Cationic polymers for non-viral gene delivery to human T cells

Brynn R. Olden[a,†], **Yilong Cheng**[a,†], **Jonathan L. Yu**[a], and **Suzie H. Pun**[a,*]

[a]Department of Bioengineering and Molecular Engineering and Sciences Institute, University of Washington, Seattle, WA 98195, USA

Abstract

The clinical success of chimeric antigen receptor (CAR) T cell immunotherapy in treating multiple blood cancers has created a need for efficient methods of *ex vivo* gene delivery to primary human T cells for cell engineering. Here, we synthesize and evaluate a panel of cationic polymers for gene delivery to both cultured and primary human T cells. We show that a subset of comb- and sunflower-shaped pHEMA-*g*-pDMAEMA polymers can mediate transfection with efficiencies up to 50% in the Jurkat human T cell line with minimal concomitant toxicity (>90% viability). We then optimize primary human T cell transfection conditions including activation time, cell density, DNA dose, culture media, and cytokine treatment. We demonstrate transfection of both CD4^{+} and CD8^{+} primary human T cells with messenger RNA and plasmid DNA at efficiencies up to 25 and 18%, respectively, with similarly high viability.

Graphical abstract
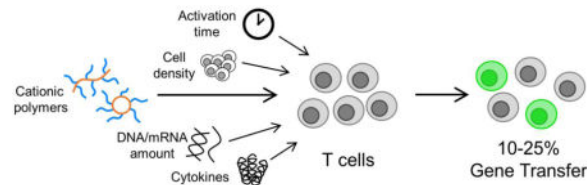
Keywords

Nonviral gene delivery; Cationic polymers; T lymphocytes

1. Introduction

T lymphocytes are key cells of the adaptive immune system that facilitate recognition and clearance of disease. In cancer, T cells primed with cancer antigens can often effectively

*Corresponding author. spun@u.washington.edu.
†Equally contributing authors

Declaration of Interest

A patent application has been fled on the sunflower and comb polymers by the University of Washington.

Appendix A. Supplementary Information

eliminate cancer cells [1]. However, tumors can hinder T cell activity by creating a poorly immunogenic environment and expressing anti-inflammatory cytokines and exhaustion signals that push T cells towards a regulatory or exhausted phenotype [2]. Over the last decade, scientists and clinicians have developed new immunotherapies that can overcome these barriers to allow T cells to better recognize and clear tumors [3,4]. One therapy that has shown remarkable promise, especially in hematologic cancers, is a cellular therapy using autologous, genetically-modified T cells that express a chimeric antigen receptor (CAR) [5]. These CARs consist of an extracellular domain that recognizes a surface epitope on cancer cells, and an intracellular domain that contains co-stimulatory signals that trigger T cell expansion, inflammatory cytokine production, and cytotoxic action against cancer cells [6].

To generate a CAR T cell product, primary human T cells must be genetically modified to express the CAR protein. Most clinical trials for CAR T cells use gammaretroviral and lentiviral methods for gene delivery [7]. These viruses are efficient at gene delivery, have low intrinsic immunogenicity, and integrate into the host genome, resulting in permanent CAR expression [8]. However, viral vectors have important limitations: DNA cargo size, potential for insertional mutagenesis, cost and challenge of production, lot-to-lot variability, and increased regulatory requirements make them a costly choice for use in CAR T cell manufacturing [9]. Further, construction and production of viral vectors is time-intensive and often a limiting factor in screening CAR constructs at the research phase. In addition, lentiviral vectors are limited to a cargo size of ~10 kb, which could limit the future ability to introduce additional functionalities into CAR T cells including kill-switches, triggered cytokine production, and multiplexed gene delivery [10]. Non-viral gene delivery methods like electroporation and chemical transfection reagents are an attractive alternative due to their reduced cost, their easy adaptation to various cargo, and their improved safety profile compared to viral vectors but historically have low gene transfer efficiencies in T cells [11,12].

Cationic lipid and polymer-based reagents have been developed extensively as gene delivery vehicles [13]. Unlike viruses, these materials are not limited to a size or type of genetic cargo they can carry. This makes them attractive delivery vehicles, because they can deliver large plasmids (pDNA), as well as messenger RNA (mRNA) and small interfering RNA (siRNA). Polymer-based reagents are especially of interest due to their controllable chemical diversity and shelf stability. Polymers for gene delivery are typically made of cationic monomers that contain primary, secondary, and/or tertiary amine groups that can complex with negatively charged nucleic acid to form condensed polymer-nucleic acid nanoparticle complexes, called "polyplexes" [14]. Polyethylenimine (PEI) and poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) are two classes of cationic polymers that have been studied extensively [15,16]. Branched PEI contains primary, secondary, and tertiary amines at an approximate 1:2:1 ratio whereas pDMAEMA only contains tertiary amines. Polyplexes are formulated with an excess positive charge to promote electrostatic binding to the negative cell membrane [17]. Highly cationic polyplexes are taken up into cells by interaction with surface proteoglycans, followed by internalization via endocytosis [18].

