Poly(beta-amino ester)s as high-yield transfection reagents for recombinant protein production

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Transient transfection is an essential tool for recombinant protein production, as rapid screening for expression is conducted without stable integration of genetic material into a target cell genome. Poly(ethylenimine) (PEI) is the current gold standard for transient gene transfer, but transfection efficiency and resulting protein yield are limited by the polymer's toxicity. This study investigated the use of an alternative class of cationic polymers, poly(beta-amino ester)s (PBAEs), for transient transfection of human embryonic kidney 293F (HEK) and Chinese hamster ovary-S (CHO) cell suspensions. In both HEK and CHO cells, several PBAEs demonstrated superior transfection efficiency and production of a cytosolic reporter compared to

PEI. This result extended to secreted proteins, as a model PBAE increased the production of three secreted antibodies compared to PEI at scales ranging from 20-2,000 mL. In particular, non-viral gene transfer using the lead PBAE/plasmid DNA nanoparticles led to robust transfection of mammalian cells across different constructs, doses, volumes, and cell types. These results show that PBAEs enhance transfection efficiency and increase protein yield compared to a widespread commercially available reagent, making them attractive candidates as reagents for use in recombinant protein production.

Keywords: protein production, transient transfection, poly(beta-amino ester)s, transfection reagents

Current research into chemical-based transfection methods focuses largely on optimizing agents for use in the development and production of recombinant proteins. Transient transfection, in which introduced genetic material is not incorporated into the host genome, is especially useful during the high-throughput design and screening of proteins (e.g., candidate biologics) wherein stable expression is not needed. While culture conditions and plasmid design have been popular targets for optimization in transient transfection workflows (Backliwal et al., 2008; Galbraith, Tait, Racher, Birch, & James, 2006), further research into improved transfection reagents has even greater potential for boosting protein yields. Chemical-based transient transfection relies on condensation and encapsulation of plasmid DNA by a biocompatible material into particles which are taken up by target cells; differences in particle size can affect the method of cellular uptake, leading to differences in transfection efficiency (Kim, Sunshine, & Green, 2014). Particles must then escape the endosome and the encapsulating material must degrade to allow for DNA release, nuclear translocation, transcription and subsequent export, and finally translation and processing into fully formed protein (Karlsson, Rhodes, Green, & Tzeng, 2020).

Transfection reagent structure and buffering capacity have been demonstrated to influence DNA uptake and escape, making these properties particularly consequential in reagents for transient transfection workflows (Sunshine, Peng, & Green, 2012). Maximizing protonability, for example, facilitates endosomal swelling and consequent rupture via the "proton sponge" effect (Boussif et al., 1995; Bus, Traeger, & Schubert, 2018). Cationic polymers have typically been among the most promising transfection reagents; their charge-based association with DNA into particles offers protection from degradation and offers sufficient buffering capacity to facilitate endosomal escape following cellular uptake (Sunshine et al., 2012).

Poly(ethyleneimine) (PEI) is a commercially available cationic polymer used extensively as a transfection reagent that has a high density of protonatable amines, giving rise to high buffering capacity and efficient endosomal escape (Boussif et al., 1995). PEI of average molecular weight 25 kDa is most frequently used in transfection workflows, but its toxicity limits transfection efficiency and, consequently, protein yield (Breunig, Lungwitz, Liebl, & Goepferich, 2007; Yang, Li, Goh, & Li, 2007). Previously, PEI has been conjugated to polyethylene glycol (Petersen et al., 2002) and arginine modified oligo(-alkylaminosiloxane) [P(SiDAAr)n] (Morris & Sharma, 2010) to mitigate cytotoxicity.

A promising alternative to PEI, poly(beta-amino ester)s (PBAEs) are a class of cationic polymers used to facilitate efficient gene transfer*in vitro* (Bishop, Kozielski, & Green, 2015). PBAEs are composed of an acrylate base monomer, an amine sidechain, and a terminal end-capping group, each of which can be varied to create a vast library of materials (Akinc, Lynn, Anderson, & Langer, 2003). Hydrolyzable ester linkages allow for degradation of the PBAEs in transfection conditions which allows for use of the polymers at high weight ratios relative to other non-biodegradable materials, maximizing density of buffering amines to facilitate endosomal escape (Sunshine et al., 2012). Their biodegradability also obviates the need for medium replacements or additions, themselves contributors to cell death, which are common where PEI is utilized (Galbraith et al., 2006). These linear polymers are synthesized from inexpensive, commercially available reagents using a two-step polymerization method (Fig. S1A) and are stable long term when stored dry at -20°C (Wilson et al., 2019).

