High-efficiency transfection of *Acanthamoeba castellanii* using a cationic polymer

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Abstract

The free-living amoeba *Acanthamoeba castellanii* is an ecologically, clinically, and evolutionarily important microorganisms. *A. castellanii* amoebae are directly pathogenic to humans, and serve as reservoirs for bacterial pathogens (e.g., *Legionella pneumophila*), but also regulate the proliferation of other microorganisms in the soil. Despite their importance, no reliable genetic system has been developed, hampering the use of *A. castellanii* and related species as model organisms. Transfecting *A. castellanii* with plasmids is possible with commercial kits, but is expensive, inefficient, and vulnerable to product discontinuation. In this contribution, we present a method for efficient transfection of *A. castellanii* with readily available and inexpensive polyethylenimines. We systematically explore the parameters of the method, obtaining up to 100-fold higher efficiency than currently used reagents. The method presented here provides a robust step towards a full genetic toolbox for *A. castellanii*, hence expanding its use as a model organism.

Keywords *Acanthamoeba* · Transfection · Cationic polymers · Polyethylenimines · PEI

Introduction

Acanthamoeba castellanii are small free-living amoebae, ubiquitous in a variety of environments. They are human pathogens responsible for two major infections – *Acanthamoeba* keratitis and granulomatous amoebic encephalitis (GAE)[1–3]. They also serve as reservoirs for other microorganisms such as bacteria, viruses and other protists, in either a parasitic or mutualistic relationship[1]. *A. castellanii* has been used as a model organism to study cytoskeleton motility, host-pathogen interactions, gene expression, as well as evolution of pathogenicity[3,4]. However, this species is still underutilised relative to other amoebae, such as *Dictyostelium discoideum*[3]. One of the reasons for this is its genomic complexity, which has hampered development of molecular biology tools[5,6]. The ability to successfully introduce and express exogenous genes in these amoebae is of particular interest and importance.

Transfection of *Acanthamoeba* with plasmid vectors has been successfully reported using physical methods (electroporation)[7], and more frequently, with chemical methods, using the commercially available reagents SuperFect® (Qiagen)[8,9], and ViaFect™ (Promega)[10]. The reported transfection efficiency is however low, typically 5% or less[2,6]. Commercial kits offer a quick and simple technique for transfection, and protocols have been established for *A. castellanii.* Nevertheless, they are costly, which restricts the freedom to further optimise the method for specific applications. Furthermore, availability has historically been very variable, with long lead times creating complications for ongoing projects. Worse, kits might get discontinued, as the example of SuperFect® shows. Since the components for such reagents are patented, finding a replacement is difficult.

Cationic polymers such as polyethylenimines (PEIs), poly-L-lysines (PLLs), polyamidoamines (PAMAMs), and chitosans are commonly employed as gene delivery systems in eukaryotic cells[11–13]. With their high number of positively charged (e.g., amine) residues, they form polyplexes with negatively charged phosphate groups in DNA molecules via electrostatic interactions[13]. This complexation condenses the DNA into small compact particles that can be internalised by cells, and make their way to the nucleus where genes can be expressed[12,13]. PEIs are the most studied and used cationic polymers due to their high cation charge density, wide availability, and low cost[12,14,15]. Their amine groups can be polymerised in two ways to create

linear or branched isomers. The degree of polymerisation, and branching of PEIs, along with other physio-chemical properties, affect their molecular weight, polydispersity and buffering capacity, reflected in how they interact with DNA. Although PEIs have been established as efficient DNA carrier vectors for a variety of cell types, there are many factors that can impact transfection efficiency, such as the properties of the targeted cell type, the nucleic acid quantity and purity, the preparation and composition of the complexes, and the transfection conditions employed[12,15,16].

PEIs have been used for transfection of another free-living amoeba – *Naeglaria*[17], but have yet to be explored and applied to *Acanthamoebae*. In this contribution, we describe the use of PEIs to transfect *A. castellanii*. We systematically explore three types of reagents and assess their effect on transfection efficiency and toxicity. We show that PEIs can be used for high-efficiency transfection of *A. castellanii*, thereby providing a foundation for the development of a full genetic system in this ecologically important amoeba.

Results

Complexation buffer affects DNA condensation by PEI

Due to the nature of the reaction that occurs during formation of DNA-PEI polyplexes, preliminary experiments were first carried out to investigate both how different complexation media and different concentrations of PEI impacted the condensation of DNA by these reagents. The different media tested were selected for supporting *A. castellanii* growth – PYG, Ac medium, LoFlo; or for having previously been described in the literature – HBS buffer, 150 mM NaCl, ultrapure water (ddH_2O) (see methods for details). Increasing quantities of PEI were used to understand how it relates to DNA complexation.