Very few polymers have been developed as gene delivery systems specifically for primary human T cells. Low molecular weight PEI (5 kDa) has been conjugated to transferrin to increase uptake of siRNA in activated primary T cells, which resulted in 50% gene silencing [19,20]. The Freitag group polymerized 20 "arms" of pDMAEMA off a silsesquioxane core and used this star polymer to deliver pDNA or siRNA, obtaining approximately 50% knockdown from siRNA delivery, and 10-50% transfection efficiency with 40-100% viability using pDNA [21]. This group also recently reported 13% transfection efficiency of primary T cells using this material with cell viability of 80% [22]. Most recently, the Stephan group reported a nanoparticle formulation with a poly(β-amino ester) core for DNA condensation and an antibody-conjugated polyglutamic acid shell for receptor-mediated uptake that was successfully used for *in vitro* and *in vivo* gene delivery to primary murine T cells, achieving ~3% transfection efficiency *in vitro* and ~1.5% *in vivo* [23]. This same nanoparticle system was able to achieve up to ~80% *in vitro* transfection efficiency of mRNA in primary human T cells [24].

The advances in controlled radical polymerization techniques over the last decade have significantly increased the design space for gene delivery polymers [25–27]. Chemists can specifically tune molecular weight, create complex architectures, and build in environmentally-responsive components [28,29]. In our laboratory, we found that altering the architecture of a pDMAEMA polymer from linear-branched (comb) to cyclic-branched (sunflower) decreased the toxicity and increased the efficiency of gene delivery to multiple cancer cell lines [30]. Including an endosomal lytic peptide in a pH sensitive block of a statistical co-polymer improved endosomal escape of polyplexes and increased subsequent gene expression in many cell lines [31].

The aim of this study was to empirically evaluate a panel of polymer architectures developed recently by our group for efficacy as an *ex vivo* gene delivery agent to primary human T cells. Polymers identified in this study could hold potential for future applications in adoptive T cell therapy manufacturing. We were surprised to find that the trends in polymer gene transfer efficiency in our previous adherent cell line studies did not hold for either cultured (Jurkat) T cells or primary T cells. In this current work, we identified a specific architecture of comb pDMAEMA polymers that, combined with optimized transfection protocols, showed the highest gene transfer efficiency to cultured and primary human T cells.

2. Materials and methods

2.1. Synthesis of pHEMA$_{15}$-g-pDMAEMA

pHEMA$_{15}$-*g*-pDMAEMA with different degrees of polymerization (DP) of DMAEMA was synthesized by three steps listed in Supplementary Information. Polymers were characterized by gel permeation chromatography (GPC) and nuclear magnetic resonance (^{1}H NMR) (Supplemental Figure 1).

2.2. Polymer preparation

Branched polyethylenimine ($M_w \sim 25,000$) was purchased from Sigma and diluted to 65 µg/mL in sterile molecular grade H_2O for transfection studies. Linear pDMAEMA$_{290}$, comb and sunflower polymers with a core of pHEMA$_{25}$, and virus-inspired polymer for endosomal release (VIPER) were synthesized as reported previously by controlled living radical polymerization [30,31]. Polymers were diluted in sterile molecular grade H_2O to desired amine concentration for transfection studies.

2.3. Antibodies and plasmids

PE/Cy-7 anti-human CD4 (clone: RPA-T4), APC anti-human CD8 (clone: RPA-T8), and Zombie Violet fixable live-dead stain were purchased from Biolegend and titrated prior to use.

Plasmids were prepared using standard molecular biology techniques. XL10 Gold ultracompetent cells (Stratagene) were transformed with pmaxGFP plasmid (Lonza). A single colony was grown up in an overnight culture, lysed and purified using the NucleoBond Xtra Maxi Endotoxin Free kit (Macherey-Nagel). Purity and concentration were quantified by Nanodrop and a diagnostic gel. Enhanced green fluorescent protein (eGFP) mRNA was purchased from TriLink and stored in aqueous stock solutions at −80°C until use.

2.4. Cell culture conditions

Jurkat cells (human T lymphocyte line) were a kind gift from Dr. Michael Jensen (Seattle Children's Research Institute). Jurkat cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (v/v). Cells were used in transfection studies 18-24 hours after passaging.

Cryopreserved vials of healthy donor primary human T lymphocytes, isolated by magnetic activated cell sorting, were generously provided by Juno Therapeutics. Thawed cells were washed once in basal X-VIVO 15 medium (Lonza) before being cultured in X-VIVO 15 supplemented with 2% KnockOut serum replacement (ThermoFisher) and either recombinant human IL-2 (200 IU/mL) or recombinant human IL-21 (10 ng/mL) (Miltenyi) at a density of 1.5×10^6/mL. Cells were rested for 2-16 hours before activation with CD3/CD28 Human T Activator beads (DynaBeads, Gibco) at the recommended 1:1 bead:cell ratio. Cells were activated for 0-72 hours prior to transfection. All cells were maintained in a 37°C and 5% CO_2 humidified incubator.

2.5. Polyplex formulation

Polyplexes were formed immediately before transfection experiments. Plasmid DNA or mRNA (1.75 and 0.1 mg/mL, respectively) was diluted in a sterile filtered 150 mM NaCl solution (Sigma) after which cationic polymers were added at the desired amine-to-phosphate (N/P) ratio to a final volume of 45 µL. This mixture was vortexed for 10 seconds and incubated at room temperature for 20-30 minutes before transfection.