Given the high transfection efficacy observed with PBAEs in various*in vitro* contexts, we sought to investigate the use of PBAE nanoparticles for transient transfection of suspension cultures in intracellular and secreted protein production workflows (Fig. 1A). We selected four PBAEs with varying base (B), sidechain (S), and

end-cap (E) structures to evaluate in comparison with linear 25 kDa PEI: B4-S4-E6 (4-4-6); B4-S5-E7 (4-5-7); B4-S5-E39 (4-5-39); and B5-S3-E6 (5-3-6) (Fig. 1B-C, S1B). Physiochemical characterization of PBAE and PEI nanoparticles in serum-free transfection media indicated that PBAE nanoparticles maintained a smaller size in transfection conditions (approximately 200-350 nm) whereas PEI nanoparticles were prone to aggregation, resulting in sizes over 1 μm (Fig. 1D, S1C). Previous studies indicated that PEI nanoparticles were prone to aggregation in serum-free media due to interactions with salts and a lack of adsorbed proteins that can help stabilize discrete particles and prevent clustering (Ogris et al., 1998; Pezzoli, Giupponi, Mantovani, & Candiani, 2017). Analysis of surface charge revealed that PBAE nanoparticles maintained a positive zeta potential in transfection conditions, whereas PEI nanoparticles exhibited a near neutral surface charge (Fig. 1E, S1C). Shielded surface charge of PEI particles may limit interactions with a charged cell membrane, thus hindering cellular uptake.

To determine the optimal DNA dose for production of cytosolic mCherry using various polymer-based transfection agents, we selected a representative PBAE, 2-(3-aminopropylamino)ethanol end-capped poly(1,4-butanediol diacrylate-co-4-amino-1-butanol) (referred to here as 4-4-6), and compared this PBAE to 25 kDa PEI at doses ranging from 0.5 to 4 μg/mL DNA. The polymers were compared in two mammalian cell lines frequently employed for protein expression: human embryonic kidney 293F (HEK) cells and Chinese hamster ovary-S (CHO) cells. Evaluation of mCherry fluorescence over a span of 5 days indicated that peak mCherry expression occurred using the 4-4-6 polymer at 2 μg/mL and 4 μg/mL DNA doses in HEK and CHO cells, respectively (Fig. S1A). Notably, peak mCherry expression in PEI-based transfections was not comparable to that attained by 4-4-6 at any dose. Subsequent time course studies using the optimized DNA dose that compared additional PBAE structures demonstrated significantly increased mCherry expression using 4-4-6, 4-5-7, and 4-5-39 in HEK cells, and using 4-4-6 and 4-5-7 in CHO cells, compared to PEI-mediated transfection (Fig. 2A, 2C). Transfection efficiency, as measured by mCherry-positive cells on day 5, was significantly increased with all PBAEs tested in HEK cells and with 4-4-6, 4-5-7, and 4-5-39 in CHO cells (Fig. 2B, 2D, S2C). Fluorescence microscopy confirmed the increase in mCherry expression (Fig. 2E, S2D). Cell viability, assessed via MTS assay 24 h following transfection, indicated that PBAEs (especially 4-5-39 and 5-3-6) showed greater toxicity than PEI, though notably at a 20- to 30-fold higher weight ratio (Fig. S2B). Importantly, this reduced viability did not result in inferior mCherry expression relative to PEI, with 4-4-6 and 4-5-7 demonstrating superior expression in both HEK and CHO cells (Fig. 2A, C).

To demonstrate that the results of these fluorescent protein expression experiments were replicable at scales relevant to the development and production of secreted proteins, we transfected HEK cell cultures of varying volumes with DNA encoding the recombinant antibody 10H2 (Chuntharapai, Lee, Hébert, & Kim, 1994; Patent No. WO/2020/243489, 2020) using either 4-4-6- or PEI-based particles. Based on SDS-PAGE image analysis of small-scale dose titrations (Fig. S3A-D), DNA was dosed at 1 μg/mL for secreted proteins in HEK cells, with polymer weight adjusted accordingly. At volumes ranging from 20-200 mL, transfection with 4-4-6 yielded between 4.5-fold and 8.2-fold more protein than did transfection with PEI (Fig. 3A). To further demonstrate the scalability of enhanced protein expression using PBAEs, 2 L cultures of HEK cells were transfected with DNA encoding either 10H2 or the bispecific antibody BS2 (Patent No. WO/2020/243489, 2020) using either 4-4-6 or PEI. Both the 10H2 and BS2 antibodies were recovered in significantly higher quantities (4.9-fold and 5.6-fold higher, respectively) when 4-4-6 was utilized compared to PEI (Fig. 3B-C). Superiority of PBAE nanoparticles was reproducible across cell lines; transfection of CHO cells with DNA encoding the recombinant antibody 602 (Krieg, Letourneau, Pantaleo, & Boyman, 2010; Létourneau et al., 2010; Patent No. WO/2020/264321, 2020) at an optimized dose of 4 μg/mL resulted in 3.4-fold more protein recovered when 4-4-6 was used compared to PEI (Fig. S3E-G).