Agarose-gel retardation assays were performed to evaluate the DNA condensation ability of the various PEIs. After electrophoresis, the migration of the polyplexes was compared to that of naked plasmid DNA (pDNA) (Figure 1 and S1). In all conditions, increasing concentrations of PEI lead to better complexation of DNA. The various media affected the PEIs differently, more noticeably between the linear PEIs (PEI-25 and PEI-40) and the branched PEI (PEI-Br) (Figure S1). Nevertheless, a high stoichiometry of PEI to pDNA resulted in successful complexation across all buffer conditions. The retardation of the polyplexes can be due to neutralisation of the negatively charged DNA, which would then not move through the gel; polyplex size change; or a combination of both. Overall, PYG appeared to work best for all PEIs, and since it is also the preferred medium for *A. castellanii*, it was selected to carry out the remaining studies.

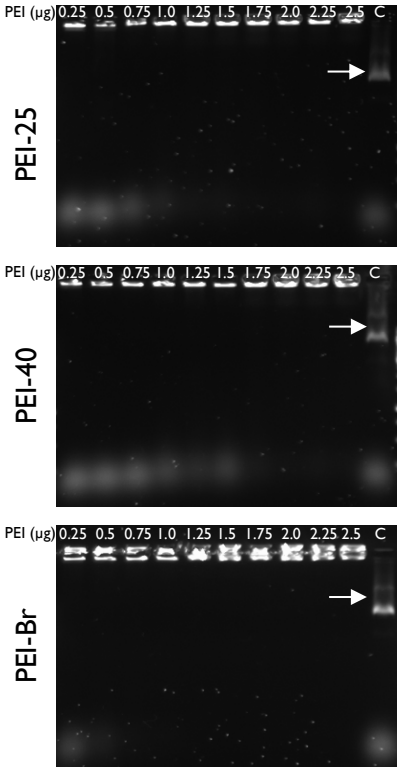

Figure 1. DNA condensation ability of PEIs in PYG. Increasing concentrations of PEI were used to complexate 0.25 µg of pDNA. The migration of the DNA-PEI polyplexes was compared to that of non-complexed pDNA, indicated by the white arrows (C, untreated control).

Evaluation of PEI toxicity on *Acanthamoeba castellanii*

In studies where PEI is used for transfection of mammalian cells, one factor always considered is how the transfection reagent alone affects the cells. A balance must be struck between a high transfection rate and cytotoxicity. To estimate the effect of PEI concentration on the amoebae, a resazurin assay was performed. In this assay the non-toxic and non-fluorescent dye resazurin is reduced to fluorescent resorufin via mitochondrial reductase. Change in fluorescence levels indicate mitochondrial activity, which is used as a proxy for the number of viable cells. The use of this assay to evaluate *A. castellanii* viability has been described[18], but the assay was further optimised here to ascertain its sensitivity for the conditions used. Measuring the fluorescence produced

by the amoebae with a 2mM resazurin solution and 6h of incubation, proved sensitive enough to discriminate even at low cell concentrations (Figure S2).

Cellular toxicity varied between the PEI reagents, and the largest contrast was, also here, between the linear and the branched PEIs (Figure 2). PEI-40 had the least toxic effect on the *A. castellanii* cells, with only a 20% decrease in cell viability at the highest concentration used (2.5 µg), followed by PEI-25 at 40% decrease, and finally PEI-Br at 80%. Altogether, the results indicate that there is a threshold at ~1.5 µg, whereafter the most substantial effects on viability are seen.

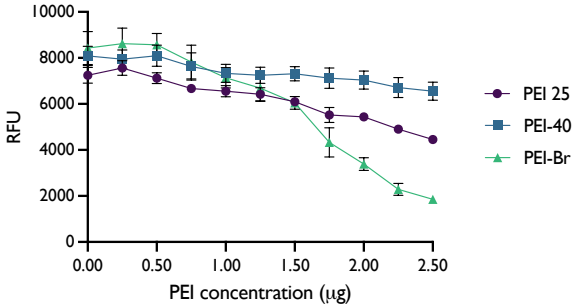

Figure 2. Cytotoxicity of PEI. Viability of *A. castellanii* as a function of concentration of PEI in the medium, evaluated by resazurin assay. Resorufin fluorescence was measured (ex: 530 nm, em: 590 nm) at 6 h post addition of the solution to the cells. Results from one of three independent experiments, data points in the graph the mean of 6 technical replicates, error bars are standard deviation.

Optimisation of transfection conditions

Transfection efficiency is highly dependent on effective formation of DNA-PEI polyplexes, i.e., all the DNA used should be condensed by the PEI, so that it can be delivered to the cell. For this to happen, there should be enough available nitrogen (N) in the PEI to cationise the phosphate (P) in the DNA, this is referred to as the N/P ratio, and it is used to find the optimal conditions for transfection. To evaluate the performance of each PEI used in this study, a combinatorial approach using a range of DNA and PEI concentrations resulting in 100 different combinations was used. The N/P ratios for these combinations ranged from ~ 0.7 to 70 (Table 1). This systematic approach was necessary since no previous work describes using PEI on *Acanthamoebae*. It was thus important to establish both the minimum and optimum effective N/P ratios at specific DNA concentrations.