2.6. Zeta potential and hydrodynamic diameters of polyplexes

Polyplexes were formulated at N/P 3, 5 & 7 with CP-25-16 polymer and 2 μg pmaxGFP plasmid DNA in molecular grade H_2O. Polyplexes were diluted to a final volume of 800 μL in 10 mM NaCl and loaded into a DTS1070 Zetasizer cell (Malvern Instruments) for dynamic light scattering and zeta potential measurements using a Zetasizer Nano ZS (Malvern Instruments).

2.7. Transfections

The same transfection protocol was used for Jurkat cells and primary human T cells. Cells were washed once in phosphate buffered saline (PBS) via centrifugation at 300×g for 5 minutes and resuspended in transfection medium 30 minutes prior to transfection. OptiMEM (Gibco), Jurkat culture medium, and T cell culturing medium were all used as transfection media in various experiments. Cells were plated at various concentrations in 250 μL transfection medium in a 24-well TC treated plate and stored in a 37°C and 5% CO_2 humidified incubator until transfection. Jurkat cells were plated at 1×10^6/mL (250 K cells per well), and T cells were plated at 2×10^6/mL (500 K cells per well), unless otherwise noted in optimization experiments.

Unless specified otherwise, 1.5 μg of nucleic acid was used per well in Jurkat transfections, and 2 μg in primary T cells transfections. Polyplexes or a control 150 mM NaCl solution were added to designated wells dropwise. Plates were returned immediately to the 37°C and 5% CO_2 humidified incubator. Cells were cultured for 48 hours prior to flow cytometry analysis.

2.8. Flow cytometry

Cells were transferred to a U-bottom 96-well plate by successive centrifugation and aspiration steps. Cells were washed once with PBS via centrifugation at 300×g for 5 minutes before being resuspended in 100 μL of a 1:500 dilution of Zombie Violet dye in PBS. Cells were incubated in the live/dead stain for 10-15 minutes at room temperature in the dark and subsequently washed twice with PBS with 1% BSA (PBSA from diluted 10% BSA stock, Miltenyi). If antibody staining was used in the experiment, cells were incubated in 100 μL of the diluted antibody cocktail for 20 minutes at room temperature, and washed twice with 1% PBSA. Cells were resuspended in 200 μL of 1% PBSA, and immediately analyzed on a MacsQuant Analyzer flow cytometer (Miltenyi) where at least 10,000 events in the "Live Cells" gate were collected.

Data analysis was performed using FlowJo software (FlowJo, LLC), with serial gating (Supplemental Figure 3). Transfection efficiency was measured as the percentage of live cells expressing GFP fluorescence.

2.9. Statistical analyses

Results are given as mean value ± standard deviation (SD). Multiple t tests, or one-way ANOVA with either Tukey's or Dunnett's multiple comparisons posthoc analysis were performed in GraphPad Prism software (Graph Pad Software).

3. Results and Discussion

3.1. Polymer panel screening in Jurkat cell line

Suspension cell lines and primary white blood cells are notoriously challenging to transfect, with no commercially available chemical reagent able to achieve high gene delivery efficiency. A panel of polymers were screened for transfection efficiency and cytotoxicity in the Jurkat human T cell line. This polymer panel included commercially available branched polyethylenimine (bPEI), as well as four distinct polymer architectures synthesized in our lab (Scheme 1). VIPER (virus-inspired polymer for endosomal release) is a diblock copolymer that contains a hydrophilic cationic block for nucleic acid loading and colloidal stability, and a pH sensitive membrane lytic block for endosomal release. This polymer is the best-performing polymer our lab has developed to date, with 80% transfection of HeLa cells with low cytotoxicity (>90% viability) [31]. Sunflower and comb polymers with a core size of 25 (pHEMA$_{25}$-g-pDMAEMA$_n$) also showed high efficiency in HeLas (50 and 40%, respectively) with low cytotoxicity (>75% viability), whereas the linear pDMAEMA polymer showed gene transfer efficiencies similar to bPEI (10%) in HeLas [30]. One polymer from each architecture was screened for transfection efficiency to Jurkat cells in both serum-containing and serum-free media using a range of polymer to DNA ratios (Supplemental Figure 4). The optimal N/P ratios for serum-containing and serum-free medium conditions were determined to be N/P 15 and N/P 5, respectively, such that the viability of cells stayed over 80% while maximizing transfection efficiency (Figure 1). The higher N/P ratio required in serum-containing media is likely due to the interaction of free polymer with serum proteins; free polymer has been reported to assist in intracellular trafficking [32–34].

Surprisingly, the trends in polymer performance in adherent cell lines was not recapitulated in Jurkat cells. Comb and sunflower polymers were the only architectures that showed appreciable transfection efficiency in serum-containing or serum-free conditions. We speculate that these differences could be due to differences in uptake and intracellular trafficking mechanisms in suspension cells compared to adherent cells, resulting in different polymer characteristics required for successful gene delivery. A 5-fold increase in transfection efficiency in serum-free media compared to serum-containing media was observed for both the comb and sunflower polymers. These results are expected, as negatively charged serum proteins can nonspecifically bind and disrupt polyplexes prior to endocytosis [35]. In addition, the cell viability was improved for transfections performed in serum-free medium, most likely due to the lower polymer concentration (N/P ratio) required for polyplex delivery. We chose to further evaluate the comb and sunflower polymers in serum-free transfection conditions for all additional Jurkat transfection studies, as the future utility of these polymers will be in well-defined *ex vivo* transfection protocols.

