viral vectors as such are known to elicit a rather significant immunogenic response, which is one of their major disadvantages together with the risk for mutagenicity.10 As alternatives, non-viral vectors such as lipids, polymers and inorganic nanoparticles have been developed and investigated.10–16 They typically demonstrate a higher packing capacity (of the polynucleotide), a lower production cost and a lower immunogenicity compared to viral vectors.1,10,16

Non-viral vectors are very promising because of the abovementioned reasons, although their transfection efficiency is typically inferior compared to viral vectors and they can be associated with cytotoxicity.16 Therefore, researchers have focused on developing new and improved carriers for gene delivery. One popular type of carrier which is frequently investigated is polymers, more specifically cationic polymers, since they can form electrostatic interactions with the negatively charged polynucleotide (i.e. DNA or RNA). Examples of cationic polymers used for gene delivery include poly(ethylene imine) (PEI), poly(L-lysine), poly(β-amino esters), chitosan, polyamidoamine dendrimers and methacrylate-based polymers with cationic side chains.17–24 Many different polymer properties can be varied in order to achieve superior results in terms of transfection efficiency and cytotoxicity, including but not limited to functional groups, molar mass, polymer architecture (linear or branched) and polymer backbone (degradable or not).16,23,25,26 Other factors, such as concentration and N/P ratio (the ratio of nitrogen-containing potentially positively charged groups of the cationic polymer over the phosphorous-containing negatively charged groups of the nucleic acids), also play a crucial role in improving transfection efficiency and cytotoxicity.23,26

Many parameters influence the cytotoxicity and the transfection efficiency of cationic polymers. Investigating these effects remains challenging and a lot of research focusses on developing new methods to study this. Two-color direct stochastic reconstruction super-resolution microscopy (dSTORM) is a novel method that allows the visualization of nanostructures (i.e. nanoscopy).27 It enables the investigation of the composition and stability of polymer-polynucleotide complexes (also called polyplexes), as well as how they behave *in vitro* while being transported within a cell and interact with intra-cellular components.28–32 Notwithstanding that this technique is very valuable to acquire an in-depth understanding of the cellular fate of the polyplexes, dSTORM only provides this information when applied in *in vitro* biological studies.

It remains hard to predict whether or not a polymer will be successful without testing them in *in vitro* biological studies.33 The interaction of polyplexes with cell membranes is an intriguing but understudied topic. Nonetheless, this is essential for comprehending some of the mechanisms by which cationic polymers and polyplexes induce cytotoxicity or transfection. Methods that allow us to study the interaction between cells and cationic polymers (and the resulting polyplexes with genetic material) would therefore be highly interesting. There are techniques, such as quartz crystal microbalance (QCM), which enable the investigation of the interaction of polyplexes with certain cell membrane components immobilized on the flat QCM sensor surface.34 However, this technique works only on a flat surface, not on spherical substrates which are much more relevant, and only investigates the interaction with one cell component. Computer simulations have also been used to model the interaction of polyplexes with cell membranes.35 While this approach is highly interesting and valuable from a theoretical perspective, it is not a method to actually measure the interaction itself.

Recently, we developed a new single-particle electrophoresis technique, which enables the investigation of the electrophoretic mobility of microparticles on a single-particle level and in a fast way.36 In a later study, we used this approach to examine the adsorption kinetics of cationic polymers on anionic particles as a function of polymer concentration. We also investigated various cationic polymers with different molar mass, functional groups and architectures, as these factors potentially influence the polymer adsorption.37 This study demonstrated the applicability of this technique to investigate the electrostatic interactions of components in solution with microparticles.

In this work, we aimed to increase the complexity of the system that can be studied with this single-particle electrophoresis technique and thereby expand upon its applicability. Concomitantly, we focused on the kinetics of the adsorption and desorption (which are expected to be dominated by electrostatic interactions) of a positively charged polyplex onto cells, since polyplex adsorption is the first step in both transfection and cytotoxicity. To this end, we used anionic microparticles as a very simple model representing a cell, since human cells have a negatively charged cell membrane.38,39

The entire transfection and cytotoxicity pathways are obviously much more complex than electrostatic interactions alone. Nonetheless, in this study, we intended to shed light on the adsorption and desorption of polyplexes on the anionic microparticle (as a model for a cell) in isolation, without taking into account all the possible steps that might follow (i.e. disruption of the cell membrane for cytotoxicity or endocytosis, endosomal escape, intracellular transport, etc. for transfection).