The transfection efficiency was quantified using a microscopy-based approach. Four micrographs of each well were taken ~18h post transfection, and the number of positive *A. castellanii* transfectants (fluorescent cells) was computed by the Cell Profiler software (Figure 3).

Table 1. N/P ratios at different combinations of DNA and PEI.

PEI (µg) \ DNA (µg)	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5
2.5	71.9	35.9	24.0	18.0	14.4	12.0	10.3	9.0	8.0	7.2
2.25	64.7	32.3	21.6	16.2	12.9	10.8	9.2	8.1	7.2	6.5
2	57.5	28.8	19.2	14.4	11.5	9.6	8.2	7.2	6.4	5.8
1.75	50.3	25.2	16.8	12.6	10.1	8.4	7.2	6.3	5.6	5.0
1.5	43.1	21.6	14.4	10.8	8.6	7.2	6.2	5.4	4.8	4.3
1.25	35.9	18.0	12.0	9.0	7.2	6.0	5.1	4.5	4.0	3.6
1	28.8	14.4	9.6	7.2	5.8	4.8	4.1	3.6	3.2	2.9
0.75	21.6	10.8	7.2	5.4	4.3	3.6	3.1	2.7	2.4	2.2
0.5	14.4	7.2	4.8	3.6	2.9	2.4	2.1	1.8	1.6	1.4
0.25	7.2	3.6	2.4	1.8	1.4	1.2	1.0	0.9	0.8	0.7

The results of the combinatorial transfection screen for two constitutive fluorescence plasmids (pGAPDH-eGFP and pGAPDH-mScarlet) illustrate a significant impact of PEI choice for transfection of *A. castellanii*. Overall, there appears to be a minimum threshold for efficient transfection, contingent on the concentration of PEI. The results for PEI-Br concur with the toxicity data, as there were only few transfectants at concentrations is above 1.5 µg (Figure 3). Most of the fluorescent cells were here observed at N/P ratios ranging ~ 2.9 - 4.5 (Table 1 and Figure 3). The linear PEIs – PEI-25 and PEI-40, had the highest transfection efficiencies, but the results were not directly correlated to higher N/P ratios. Although the results vary considerably between the two plasmids used, the overall distribution trends were similar. Moreover, there seems to be a minimum threshold for transfection, set by the concentration of PEI; above 0.5 µg and 0.75 µg for PEI-25 and PEI-40, respectively. Over this threshold, there were transfectants even at the lowest concentration of DNA. The highest efficiencies were observed at the low to modest N/P ratios, i.e., between ~5-10 (Figure 3). Interestingly, the fluorescent cell count with PEI-40 for both plasmids was at the same N/P ratio of 8.6. With PEI-25 there was a high number of fluorescent cells at also higher concentrations of PEI.

Fluorescence variation between transfected cells

Through microscopy, it was possible to observe that the level of fluorescence varied between transfectants. To explore if this was a quantifiable metric, and in which conditions it was most evident, the mean fluorescence per cell was analysed (Figure 4 and S3). A notable variation in individual cell fluorescence can be seen throughout the transfectants, although it was most pronounced on those for pGAPDH-mScarlet, and particularly at high concentrations of DNA and PEI.

To discern whether the fluorescence variability was due to the number of plasmids per cell or to differential

gene expression, the amoebae were transfected with both plasmids at the same time. From the transfection screen results, it was clear that pGAPDH-mScarlet yields more transfectants than pGAPDH-EGFP, so it was imperative to optimise ratio of EGFP:mScarlet plasmid. For this analysis, the *A. castellanii* cells were co-transfected with PEI-40, at the optimal DNA:PEI concentrations – 1.25:1.5µg, as experimentally observed (Figure 3); and using a range of plasmid ratios from only EGFP to only mScarlet, in 10 increments (Figure S4).

As expected, mScarlet yielded more transfectants. Most importantly, at 9:1 EGFP:mScarlet plasmid ratio, we observed a substantial fraction of mScarlet+ and EGFP+ co-transfectants (Figure 5 and S4). This experiment shows that *A. castellanii* can be co-transfected with more than one plasmid per cell with the current protocol, and that titration of protein expression levels can be accomplished through altering pDNA amounts.

Transfection efficiency of PEI *vs* currently used reagents

The efficiency of PEI as a transfection reagent for *A. castellanii* was next compared to that of currently used reagents: SuperFect and ViaFect. To reduce biases, the parameters for PEI transfections were kept as similar as possible to those used for the other reagents, instead of using the optimal conditions, i.e., the quantity of pDNA and the incubation period was kept the same; and the protocol followed for the other reagents was derived from been published studies on *A. castellanii*[9,10]. However, because the published methods had instructions for larger volumes (6- and 24-well plates), the manufacturers' instructions were also used to derive the quantities of pDNA and transfection reagent required for a 96-well plate. Finally, the transfection with SuperFect was done with 1µg of pDNA, and 0.2µg for ViaFect; the incubation times of the complexes with the cells for these reagents also differed – 3h for SuperFect, and 16h for ViaFect.