3.2. Optimization of pDMAEMA polymer architecture for transfection of Jurkat cells

Due to the promising T cell transfection efficiencies mediated by comb and sunflower polymers, we expanded this panel by synthesizing polymers with two core sizes and varying branch lengths per core size (Table 1). The impact of molecular weight and polymer geometry on gene transfer was then explored. Polymers with the same core size of DP 25

had similar transfection efficiencies and cytotoxicities in Jurkat cells (Figure 2 a & b). There were statistically significant differences between core geometries (linear vs. circular) at the two lower branch lengths, with comb polymers outperforming sunflower. Branch length was only significantly different within the same core geometry for the smallest molecular weight sunflower polymer (SP-25-11).

We observed a larger significant difference in transfection efficiencies of comb polymers with varying core sizes (DP 15 vs. 25), with the DP 15 core polymers exhibiting significantly reduced transfection efficiency and greater cytotoxicity (Figure 2 c & d, Supplemental Figure 5). While we did not investigate the mechanism underlying this difference in performance between the two core sizes, we hypothesize that this difference may be due to increased branching on the DP25 polymers. This hypothesis would be consistent with the results from the Müller group, where they have previously shown that star PDMAEMA with a higher number of arms were less toxic and resulted in higher transfection efficiency than polymers with fewer arms at equivalent molecular weights [36]. With these results, we chose to use comb polymers with a core size of DP 25 to optimize transfection conditions for primary human T cells, as they consistently showed the highest transfection efficiencies and viabilities across transfection studies in the Jurkat cell line.

Prior to moving into primary T cell studies, a more refined range of N/P ratios (3, 5, & 7) were evaluated around the highest performing N/P in serum-free transfection conditions. The size and zeta potential of polyplexes formed at these N/P ratios were similar (Supplemental Figure 2). N/P ratios of 5 and 7 resulted in similar viabilities and transfection efficiencies in Jurkat cells and were used for primary T cell studies (Supplementary Figure 6).

3.3. Impact of activation time on primary T cell transfection

Unlike viruses, cationic polymers do not have an active mechanism for transporting DNA into the nucleus of cells. Therefore, transfection efficiency is highly dependent on cell cycling and is most efficient during the G2/M phase of mitosis [37]. Unsurprisingly, transfections to quiescent, unactivated primary human T cells resulted in negligible gene expression (data not shown). To determine the optimal timing for transfections, primary T cells were transfected at various timepoints from 0-72 hours post-activation (Figure 3). In this study, cells were seeded at 250 K cells/well in OptiMEM and transfected with 1 μg of pDNA at N/P ratio of 5 with CP-25-16. The complete T cell medium was supplemented with 200 IU/mL IL-2. Untransfected cells went through the same manipulation steps as transfected cells, but were not treated with polyplexes.

Significant cell death (>50%) was observed in transfected and control groups manipulated prior to 24-hours. These results are consistent with common *in vitro* T cell culture practices of leaving T cells untouched for the first 2-3 days after bead-based activation, as cells are more prone to activation-induced apoptosis [38]. Maximum transfection efficiency was reached when performed 48-hours after activation, however, transfection efficiencies were 10-fold lower than those observed in Jurkat cells. We therefore sought to improve transfection efficiencies by optimizing other parameters of the transfection protocol.

3.4. Optimization of transfection conditions for primary T cells

We performed two design of experiment (DOE)-style screening experiments to identify which variables impacted primary T cell transfection and viability. In the first experiment, we screened cell density (125 K, 250 K, or 500 K cells/well), total mass of DNA delivered (1 or 2 μg), and transfection medium (OptiMEM or complete T cell medium with IL-2) (Figure 4 a & b). Higher cell densities and lower DNA doses both improved viability of cells. Transfection efficiency with CP-25-16 was slightly higher when OptiMEM was used as the transfection medium, and was highest for the highest cell density (500 K cells/well) and highest DNA dose (2 μg).

We hypothesized that higher DNA doses could be tolerated at higher cell densities, and perhaps result in even greater transfection efficiencies. In the second optimization experiment we screened cell density (500 K or 750 K cells/well), DNA dose (2 or 3 μg), and cytokine supplement (IL-2 or IL-21). We chose to include cytokine supplement to T cell culture medium as a variable because cytokines can significantly impact the phenotype and differentiation programs of cultured T cells. While IL-2 is the most common cytokine used for outgrowth of T cells, it has been shown that T cells cultured in IL-21 supplemented media can better mediate tumor regression after adoptive transfer [39].