We used the established single-particle electrophoresis technique to track and measure the electrophoretic mobility of a single microparticle over time as it interacts with polyplexes. We investigated the feasibility of this approach, as well as the influence of the polymer concentration and the N/P ratio on the adsorption kinetics of this interaction. To this end, we used poly(2-guanidinoethyl methacrylate) (PGUMA) as the cationic polymer. Because of the high pKa value of guanidine functionalities (i.e. around 12.5), we can safely assume that it is completely protonated and therefore positively charged at physiological pH.40 This allows it to interact strongly with DNA, in our case commercially available linear DNA, resulting in the polyplexes that were used in this work (Figure 1). Finally, negatively charged polystyrene (PS) microparticles were used as a cell model to study the interaction with the polyplexes.



Figure 1: Complexation of a cationic polymer with nucleic acids (e.g. DNA) results in the formation of a polyplex (A). These positively charged polyplexes will then interact with anionic particles, resulting in a polyplex-particle complex (B).

1. Materials and Methods
	1. Materials

Hydrogen chloride (37 %), acetonitrile (99.5 %), and sodium dihydrogenphosphate dihydrate (≥98 %) were obtained from Chem-Lab (Zedelgem, Belgium). Deuterium oxide (99.90 % + 0.1 % 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt, TMSP) was purchased from Eurisotop (Saint-Aubin, France). 2-aminoethyl methacrylate hydrochloride (AEMA, ≥95 %) and PS particles with a diameter of 0.99 ± 0.01 µm were bought from Polysciences (Hirschberg, Germany). Ammonium persulfate (APS, ≥98 %), triethylamine (TEA, ≥99.5 %), 1-H-pyrazole-1-carboxamidine hydrochloride (HPC, 99 %), DNA sodium salt from calf thymus (type I), N,N,N’,N’-tetramethylethylenediamine and phosphate buffered saline (PBS) tablets were all bought from Sigma-Aldrich (Overijse, Belgium). 30 % Acrylamide/Bis Solution 29:1 was bought from Bio-Rad (China). 10x pH 8.3 tris-borate-EDTA (TBE) buffer was obtained from Millipore (Darmstadt, Germany). GelRed Nucleic Acid Gel Stain (10 000x) was purchased from Biotium. DNA loading dye (6x, bromophenol blue and xylene cyanol FF) was bought from Thermo Scientific (Vilnius, Lithuania) and was diluted to 2x using double distilled water. All other chemicals were used as received. All PGUMA and DNA stock solutions were prepared at 1 mg/mL in a 0.8 mM (0.005x) PBS buffer.

* 1. Polymer synthesis, modification and characterization

The polymers were synthesized and modified according to Christiaens et al.3 In short, AEMA monomer was polymerized to poly(2-aminoethyl methacrylate) (PAEMA) via a free radical polymerization using APS as an initiator. The final monomer concentration was 0.5 M and the monomer to initiator molar ratio was 25. Both components (i.e. monomer and initiator) were dissolved in double distilled water at pH 4 (acidified using HCl) and the resulting solution was flushed using an inert gas for 30 min and then consecutively heated to 70 °C for 24 h. Purification of the resulting polymer (i.e. PAEMA) was performed by dialyzing against double distilled water at pH 4 (acidified using HCl) with 3500 g/mol cut-off membranes (SpectraPor) for 24 h, followed by freeze-drying.

Guanylation of the primary amines of PAEMA was performed by reacting the starting polymer (i.e. PAEMA) with 1 equivalent of both HPC and TEA for 24 h at room temperature. Afterward, the polymer (i.e. PGUMA) was purified using dialysis against double distilled water at pH 4 (acidified using HCl) with 3500 g/mol cut-off membranes (SpectraPor) for 24 h, followed by freeze-drying.