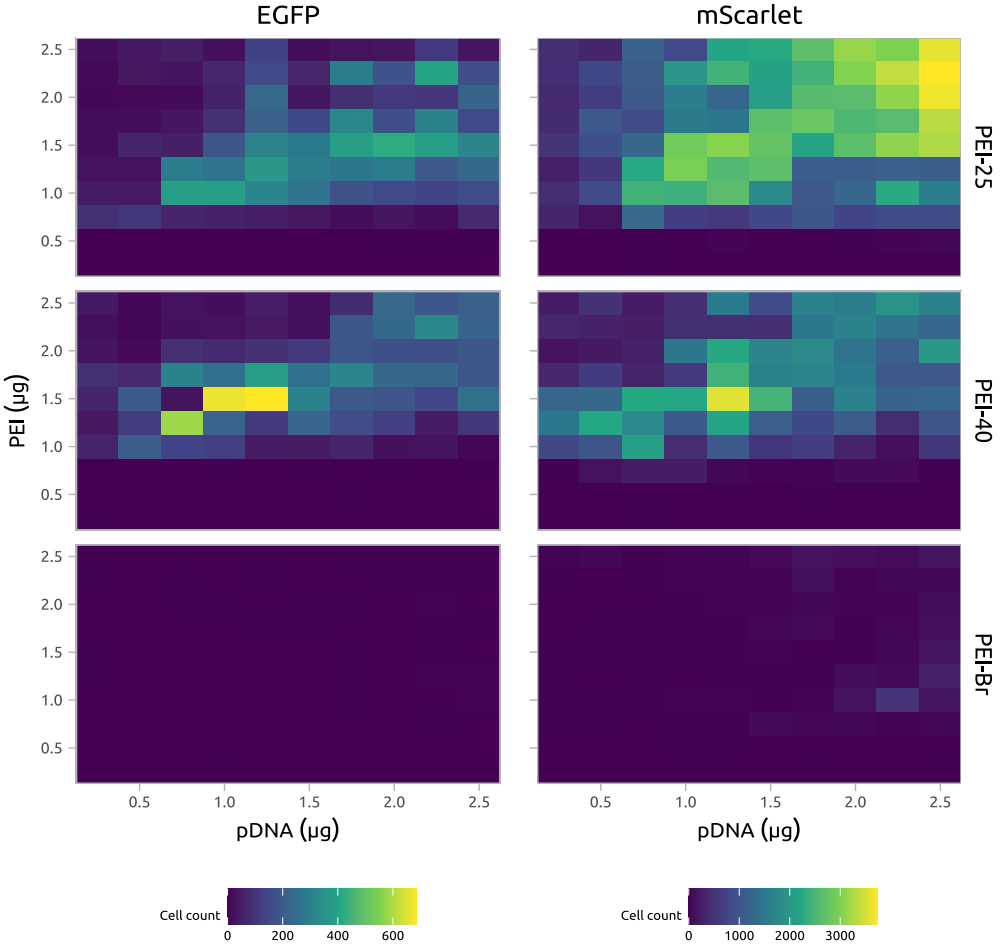

Figure 3. Effect of DNA and PEI concentrations on transfection efficiency. The left and right panels show the results for cells transfected with the plasmid encoding the EGFP and the mScarlet proteins, respectively. The top, middle and bottom panels correspond to PEI-25, PEI-40 and PEI-Br, respectively. The x- and y-axis represent the concentration of DNA and PEI, respectively. The colour of the tiles represents the sum of fluorescent cells detected on the four spots of the well. Note that scales are different for the left and right panels.