Taken together, experiments with both cytosolic and secreted proteins demonstrated that PBAEs lead to significantly enhanced protein yields compared to leading commercial reagent PEI in two cell lines that are widely used for protein production. Storage stability and straightforward synthesis from inexpensive chemical monomers further strengthen their attractiveness for use in recombinant protein production across batch scales. Overall, the favorable properties of PBAEs combined with the results herein suggest that these polymers hold promise as superior reagents for transient transfection that can significantly improve protein

production workflows.

Materials and Methods

Tissue culture

HEK 293F (HEK) cells (Thermo Fisher Scientific) were cultured in FreeStyle 293 Expression Medium (Thermo Fisher Scientific) supplemented with penicillin-streptomycin (0.2 U/mL) (Thermo Fisher Scientific). CHO-S cells (Thermo Fisher Scientific) were cultured in FreeStyle CHO Expression Medium (Thermo Fisher Scientific) supplemented with L-glutamine (8 mM, Thermo Fisher Scientific) and penicillin-streptomycin (0.2 U/mL). All cells were grown at 37°C and 5% $CO_2$with rotation at 125 rpm in a humidified atmosphere. Transfections were incubated in the same growth conditions. HEK cells were not subject to more than 25 passages, whereas CHO cells were studied below 10 passages to mitigate cell clumping. All cells were grown to a density of 1.2×10^6 cells/mL and diluted to 1.0×10^6 cells/mL on the day of transfection.

PBAE synthesis

1,4-Butanediol diacrylate (B4), 3-amino-1-propanol (S3), 5-amino-1-pentanol (S5), 1-(3-Aminopropyl)-4-methylpiperazine (E7) (Alfa Aesar), 2-(3-Aminopropylamino)ethanol (E6), 1-(2-Aminoethyl)piperazine (E39) (Sigma Aldrich), 1,5-Pentanediol diacrylate (B5) (SantaCruz Biotechnology), and 4-amino-1-butanol (S4) (Fisher Scientific) were purchased and stored as directed. Base monomer (B4 or B5) was mixed with sidechain monomer (S3, S4, or S5) at a 1.08:1 ratio and the reaction was allowed to proceed for 24 h with stirring at 85°C. The neat acrylate terminated B-S polymer was then resuspended in THF (final concentration 200 mg/mL) and combined with 0.5M end-capping monomer (E6, E7, or E39) for 2 h with stirring at room temperature. The resulting polymer was precipitated twice in diethyl ether and dried under vacuum for 48 h, then resuspended to 100 mg/mL in DMSO and stored at -20°C in desiccant.

Nanoparticle formation and characterization

PBAE and plasmid DNA were each dissolved in sterile 25 mM magnesium acetate buffer, pH 5.2, then combined to yield a weight ratio of 60:1 PBAE:DNA and incubated at room temperature for 15 minutes to allow for nanoparticle self-assembly. Linear PEI of average molecular weight 25 kDa (Polysciences) was prepared according to manufacturer's instructions and stored at -80°C. To prepare particles, PEI was thawed, diluted in OptiPRO SFM, and incubated for 15 minutes at room temperature before being combined with plasmid DNA, also diluted in OptiPRO SFM, for resulting weight ratios of 2:1 PEI:DNA for HEK cell transfections or 3:1 PEI:DNA for CHO cells. The PEI:DNA mixtures were then incubated at room temperature for 15 minutes to allow for nanoparticle self-assembly. To characterize the resultant particles, 1:20 dilutions were prepared in FreeStyle 293 Expression Medium (HEK cells) or FreeStyle CHO Expression Medium (CHO cells). Size, as measured by hydrodynamic radius determined via dynamic light scattering, and surface charge, as measured by zeta potential determined via electrophoretic light scattering, were measured using a Malvern Zetasizer Pro (Malvern Panalytical). Measurements were reported as mean values from n =3 independently prepared replicates.