The chemical structure of the resulting polymers was confirmed with proton nuclear magnetic resonance (1H NMR) spectroscopy. All NMR spectra were obtained using the Brüker Avance II Ultrashield 400 MHz NMR spectrometer. Deuterium oxide (with 0.1 % of 3-(trimethylsilyl)propionic-2,2,3,3-d 4 acid sodium salt) was used as the solvent for these measurements. Analysis of the spectra was performed using the Mestrenova Software.

The molar mass of PAEMA was determined using size exclusion chromatography (SEC). The setup includes a Waters 600 controller with an isocratic pump, Waters 610 fluid unit, Rheodyne injection unit with 20 µL loop, two Shodex OHpak SB-806m HQ columns, a Waters 410 differential refractometer and Waters 996 photodiode array detector. An aqueous buffer containing 4 % m/v disodium hydrogen phosphate and 3 % m/v acetonitrile was used as the mobile phase. The samples were dissolved in this buffer at a concentration of around 10 mg/mL. Monodisperse dextran standards were used to obtain a calibration curve for the SEC. Data analysis was performed using the Empower2 software.

* 1. Polyplex formation and characterization

For the preparation of the polyplexes, 1 mg/mL stock solutions of both the DNA and the polymer in 0.005x PBS were used. The required amount of DNA solution was added to an Eppendorf, followed by dilution with a volume of 0.005x PBS to obtain a final DNA concentration of 10 µg/mL. This solution was homogenized by trituration (i.e. pipetting up and down), followed by the addition of the required volume of polymer solution and again mixing by trituration. The required volume of the polymer depended on the N/P ratio. We calculated the average molar mass of the polymer repeating unit based on the degree of guanylation (as determined by 1H NMR spectroscopy). Since the pH we are working with (i.e. around 7.5) is much lower than the pKa of the guanidine functions (i.e. around 12.5), we assumed full protonation of the polymer and therefore took this value as the mass per charge for the polymer. For DNA, we used a mass per charge of 325 g/mol. Based on these two values, we could determine the volumes of the polymer and DNA stock solutions required to obtain various N/P ratios (i.e. 2, 10 and 20).

The size and zeta potential of the obtained polyplexes were measured using dynamic light scattering (DLS). All DLS measurements were performed using a Malvern Zetasizer Nano-ZS device equipped with a 4 mW He-Ne laser at 633 nm. The polyplex solutions were transferred from the Eppendorf to the disposable cuvettes for size measurements and then to disposable folded capillary cells for the zeta potential measurements. The polyplexes were used for further experiments on the same day as they were made. Their stability was verified using DLS to make sure they did not change before performing the single-particle electrophoresis experiments.

To verify if full complexation of the DNA occurs at different N/P ratios, gel electrophoresis experiments were performed. To this end, 20 µL of the polyplex solutions was mixed in a 1:1 ratio with a 2x loading dye. Subsequently, 20 µL was injected in the wells of a 4 % polyacrylamide gel and allowed to run for 3 hours at 100 V using a 10 % TBE running buffer. Naked DNA was used as control. After 3 hours, the gels were stained for 10 min at room temperature using GelRed and visualized using the VisionWorksLS software.

* 1. Single-particle electrophoresis: set-up and analysis

An in-house single-particle electrophoresis (SPE) technique was used to measure the electrophoretic mobility of the individual PS particles, as describes elsewhere.36,37 This technique, based on particle tracking velocimetry, avoids electroosmosis effects by measuring at a high frequency (300 Hz) oscillatory electric field and in the middle of the microchannel (200 ± 40 µm).37 The set-up includes a custom-built inverted microscope and a high-speed CMOS camera (Andor Instruments, Zyla 4.2 sCMOS), a green LED (Thorlabs, 530 nm, 350 mW) for bright field illumination, and a 40x objective (Nikon, Plan Fluor, NA=0.75) for collecting the light.