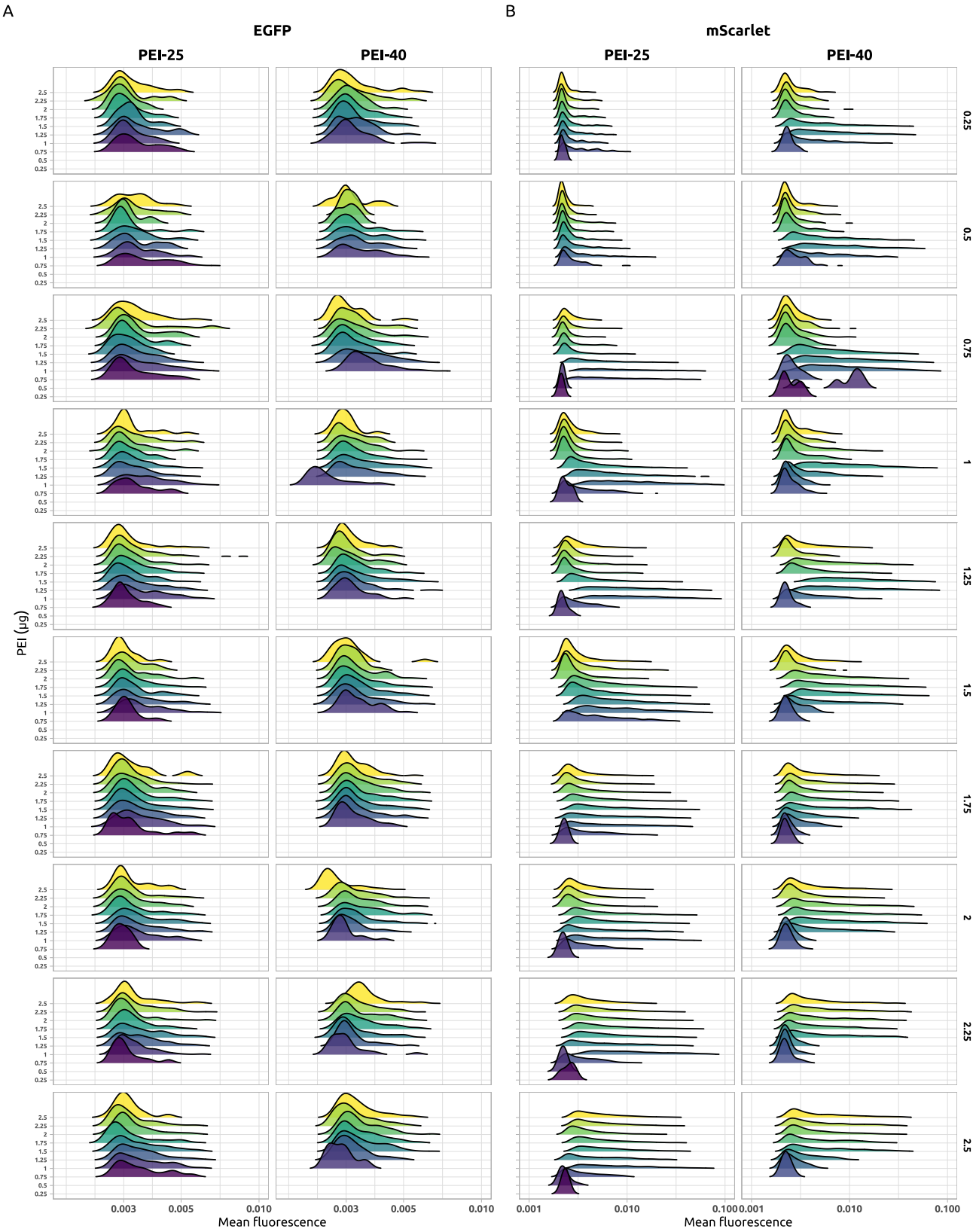

Figure 4. Fluorescence distribution per cell upon transfection with different DNA and PEI concentrations. (**A**) Transfection of EGFP plasmid with PEI-25 and PEI-40. (**B**) Transfection of mScarlet plasmid with PEI-25 and PEI-40. The rows of panels correspond to, from top to bottom, increasing concentration of plasmid DNA. Within each panel, each ridge corresponds to increasing concentrations of PEI (bottom to top), each marked with a different colour. The ridge shows the distribution of the cell fluorescence per condition (x-axis, logarithmic scale). Note that x-axis scale is different for **A** and **B.**

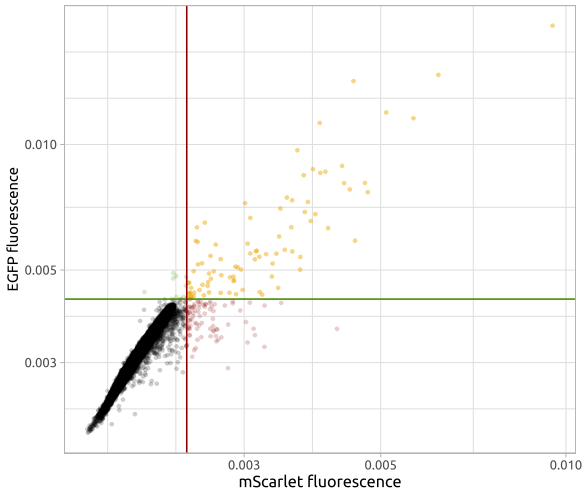

Figure 5. Distribution of transfectants according to their fluorescence. Transfection with 10% pGAPDH-mScarlet. Co-transfectants with both EGFP and mScarlet plasmids are yellow dots; red and green are EGFP- and mScarlet-single plasmid transfectants, respectively.; black dots are non-transfected cells. X- (red/mScarlet) and Y-intercept (green/EGFP) lines represent the threshold fluorescence for cell to be considered a positive transformant.