Cytosolic mCherry protein expression and transfection efficiency

4-4-6 and PEI nanoparticles encapsulating the CAG-mCherry plasmid (Addgene #108685, (Mishra et al., 2020)) were formed as described above and added to HEK or CHO cells diluted for transfection (2 mL culture in 6-well plate). Optimized DNA concentrations were determined based on mean fluorescence over 5 days (580± Ex/630Em) using a BioTek SynergyMX plate reader (BioTek Instruments, Inc.) by reading a 3 × 3 grid distributed across the well surface area. For transfection efficacy studies, HEK or CHO cells were dosed with PBAE or PEI nanoparticles formed as described above, delivering 2 μg/mL (HEK) or 4 μg/mL (CHO) mCherry DNA (n =5). Cells were collected on day 5 and CHO cells were treated with Accutase (Sigma Aldrich), according to manufacturer's instructions. Cells were then resuspended in phosphate-buffered saline (PBS, pH 7.4) containing 2% fetal bovine serum (FBS) and stained with LIVE/DEAD Fixable Green Dead Cell Stain Kit (Invitrogen), according to manufacturer's instructions. mCherry expression in live cells was

assessed using an Attune NxT Flow Cytometer (ThermoFisher Scientific). Experiments were performed a minimum of twice with consistent results.

Cellular viability

Viability was assessed via MTS CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) 24 h following transfection, according to the manufacturer's instructions. PBAE and PEI treated conditions were normalized to an untreated control sample. Experiments were performed with *n=5* and conducted twice with similar results.

Fluorescence microscopy

10x images were acquired with a Zeiss Axio Observer with Axiovision 5 software (Zeiss United States) and composite images were generated in ImageJ.

Secreted protein expression and purification

The 10H2 and BS2 antibodies (Patent No. WO/2020/243489, 2020) were expressed via transient co-transfection of gWiz plasmids (Genlantis) encoding their respective heavy and light chains (1:1 ratio) in HEK cells (2 μg DNA/mL culture). The 602 antibody (Patent No. WO/2020/264321, 2020) was expressed in CHO cells (4 μg DNA/mL culture). The optimized DNA concentration for transfection was determined by dose titration in small-scale transfections by adding 4-4-6- and PEI-based nanoparticles (formulated as described above) to 2 mL cells in 6-well plate format. Cells were incubated as described above for 96 to 120 h. Cell supernatants were then incubated with Protein G agarose beads (Thermo Fisher Scientific) for 3 hours at room temperature. Beads were washed and bound protein was eluted using 0.1M glycine, pH 2, and measured by SDS-PAGE analysis. Band quantification was performed using ImageJ software. For larger scale (>2 mL) transfections, 4-4-6- and PEI-based nanoparticles were formed as described above, added to cells in a shaking flask, and incubated as described above for 96 to 120 h. Cell supernatants were then incubated with Protein G agarose beads either at room temperature for 3 hours or at 4°C overnight. Secreted protein was harvested from cell supernatant by Protein G affinity chromatography, and 10H2 and BS2 samples were further purified using a Superdex 200 sizing column equilibrated in PBS on a fast protein liquid chromatography (FPLC) instrument (Cytiva). Yield was quantified by measuring absorbance at 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Statistics

Statistical analysis was performed in Prism 8 (GraphPad). Differences between multiple groups was determined via one-way ANOVA with Dunnett post-test comparing all treatment groups to PEI treatment. Differences between two groups was calculated using Student's t test. Significance designated: *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001.

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References

Akinc, A., Lynn, D. M., Anderson, D. G., & Langer, R. (2003). Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery. *Journal of the American Chemical Society* , *125* (18), 5316–5323. https://doi.org/10.1021/ja034429c

Backliwal, G., Hildinger, M., Chenuet, S., Wulhfard, S., De Jesus, M., & Wurm, F. M. (2008). Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers excee-

ding 1 g/l by transient transfection under serum-free conditions.*Nucleic Acids Research* , *36* (15), e96. https://doi.org/10.1093/nar/gkn423

Bishop, C. J., Kozielski, K. L., & Green, J. J. (2015). Exploring the role of polymer structure on intracellular nucleic acid delivery via polymeric nanoparticles. *Journal of Controlled Release* ,*219* , 488–499. https://doi.org/10.1016/j.jconrel.2015.09.046