The sample, which consisted of a colloidal suspension of PS particles and polyplexes, was dispersed in a commercial microchannel (Ibidi, uncoated 1.5 µ-Slide VI 0.4) that was mounted on top of an XYZ nano-translation stage. To create an AC electric field in the x-direction, a sinusoidal voltage was generated using an NI-DAQ and amplified 10 times (80 V peak-to-peak and a frequency of 300 Hz). This voltage was applied via two electrodes across the two reservoirs of the microchannel. The resulting AC electric field (peak electrical field strength of 5.1 x 10³ V/m)36 caused the particles to perform an oscillating motion, which was recorded using the CMOS camera measuring at 850 Hz. A custom-made program in LabVIEW software (National Instruments) was used to manage the entire setup, including the synchronization between the image acquisition and the applied voltage. This program also allowed for easy measurement and saving of experimental data, which consisted of a set of movies of individual particles (with one particle in the field of view). A customized Matlab software described in previous work36 was used for post-processing analysis of the data. In essence, the amplitude of the oscillating particle is extracted and used to calculate the electrophoretic mobility.

The SPE setup was used for adsorption kinetics experiments by measuring the electrophoretic mobility of a single PS particle over time as it interacts with polyplexes molecules. The samples consist of a fixed concentration of PS particles of 2 x 106 part./mL and polyplexes at different concentrations and N/P ratios. For these experiments, two separate stock solutions of PS particles and polyplexes were prepared and then mixed directly inside the microchannel. The measurement program logged the start time of the experiment immediately after the mixing procedure. The micro- and nano-stages were used to locate an isolated particle, which was manually tracked until the end of the experiment. Electrophoretic mobility measurements were carried out roughly every 30 s by applying the electric field for about 1.5 s and recording a movie of the oscillating particle. During each electrophoresis measurement, no particle tracking with the translation stages was performed. Between the mobility measurements, the field was zero. The total time for a single-particle experiment ranged from 600 s to 1000 s.

1. Results and Discussion

To investigate the adsorption and desorption of polyplexes on anionic particles, we first needed to produce and characterize the polyplexes, which in turn requires the synthesis of the polymer (i.e. PAEMA) and its subsequent modification towards PGUMA.

* 1. Synthesis and characterization of polymers and polyplexes

The first step in the synthesis of PGUMA is the synthesis of PAEMA. Using the protocol described in the Materials and Methods section, we obtained a polymer with a number-average molar mass (Mn) of 32.7 kg/mol and a polydispersity of 3.5 (as measured with SEC). The degree of modification of the amine functionalities towards guanidine groups was determined via 1H NMR spectroscopy (Figures S1 and S2) and we obtained a guanylation degree of 91 % (Figure 2). This resulted in PGUMA with a molar mass of 40.2 kg/mol, which was calculated based on the molar mass of PAEMA as obtained through SEC, taking into account the mass increase due to guanylation (and assuming that the degree of polymerization remains unchanged). These results are in line with expectations based on earlier reports from our group using the same synthesis method.3,37



Figure 2: Chemical structure of the in-house synthesized PGUMA in its non-protonated form. The synthesis starts from PAEMA, after which the amine functionalities are guanylated. In this work, the obtained degree of guanylation was 91 %.

Polyplexes of PGUMA and commercially available linear DNA were prepared using different N/P ratios and analyzed with gel electrophoresis to investigate from what N/P ratio onwards full complexation is achieved (Table 1). This revealed that the DNA is fully complexed at N/P ratios of 2 or above, as evidenced by the absence of a DNA band (Figure 3). At an N/P ratio of 1 or lower, a DNA band is still visible, indicating that full condensation is not yet achieved. Additionally, for three different N/P ratios (i.e. 2, 10 and 20), we also measured the size and zeta potential of the polyplexes using DLS (Table 1). For all these polyplexes, the zeta potential is positive (i.e. > 20 mV), suggesting that the DNA is neutralized by the polymer and the polyplexes are successfully formed, confirming the gel electrophoresis results. The Z-average size of all polyplexes, calculated from the light scattering intensity fluctuations caused by Brownian motion, is in the range between 66 and 105 nm, with the polyplexes with N/P ratio 20 being the smallest and the polyplexes with N/P ratio 10 being the largest (cfr. Table 1 and Figure S3). Using N/P ratio 2 results in polyplexes with intermediate sizes (i.e. 80 nm).