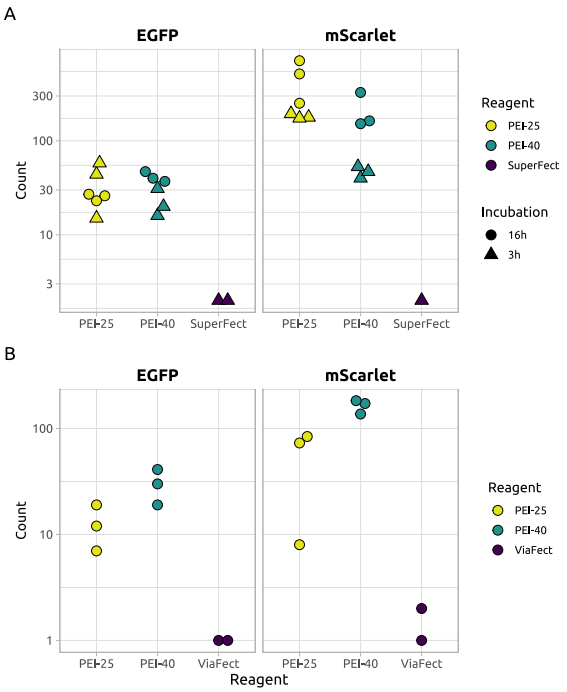

Figure 6. Comparison of the transfection efficiency between PEI-25 and 40 *vs* SuperFect or ViaFect. (**A**) Fluorescent cells counted per well after transfection of *A. castellanii* with pGAPDH-EGFP or -mScarlet using PEI-25, PEI-40 or SuperFect. PEI complexes were incubated for 3 or 16h, whilst SuperFect was incubated for only 3h, according to manufacturer instructions. (**B**) shows the same comparison between PEIs and ViaFect. The x-axis and different dot-colours correspond to the different reagents used, and the y-axis to the total transfectant count. The dot /triangle shape represents the incubation period of the reagent with the cells. Each dot is a technical replicate.

The results from the combinatorial experiments (Figure 3) were mined to find the right quantity of PEI-25 and -40 to add to these quantities of pDNA.

In both comparisons, both PEIs were vastly more effective at transfecting the amoebae than either commercial reagent (Figure 6). When compared against SuperFect, the efficiency both PEIs were at least 5x more efficient, up to ~100x more efficient when using PEI-25 and the mScarlet plasmid (Figure 6A). As the transfections using PEIs had not been tested with shorter incubation periods, replica transfections were performed where the *A. castellanii* were incubated with complexes for 3 or 16 h. Notably, the results indicate that DNA:PEI complexes can be transfected with broadly similar efficiency even within much shorter incubation periods.

In the comparison with ViaFect, there were considerably fewer PEI transfectants, but this is congruent with the previous results in this study. Nevertheless, even under these sub-optimal conditions both PEIs performed vastly better than ViaFect (Figure 6B).

Discussion

A. castellanii is a free-living protist, ubiquitously and abundantly present in fresh water and soil habitats. It is one of the most often isolated amoebal species worldwide[19,20]. Its status as an emerging human pathogen is due not only to the infections it causes as an opportunistic human pathogen, but also because of its role as a reservoir for bacteria, viruses, and fungi[19,20,21]. Furthermore, due to the cellular and functional similarities between *A. castellanii* and mammalian phagocytic cells, the former are considered as training grounds for these pathogens to later infect humans.[19,20,22]

All of these factors make *A. castellanii* an important and interesting model organism, however, but because of the shortage of molecular biology techniques (among others), it has not been as widely used as other amoebae[19–22]. Here, we demonstrate that the readily available and inexpensive cationic polymer PEI can be used to transfect *A. castellanii* with plasmids. We have systematically explored the use of three different types of PEIs, their concentration, and the concentration of DNA on the efficiency of transfection, and their effects on cell viability. The cytotoxicity data show that even at high concentrations of PEI-40 and PEI-25 are relatively non-cytotoxic. Yet, it is important to be wary that these high levels of PEI could potentially affect the cells in other ways not assessed here. Other cell characteristics, such as morphology, should be considered in future experiments.

The combinatorial approach allowed us to test a broad range of PEI and pDNA concentrations in a high-

throughput manner. However, due to both molecules' properties, the N/P ratio range tested was not as comprehensive, with most of the ratios falling between 4.5-11. Still, this approach was sufficient to highlight how at the extremes of the N/P ratio distribution there are none or very few transfectants. It also highlights how the actual concentration of the reagents in the solution, and not just N/P ratio, plays a role in efficient transfection. Together, these observations provide a comprehensive guide to choose optimal transfection parameters for *A. castellanii*.

By delving into the causes of variation in fluorescence levels between transfected cells, we were able to show that co-transfection of more than one plasmid per cell is both possible and prevalent. The range of fluorescence distribution was also affected by the quantity of PEI used, making it possible to modulate the level of co-transfection. This could be used to normalise or titrate the number of plasmids transfected per cell, should it be desirable to have a homogenous, or a graded, protein expression within the population.