Cells cultured in IL-21 showed an overall higher cell viability and comparable or higher transfection efficiency by CP-25-16 for all cell densities and DNA doses tested (Figure 4 c & d). Increasing the DNA dose to 3 μg significantly reduced the viability of cells cultured in IL-2 and reduced the viability of IL-21 cultured cells. Increasing the DNA dose to 3 μg did not have the desired effect of increasing transfection efficiency. Instead, cells treated with this higher dose showed lower transfection efficiencies for all conditions tested.

The maximum transfection efficiency achieved for delivery of plasmid DNA to primary T cells was 18%. Optimal conditions were 500-750K cells per well in a 24-well-plate format in complete T cell medium supplemented with IL-21, and transfecting cells in OptiMEM with a total dose of 2 μg of DNA 48-hours after activation. These transfection parameters were used to probe the initial utility of these comb polymers for applications relevant to adoptive T cell therapy.

3.5. Delivery of various nucleic acid cargoes to primary T cells

Many future applications of cationic polymers as gene transfer agents for T cell-based therapies will require the use of an integrating technology like transposon/transposase for stable gene expression or CRISPR/Cas9 systems for gene editing [40,41]. The *Sleeping Beauty* transposon/transposase system has already been used with electroporation to generate stably expressing CAR T cells [11,42,43]. These systems require the parallel delivery of two nucleic acid cargoes: a plasmid or mRNA to express the editing enzyme (Cas9 or transposase) and a plasmid with the gene for insertion (transposon or DNA template). The delivery platform with the best safety profile delivers the editing enzyme gene via mRNA and the gene for insertion by plasmid DNA [44]. The transient expression of the enzyme nearly eliminates the possibility of repeat template excision/re-integration genotoxicities, and prevents the enzyme from integrating itself into the host genome.

To show the utility of cationic polymers for this application, we successfully delivered mRNA and pDNA to primary T cells using the optimized transfection protocol (Figure 5 a & b). We observed a higher level of gene expression from cells transfected with mRNA compared to plasmid DNA, suggesting that nuclear transport is a contributing barrier to higher gene expression with this reagent.

3.6. Delivery to both CD4⁺ and CD8⁺ primary T cells

We wanted to ensure that these comb polymers could transfect both CD4$^+$ "helper" and CD8$^+$ "cytotoxic" T cells, as both subtypes are used in CAR T cell therapy, and initial polymer screening was performed on the CD4$^+$ Jurkat cell line. We stained transfected T cells with fuorescently tagged antibodies for CD4 and CD8 and observed an equivalent proportion of transfected cells in each subset (Figure 5 c & d). There was no difference in transfection efficiency of pDNA and only a small statistical difference in transfection efficiency of mRNA between CD4$^+$ and CD8$^+$ T cells, with a slightly higher level of gene delivery to CD4$^+$ T cells.

These results support the continued development of comb polymers for *ex vivo* non-viral genetic reprogramming of primary human T cells for the application of CAR T cell manufacturing. Additional polymer and polyplex engineering would be required to create a gene delivery platform for *in vivo* delivery to prevent non-specific interactions and toxicities caused by the positive charges on the surface of the polyplex. However, the flexible cargo loading of this polymer system makes it attractive for *ex vivo* gene delivery applications.

4. Conclusion

One of the major opportunities for cost reduction and safety improvement in CAR T cell manufacturing is moving from viral to non-viral methods of genetically reprogramming patient T cells. These studies begin to evaluate shelf-stable, easy to manufacture, untargeted cationic polymers as alternative non-viral gene delivery vehicles for CAR T cell manufacturing. We identified a class of pHEMA-*g*-pDMAEMA comb and sunflower polymers that can transfect the Jurkat T cell line at high efficiencies of 25-50% in serum-free medium. This could be a very useful tool for the screening experiments performed in the development of new chimeric antigen receptors. Extensive and iterative protein engineering is required for developing new chimeric antigen receptors [45,46]. Having a quick and efficient method to screen expression and binding affinity of various CARs in a T cell line, instead of creating a new viral vector for each one, could significantly reduce the time it takes to develop and perform early validation studies on new CAR constructs. Jurkat cells, like all suspension cell lines, are notoriously challenging to transfect. Though untested, these comb and sunflower polymers may also be able to transfect other suspension cell lines with high efficiency.

Our data demonstrate that these polymers can also transfect primary human T cells with pDNA and mRNA at modest efficiencies up to 18 and 25%, respectively, while maintaining high cell viability (>75%). These efficiencies were reached through DOE-style optimization experiments that yielded a 4-fold increase over initial transfection values. These results support the continued development of comb polymers for non-viral genetic reprogramming