Figure 3: The gel electrophoresis experiments demonstrate that polyplexes are successfully formed at N/P ratios of 2 and higher. Naked DNA was used as a control.

While the Z-average size yields valuable information, it is important to also take into account the number-average sizes of polyplexes. Larger particles scatter more light, which biases the Z-average (which is based on the intensity) to larger sizes. As a result, the number-average sizes are smaller in comparison (cfr. Table 1 and Figure S4). The smallest polyplexes, with a size of 33 nm, are those with N/P ratio 10, while the biggest are those made with N/P ratio 2. Even though three data points are not sufficient to determine any definitive trend, it would seem that the highest N/P ratio typically results in smaller polyplexes, especially when considering the number-average sizes, although the Z-average size of N/P ratio 10 polyplexes is the highest. Nonetheless, even though the sizes are statistically significantly different in most cases, they do not differ that much and extracting a definitive trend with regards to the effect of the N/P ratio on the polyplex size remains difficult, which is similar to observations described in literature.3,7,23,41–43

Table 1: Z-average and number-average sizes and zeta potential of polyplexes made at different N/P ratios.

|  |  |  |  |
| --- | --- | --- | --- |
| N/P ratio | Z-average size (nm) | Number-average size (nm) | Zeta potential (mV) |
| 2 | 80 ± 1 | 46 ± 2 | 22 ± 2 |
| 10 | 105 ± 1 | 33 ± 10 | 33 ± 1 |
| 20 | 66 ± 1 | 35 ± 1 | 26 ± 2 |

* 1. Adsorption kinetics of polyplexes on anionic particles

Once the polyplexes were successfully obtained and characterized, we proceeded with the main objective of this research: investigating the adsorption and desorption of these polyplexes onto anionic particles. To this end, we used our in-house developed single-particle electrophoresis technique, of which a detailed description can be found in our previous publications.36,37

To ensure that polyplexes are indeed interacting with the anionic PS particle, an equilibrium experiment was carried out using polyplexes with the lowest N/P ratio (i.e. N/P ratio 2). The sample, containing the polyplexes as well as the PS particles, was prepared and incubated overnight, after which the electrophoretic mobility of 20 individual particles was measured. This resulted in an equilibrium electrophoretic mobility of μ = (+3.5 ± 0.1) x 10-8 m² V-1 s-1. Since the electrophoretic mobility of the bare PS particle in this buffer is μ0 = (-5.5 ± 0.2) x 10-8 m² V-1 s-1, this indicates that there is indeed a shift in electrophoretic mobility due to the adsorption of positively charged polyplexes. Knowing that the polyplexes interact with the anionic PS particles, the next step was to study the adsorption/desorption dynamics.

To investigate the adsorption and desorption kinetics of positively charged polyplexes on anionic particles, we measured the electrophoretic mobility of individual particles over time. Additionally, by varying both the N/P ratio and the polyplex concentration, we studied the effect of these parameters on the adsorption and desorption process. While the actual concentration of the polyplexes themselves is unknown, we hypothesize that the concentration of polyplexes will vary and correlate with the concentration of the polymer (n). Consequently, the dependency of the adsorption and desorption on this parameter n will be investigated in this work. As previously discussed, we used the N/P ratios 2, 10 and 20, while the polymer concentration was varied between 1253 and 3133 ng/mL. To ensure that we are working within the reaction-limited regime and not in the diffusion-limited regime, we performed simulations to calculate the required time to reach the electrophoretic mobility plateau (i.e. the equilibrium electrophoretic mobility) purely based on diffusion (Supporting information, Figure S5). Given that these times are smaller than the times observed during the experiments (cfr. Figure 4), we can conclude that we are predominantly working in a reaction-limited regime, similar to our previous study.37

When we investigate the typical shape of the obtained adsorption curves (Figure 4), we see that in a first phase, which is dominated by association, the particle electrophoretic mobility increases linearly due to adsorption of polymer over time. As the number of adsorbed polyplexes onto the particle increases, the desorption rate increases as well. After a certain period of time, saturation occurs and the electrophoretic mobility reaches a plateau value. At this point, the adsorption and desorption of the polyplexes happen at the same rate and an equilibrium is obtained, resulting in the equilibrium electrophoretic mobility.