The differing transfection efficiency between the two fluorescence plasmids remains unclear. Two hypotheses may explain this phenomenon: (i) mScarlet is a brighter fluorescent molecule, compared to EGFP (70 vs 33.54, according to www.fpbase.org), and so it may be more easily detected; (ii) alternatively, while EGFP was codon-optimised for expression in humans, mScarlet sequence was codon optimised for *A. castellanii*, which could improve the expression of the latter specifically in *A. castellanii.* This effect has been described before, as Bateman observed that: "*(...) high levels of monomeric red fluorescent protein (mRFP) expression could be serendipitously obtained.*", when using pTPBF-mRFP *versus* pTPBF-EGFP[23]. Another report mentions using a 1.7 ratio of mVenus- over tdTomato-expressing plasmid, when co-transfecting, to account for differences in brightness and quantum yield of the fluorescent proteins[24,25]. Temperature, an important element to consider when choosing fluorescent proteins[25], might also provide some insight in this phenomenon; since EGFP has been optimised for expression in human cells, it stands to reason that this protein would also be best expressed at 37°C, and not at 30°C. Ultimately, no generalisable conclusions regarding if one plasmid is always "better" than another can be drawn from the present work. Importantly, however, we demonstrate that PEI transfection provides a vastly improved protocol over current commercial standards, both for an EGFP and an mScarlet fluorescence plasmid.

Not investigated here, but of interest, is whether PEI is also effective for transfection of siRNA in *A. castellanii*. There are many studies demonstrating PEI-mediated gene knockdown with siRNA in other cell types. In studies where *A. castellanii* is transfected with siRNA, the reagents used are the same as those used for transfection with plasmid DNA, so it can be surmised that PEI would also work to achieve this in *A. castellanii*.

In summary, we here provide here evidence for high-efficiency transfection protocol in *A. castellanii* using the cationic polymer polyethylenimine, and give a comprehensive account of how to optimise the relevant transfection conditions.

Materials and Methods

Acanthamoeba culture

Acanthamoeba castellanii (ATCC 30010) were cultured in PYG medium with additives (ATCC medium 712) at 30°C. Cultures were split three times per week.

Plasmid DNA

The pGAPDH-EGFP plasmid used in this study was first described by Bateman[23], and it was kindly sent by Prof. Yeongchul Hong (Kyungpook National University, Korea). To make pGAPDH-mScarlet, the EGFP fluorescence reporter gene was removed by enzymatic digestion of the plasmid with *Nde*I and *Xba*I restriction enzymes. The plasmid backbone was gel purified, and used for ligation with mScarlet cut with the same restriction enzymes as above. The sequence of the mScarlet gene[26] was codon optimised according to the codon profile of *A. castellanii*, using a codon optimisation tool (https://dnahive.fda.gov/dna.cgi?cmd=codon_usage&id=537&mode=cocoputs)[27]. The restriction sites – *Nde*I and *Xba*I – were added at the 5' and 3' ends, and the sequence was synthesised by Integrated DNA Technologies®.

Transfection reagents

The 25kDa PEI 25™ (PEI-25) and 40 kDa PEI Max® (PEI-40) polymers were acquired from Polysciences, and were prepared according to manufacturer instructions to get a stock solution of 1 mg/mL. Branched PEI (PEI-Br) was purchased from Sigma-Aldrich (408727), and was diluted in ultrapure water to a concentration of 1 mg/mL.

Plasmid DNA-PEI affinity in different media

To test the effect of different media and buffers on the ability of the PEI to form complexes with the plasmid DNA at different N/P ratios, increasing amounts of PEI (0.25 – 2.5 µL) and 0.25 µg of pDNA were each diluted in 10 µL of the indicated complexation buffers, mixed together by pipetting, and incubated at RT for 15 minutes. After the incubation period, 30 µL of the respective buffer was added to the PEI-pDNA complex mixture. An aliquot of this was used to load a 0.7% agarose gel (with GelRed), and electrophoresis was carried out for 1h at 100V. The different media used were: Peptone Yeast Glucose (PYG) (ATCC 712); Ac medium (PYG medium minus protease peptone, yeast extract, and glucose); LoFlo (ForMedium); HEPES buffered solution (HBS); 150 mM NaCl solution; and ultrapure water (ddH_2O).

Viability Assay standardisation

To assess the toxicity of the transfection reagents, viability assays were performed using a resazurin assay[18]. Resazurin sodium salt (Sigma) was diluted in PBS to 2 mM, filter-sterilised, and kept at 4°C, protected from light.