of primary human T cells for the application of CAR T cell manufacturing. Future studies will seek to understand the additional barriers to non-viral gene delivery in primary T cells and identify alternative protocol changes that will further increase transfection efficiency to a level approaching those achieved by viral gene delivery methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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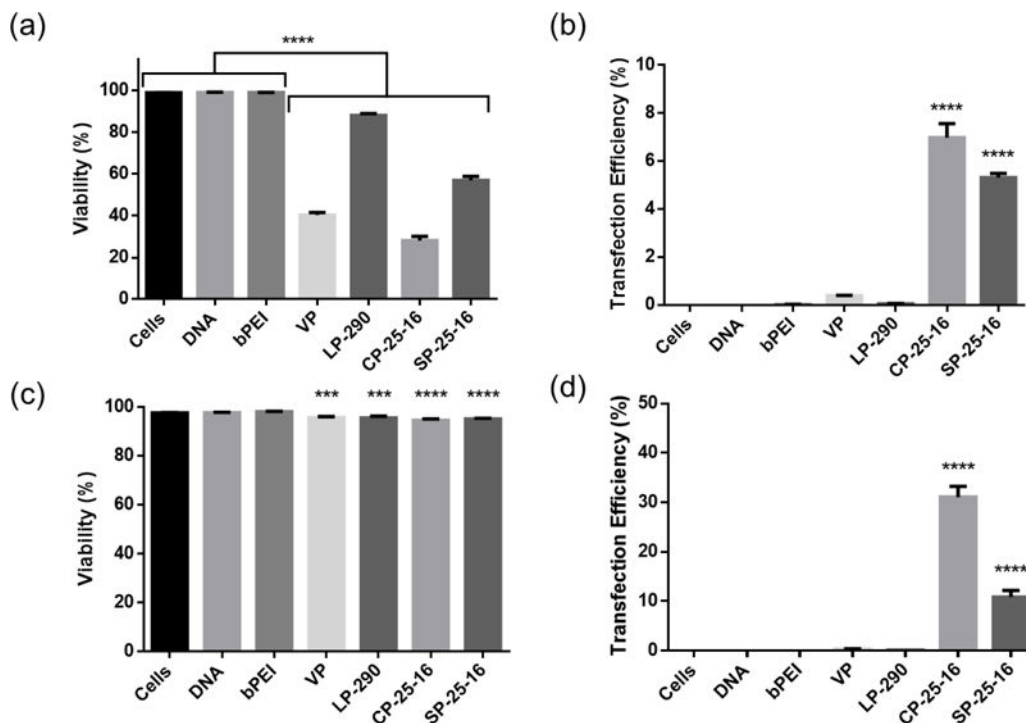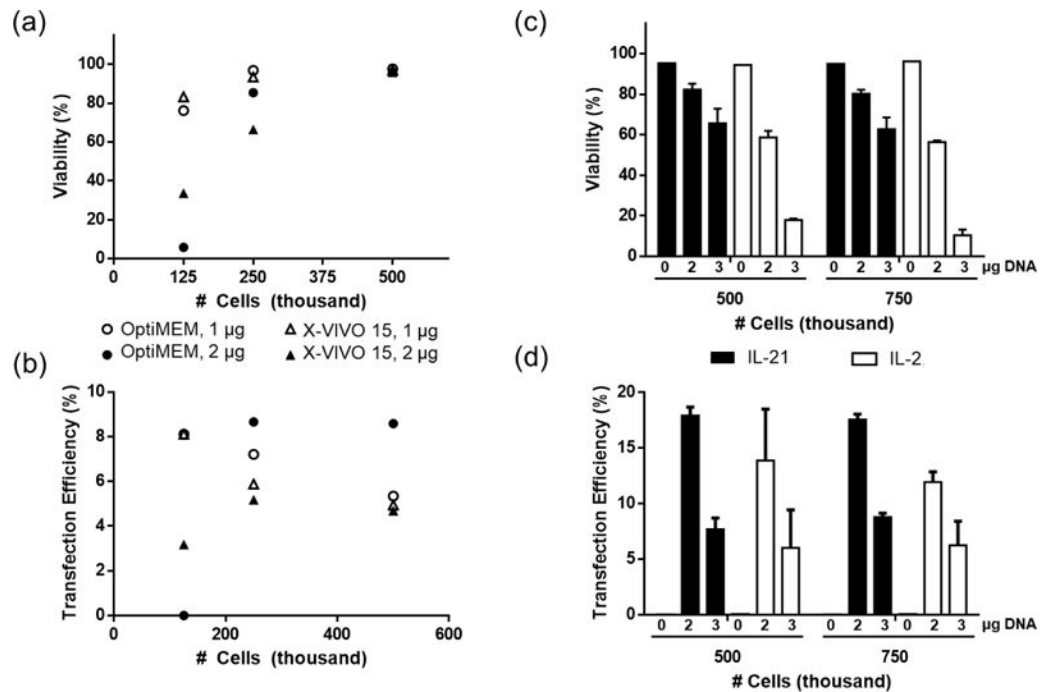
Figure 1.
Polymer transfections delivering pmaxGFP plasmid in serum and serum-free medium to Jurkat T cell line. Toxicity (a) and transfection efficiency (b) of cationic polymers in Jurkat human T cell line in serum containing medium at N/P 15. Toxicity (c) and transfection efficiency (d) in serum-free medium at N/P 5. Transfection efficiencies are expressed as percentage of GFP-positive cells. Data are shown as mean ± SD (n=3; 1-way ANOVA with Tukey's multiple comparisons, *p<0.05, *** p<0.001, ****p<0.0001).
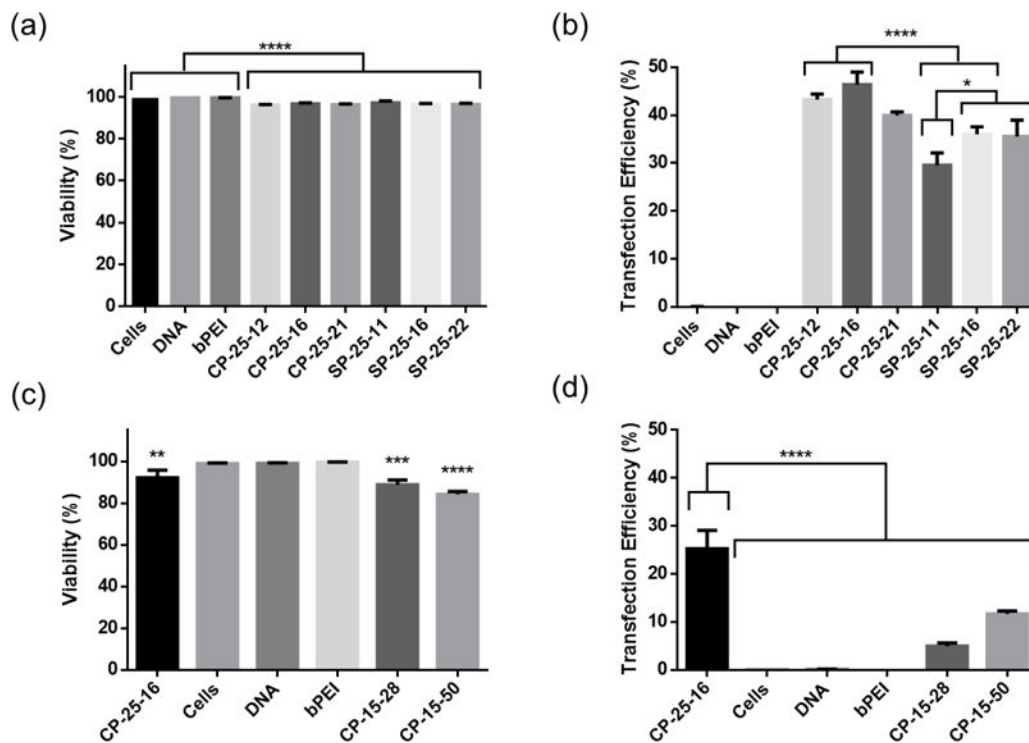
Figure 2.
Impacts of polymer architecture on transfection of pmaxGFP plasmid to Jurkat T cell line. Toxicity and transfection efficiency of cationic polymers with varying core shape and branch length (a & b) or core size and branch length (c & d). Transfection efficiencies are expressed as percentage of GFP-positive cells. Data are shown as mean ± SD (n=3; 1-way ANOVA with (a, c & d) Dunnett's or (b) Tukey's multiple comparisons, * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001).
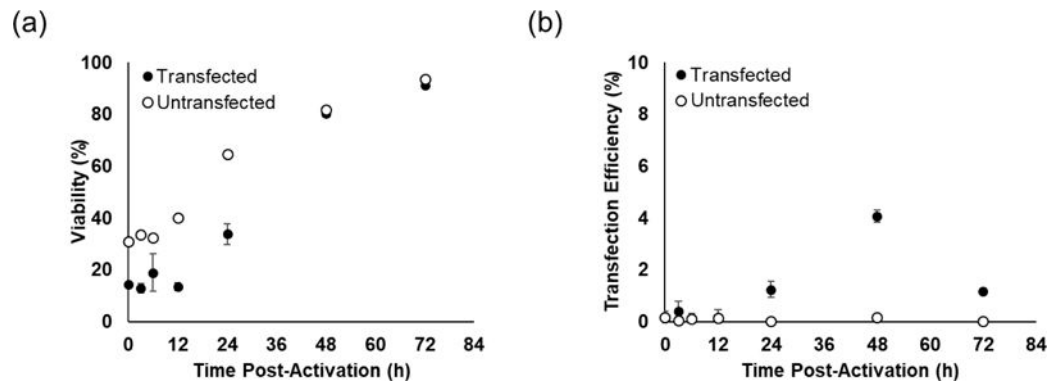