Figure 4: The electrophoretic mobility curves of PS particles interacting with polyplexes with different N/P ratios: 2 (A), 10 (B) and 20 (C). For each N/P ratio, various polymer concentrations were used (n = 3133 ng/mL (green), n = 2088 ng/mL (red), n = 1566 ng/mL (yellow), n = 1392 ng/mL (orange) and n = 1253 ng/mL (blue)). The least-square fitting of equation 1 is indicated with a dashed line for the data corresponding to n = 1566 ng/mL.

The first thing we notice when we compare the different adsorption curves (Figure 4) is that the particle-polyplex interaction is heavily influenced by the polymer concentration. According to the theory described in our previous work, the rate of the adsorption and desorption is mostly determined by the concentration of target particles.37 Since the concentration of PS particles is kept constant, the reaction kinetics are therefore expected to be dominated by the concentration of polyplexes. Indeed, we observe an increase in reaction rate as the polymer (and therefore the polyplex) concentration increases. Because we observe the same trends for all different N/P ratios, irrespective of the dilution, this suggests that there is indeed a correlation between the polymer concentration and the concentration of the polyplexes, at least in the investigated concentration range. This validates our earlier assumption.

Remarkably, we found that the effect of the N/P ratio on the electrostatic interaction rate is very small if the polymer concentration is kept constant (cfr. Figure 4). Furthermore, the adsorption curves for the two lowest polymer concentrations (i.e. 1253 and 1392 ng/mL) show that the electrophoretic mobility of the PS particle is almost unchanged during the duration of the experiment (i.e. around 10 minutes), indicating that there is no measurable interaction occurring.

To gain more insight into the kinetics of the polyplex adsorption and desorption on the PS particles, the results are fitted with the Langmuir theory, similar to what we performed in our previous work.37 The data corresponding to n = 1566 ng/mL (yellow data points in Figure 4) was subjected to a least-square fit according to the following equation:

$µ\left(t\right)=\left(µ\_{s}-µ\_{0}\right)\frac{K∙n}{1+K∙n}\left(1-e^{-t∙k\_{obs}}\right)+µ\_{0}$ (1)

Since the exact polyplex concentration is unknown but is expected to be correlated to the polymer concentration (vide supra), we used this latter parameter as input for the fitting. Furthermore, for the electrophoretic mobility of the bare PS particle in the buffer, we used μ0 = -5.5 x 10-8 m² V-1 s-1, while for the effective saturation mobility, μs = +3 x 10-8 m² V-1 s-1 was used, since this is the highest electrophoretic mobility value obtained for the highest polymer concentration. There are two unknowns in Equation 1, namely the equilibrium constant K (mL g‑1) and the observed rate constant kobs (s‑1). These are defined as $K=\frac{k\_{on}}{k\_{off}}$ and $k\_{obs}=k\_{on}n+k\_{off}$, respectively, where kon (mL g‑1 s­‑1) is the association rate constant and koff (s-1) is the desorption rate constant. The results of this fitting are provided in Table 2.

Table 2: The association rate, dissociation rate and equilibrium constant values for different N/P ratios, resulting from the least-square fitting of Equation 1 to the data presented in Figure 4 corresponding to a polymer concentration of 1566 ng/mL (yellow data points).

|  |  |  |  |
| --- | --- | --- | --- |
| N/P ratio | kon x 10-6 [(ng/mL)-1s-1] | koff x 10-3 [s-1] | K x 10-4 [(ng/mL)-1] |
| 2 | 3.4 | 1.4 | 25 |
| 10 | 4.8 | 1.5 | 30 |
| 20 | 4.9 | 0.9 | 50 |

These results confirm that the interactions have rates of association and desorption of a comparable order of magnitude for all N/P ratios, notwithstanding some small variations. Indeed, polyplexes obtained at higher N/P ratios generally resulted in a higher association rate constant and a lower dissociation rate constant, indicating that the interaction is stronger and occurs faster. This is also reflected in the equilibrium constant K, which increases as the N/P ratio increases. This is in line with the expectations, since a polyplex with higher N/P ratio typically has more positive charges and will therefore have a stronger and faster interaction.