To determine both the optimal cell number and incubation period with the resazurin salt solution for the assay, $2.5x10^3$-$8.0x10^4$ *A. castellanii* cells were seeded per well, in triplicates, in a black 96 well plate, in a final volume of 100 µL of PYG. The plate was incubated at 30°C for 16 h. After this time, 10 µL of the resazurin solution was added to each well, the plates were further incubated, and fluorescence measurements were taken at 2, 4 and 6 h. Resorufin fluorescence was measured at 530 nm (excitation) and 590 nm (emission) at 5 different locations in the well, using a Tecan Spark fluorometer.

PEI toxicity assays

A. castellanii tolerance for each PEI reagent was assessed using the resazurin assay. Briefly, $2x10^4$ cells were seeded per well in a black 96 well plate, and incubated overnight at 30°C. The following day the medium was replaced by 50 µL of PYG containing increasing concentrations of PEI (0.25-2.5 µL). The plates were further incubated for 24 h. The medium was then removed, and 100 µL PYG was added before initiating the resazurin assay (described above).

Nitrogen/Phosphate ratio theoretical approximation

The ratio of the amount of nitrogen in PEI per phosphate in pDNA in a reaction mixture (N/P ratio) was calculated, as it plays an important role in polyplex formation and transfection efficiency. The theoretical amine (nitrogen) groups in PEI are derived from the chemical formula for a single repeat unit (monomer) of the polymer. Using the 25kDa lPEI (-$NHCH_2CH_2$-) as an example, the molecular weight (MW) of a monomer would be 43 g/mol. Therefore, 1 µg would contain 23 nmol of nitrogen. The number of phosphate groups in the DNA are calculated using the equations (1) and (2)[11,12,16]:

$$(1)\ P\ moles = dsDNA\ moles \times dsDNA\ length\ (bp) \times 2$$

where

$$(2)\ dsDNA\ moles = \frac{dsDNA\ moles\ mass\ (g)}{dsDNA\ length\ (bp) \times MWbp}$$

Hence, 1 µg pGAPDH-EGFP (5831 bp) would have 3.2 nmol of phosphate. The N/P ratio of this reaction is then calculated with the following equation (3):

$$(3)\ N/P = \frac{23\ nmol}{3.2\ nmol} \approx 7.2$$

Acanthamoeba transfection

A day before transfection, $1x10^4$ cells were seeded per well in a 96 black well plate, in 100 µL of PYG with 1% Penicillin-Streptomycin (PS). On the day of transfection, the required amounts of pDNA and PEI reagents were first diluted in 10 µL of PYG, then mixed together by pipetting, and incubated at room temperature for 20 mins. During this incubation period, the amoebae plates were prepared by aspirating the PYG, and washing 1x with PBS. Lastly, the total volume of the pDNA-PEI reaction (50 µL) was added per well, drop-wise, and the plates were then incubated at 30°C, for 16 h.

Transfection with SuperFect and ViaFect

For comparison between PEI-25 and PEI-40 and the commercial reagents – ViaFect and SuperFect, *A. castellanii* cells were prepared and seeded as described above. The ratios of pDNA to transfection reagent used were based on published methods[9,10] and the manufacturers' protocols. For transfections with SuperFect, 1µg pDNA was mixed in 30µl of PYG, before adding 5µl of SuperFect. This reaction was incubated at room temperature for 20 mins, then 150µl of PYG were mixed in, and finally it was added to the cells. The cells were then incubated for 3h at 30°C, after which the SuperFect-DNA solution was carefully aspirated, the cells were washed once, and fresh PYG+PS was added, followed by an overnight incubation. For the paired comparison assays, 1 µg pDNA was transfected with 1.5 µg of PEI-25/PEI-40, as described, cells were incubated with the DNA:PEI complexes, for 3h or 16h, before replacing the medium

with PYG+PS. ViaFect transfections were performed in a similar manner, 0.2 µg pDNA and 1µl reagent were mixed in 50 µl PYG; this solution was incubated with the cells for 3 h, before adding 100 µl of PYG and incubating further, for a total of 16 h. To compare the PEIs with ViaFect, 0.2 µg pDNA was mixed with 1 µg PEI-25/PEI-40, and incubated with the cells for 16 h.

Detection of transgene expression

To assess the efficiency of transfection, a microscopy-based quantification approach was taken. Using a Nikon T*i* Eclipse microscope, 4 pictures were taken at different locations per well, with a 10x objective, covering a total area of approximately 38% of the well. Micrographs were taken both in the DIC channel, and in the GFP or RFP channels. The images were processed and analysed with the Cellpose 2.0 segmentation software [28] in CellProfiler analyst [29]. Data was further analysed with R[30] and figures were produced with the R package ggplot2[31] and GraphPad Prism (version 9.4.0 for Mac).

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Author contributions

A.B.M.: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing - Original draft. V.E.: Visualisation, Software. J.E.: Methodology, Software. M.E.S.: Methodology, Resources. L.G.: Formal Analysis, Visualisation, Resources. All authors reviewed and edited the manuscript.

Declaration of interests

The authors declare no competing interests.

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