Figure 3.
Impact of activation time prior to transfection on primary T cell viability (a) and transfection efficiency (b). Cells were transfected with 1 μg of pmaxGFP pDNA at N/P ratio of 5 using CP-25-16. Transfection efficiencies are expressed as percentage of GFP-positive cells. Data are shown as mean ± SD (n=3).
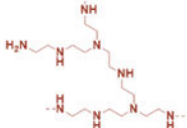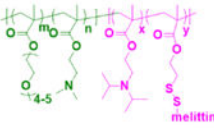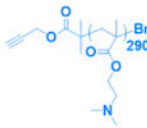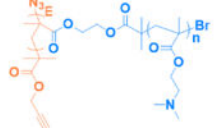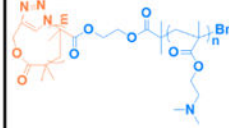
Figure 4.
Design of Experiments (DOE) optimization of primary T cell transfection conditions. (a & b) Screening effects of transfection medium, cell density, and DNA dose on viability and transfection efficiency (n=1). (c & d) Screening effects of cytokine supplement in culture medium, cell density, and DNA dose (n=3). Cells were transfected with pmaxGFP pDNA at N/P ratio of 7 using CP-25-16. Transfection efficiencies are expressed as percentage of GFP-positive cells. Data are shown as mean ± SD.
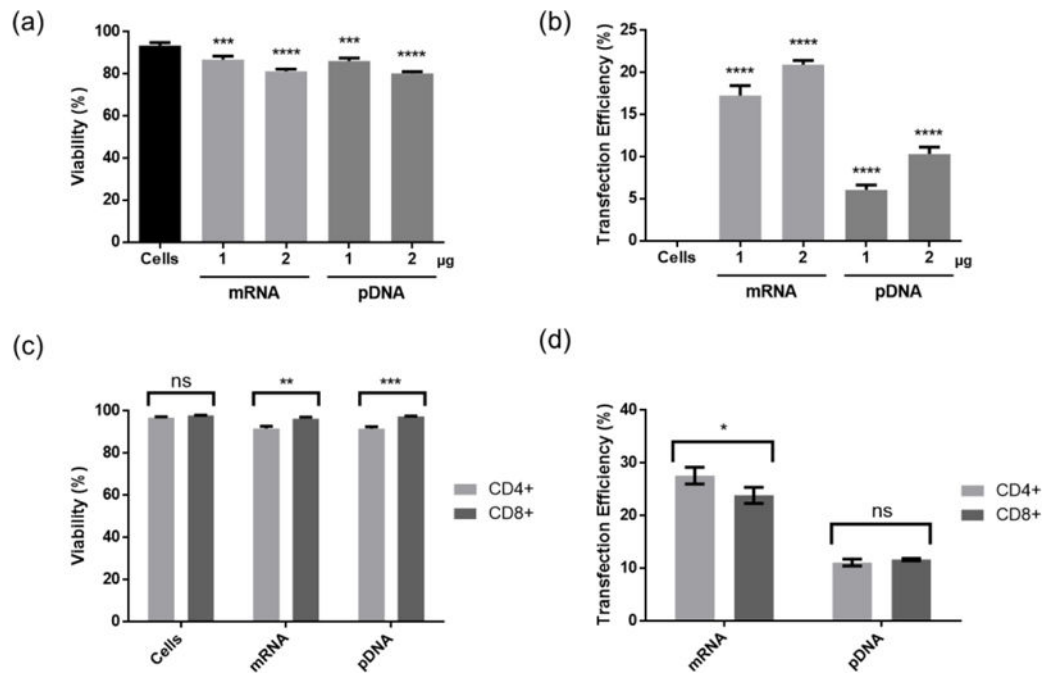
Figure 5.
Viability (a) and transfection efficiency (b) of eGFP expressing pDNA and mRNA to a mixture of CD4^{+} and CD8^{+} T cells at optimized conditions. Viability (c) and transfection efficiency (d) in CD4^{+} and CD8^{+} T cells. Cells were transfected with pDNA at N/P ratio of 7 using CP-25-16. Transfection efficiencies are expressed as percentage of GFP-positive cells. Data are shown as mean ± SD (n =3; (a & b) 1-way ANOVA with Tukey's multiple comparisons, (c & d) multiple t tests, *p<0.05, * *p<0.01, ***p<0.001, ****p<0.0001).