Even though there are clear differences in the adsorption/desorption kinetics due to the N/P ratio, the effect of the polyplex concentration is still dominating (cfr. Figure 4). Therefore, we can conclude that this is the most important factor influencing the adsorption/desorption kinetics, while the N/P ratio is what defines the subtleties. When the polymer concentration is too low (i.e. n ≤ 1392 ng/mL), there is no significant change in electrophoretic mobility of the particles. We hypothesize that this is due to adsorption of polyplexes to the walls of the recipients we used during these experiments, leading to an overestimation of the actual concentration. This is in line with our previous findings and has been described in literature.37,44 This also suggests that a critical polymer concentration is required to overcome this issue, which in this case would be around 1500 ng/mL.

The ability of single-particle electrophoresis to investigate the effect of key parameters (polyplex concentration and N/P ratio) on the adsorption and desorption of polyplexes onto negatively charged particles has been demonstrated with this research. This technique could shed light on the importance of these parameters when studying cytotoxicity and transfection and enable selecting the best cationic polymers for gene delivery purposes. However, to draw conclusions relevant for applications, more research is needed, and a more accurate cell model should be considered. We used PS particles as a cell model, but ideally softer particles or vesicles could be used to mimic the cell better. Furthermore, immobilizing receptors or other cell membrane components on the particles might result in a model system which is a better approximation for the cell. Nonetheless, this work clearly demonstrates that the single-particle electrophoresis technique can measure the electrophoretic mobility of microparticles over time while they interact with polyplexes, and that the effect of several parameters (including polymer concentration and N/P ratio) can be investigated.

1. Conclusions

In this study, we used single-particle electrophoresis to investigate the very first step in the transfection and cytotoxicity process occurring during gene delivery, namely the electrostatic interaction between the polyplexes and the cell. Anionic PS microparticles were used as a simple cell model, since cells are also negatively charged. We studied this interaction for polyplexes with different N/P ratios and with different polymer concentrations to investigate the effect of these parameters on the adsorption/desorption kinetics. We found that the observed change in electrophoretic mobility is dominated by the polymer concentration (which in turn is related to the polyplex concentration), while the N/P ratio has a much smaller influence on the adsorption and desorption. Nonetheless, we observe that the interaction is typically stronger and faster for higher N/P ratios, as is reflected in the association rate, dissociation rate and equilibrium constants (determined by fitting the data to the Langmuir model).

This research demonstrates that single-particle electrophoresis can be a useful tool to study part of the processes involved in cytotoxicity and transfection and could have strong implications regarding which parameters are the most important. This opens new research potential for screening methods, with the final goal to be able to screen polymer libraries under different conditions to evaluate their potential for gene delivery applications, without the need for elaborate *in vitro* biological assays.

1. Supporting information

The supporting information contains 1H NMR spectra of the synthesized polymers (i.e. PAEMA and PGUMA), DLS data of the polyplexes and the simulation data that demonstrate we are predominantly operating in a reaction-limited system.

1. Acknowledgements

L. Van Daele would like to thank the Fund for Cardiac Surgery and the Research Foundation Flanders (FWO) (grant number 1SA2720N) for funding his PhD research. The research of Íngrid Amer Cid is funded by the Research Foundation-Flanders (FWO) through the Strategic Basic Research grant 1SA5919N. P. Dubruel would like to acknowledge the BOF-special research fund for the GOA financing (2022–2026). Prof. Richard Hoogenboom is acknowledged for the use of the DLS device.

1. Data availability

Data will be made available on request.

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