Branched PEI (bPEI)	VIPER (VP)	Linear (LP)	Comb (CP)	Sunflower (SP)
bPEI	VP	LP-290	CP-25-12 CP-25-16 CP-25-21 CP-15-28 CP-15-50	SP-25-11 SP-25-16 SP-25-22

Scheme 1.

Schematic and chemical structures of polymers evaluated in gene delivery studies.

Abbreviated polymer names denote degrees of polymerization (DP) for each block.

Table 1

Characterization of synthesized polymers.

Composition[a]	Code	M_n [kDa][a]	M_n [kDa][b]	PDI[b]
l-pDMAEMA$_{290}$	LP-290	46	53.5	1.09
l-pHEMA$_{25}$-g-(pDMAEMA$_{12}$)$_{25}$	CP-25-12	54	192	1.48
l-pHEMA$_{25}$-g-(pDMAEMA$_{16}$)$_{25}$	CP-25-16	70	263	1.67
l-pHEMA$_{25}$-g-(pDMAEMA$_{21}$)$_{25}$	CP-25-21	89	371	1.75
l-pHEMA$_{15}$-g-(pDMAEMA$_{28}$)$_{15}$	CP-15-28	70	209	1.43
l-pHEMA$_{15}$-g-(pDMAEMA$_{50}$)$_{15}$	CP-15-50	122	481	1.62
c-pHEMA$_{25}$-g-(pDMAEMA$_{11}$)$_{25}$	SP-25-11	50	176	1.44
c-pHEMA$_{25}$-g-(pDMAEMA$_{16}$)$_{25}$	SP-25-16	70	287	1.81
c-pHEMA$_{25}$-g-(pDMAEMA$_{22}$)$_{25}$	SP-25-22	93	413	1.88
p(OEGMA$_{11}$-DMAEMA$_{56}$)-*b*-p(DIPAMA$_{33}$-(PDSEMA-*g*-melittinb)$_1$)	VP-67-34	23	25	1.03

[a]Determined by ^{1}HNMR;

[b]Obtained by GPC.