Boussif, O., LezoualC'H, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., & Behr, J. P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America* , *92* (16), 7297–7301. https://doi.org/10.1073/pnas.92.16.7297

Breunig, M., Lungwitz, U., Liebl, R., & Goepferich, A. (2007). Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. *Proceedings of the National Academy of Sciences of the United States of America* , *104* (36), 14454–14459. https://doi.org/10.1073/pnas.0703882104

Bus, T., Traeger, A., & Schubert, U. S. (2018). The great escape: how cationic polyplexes overcome the endosomal barrier. *Journal of Materials Chemistry B* , *6* (43), 6904–6918. https://doi.org/10.1039/C8TB00967H

Chuntharapai, A., Lee, J., Hébert, C. A., & Kim, K. J. (1994). Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. *The Journal of Immunology* , *153* (12), 5682–5688.

Galbraith, D. J., Tait, A. S., Racher, A. J., Birch, J. R., & James, D. C. (2006). Control of Culture Environment for Improved Polyethylenimine-Mediated Transient Production of Recombinant Monoclonal Antibodies by CHO Cells. *Biotechnology Progress* , *22* (3), 753–762. https://doi.org/https://doi.org/10.1021/bp050339v

Karlsson, J., Rhodes, K. R., Green, J. J., & Tzeng, S. Y. (2020). Poly(beta-amino ester)s as gene delivery vehicles: challenges and opportunities. *Expert Opinion on Drug Delivery* , *17* (10), 1395–1410. https://doi.org/10.1080/17425247.2020.1796628

Kim, J., Sunshine, J. C., & Green, J. J. (2014). Differential polymer structure tunes mechanism of cellular uptake and transfection routes of poly(β-amino ester) polyplexes in human breast cancer cells.*Bioconjugate Chemistry* , *25* (1), 43–51. https://doi.org/10.1021/bc4002322

Krieg, C., Letourneau, S., Pantaleo, G., & Boyman, O. (2010). Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells. *Proceedings of the National Academy of Sciences* , *107* (26), 11906–11911. https://doi.org/10.1073/pnas.1002569107

Létourneau, S., Van Leeuwen, E. M. M., Krieg, C., Martin, C., Pantaleo, G., Sprent, J., ... Boymana, O. (2010). IL-2/anti-IL-2 antibody complexes show strong biological activity by avoiding interaction with IL-2 receptor α subunit CD25. *Proceedings of the National Academy of Sciences of the United States of America* , *107* (5), 2171–2176. https://doi.org/10.1073/pnas.0909384107

Mishra, B., Wilson, D. R., Sripathi, S. R., Suprenant, M. P., Rui, Y., Wahlin, K. J., ... Zack, D. J. (2020). A Combinatorial Library of Biodegradable Polyesters Enables Non-viral Gene Delivery to Post-Mitotic Human Stem Cell-Derived Polarized RPE Monolayers. *Regenerative Engineering and Translational Medicine* , *6* (3), 273–285. https://doi.org/10.1007/s40883-019-00118-1

Morris, V. B., & Sharma, C. P. (2010). Enhanced in-vitro transfection and biocompatibility of l-arginine modified oligo (-alkylaminosiloxanes)-graft-polyethylenimine. *Biomaterials* ,*31* (33), 8759–8769. https://doi.org/10.1016/j.biomaterials.2010.07.073

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., & Wagner, E. (1998). The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Therapy* , *5* (10), 1425–1433. https://doi.org/10.1038/sj.gt.3300745

Petersen, H., Fechner, P. M., Martin, A. L., Kunath, K., Stolnik, S., Roberts, C. J., ... Kissel, T. (2002). Polyethylenimine-graft-poly(ethylene glycol) copolymers: Influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjugate Chemistry* , *13* (4), 845–854. https://doi.org/10.1021/bc025529v

Pezzoli, D., Giupponi, E., Mantovani, D., & Candiani, G. (2017). Size matters for in vitro gene delivery: investigating the relationships among complexation protocol, transfection medium, size and sedimentation. *Scientific Reports* , *7* (1), 44134. https://doi.org/10.1038/srep44134

Spangler, J., Tomala, J., Isaac, L. M., Ludwig, S., & Leonard, E. K. (2020). *Patent No. WO/2020/264321* . The Johns Hopkins University.

Spangler, J., Wirtz, D. G., Yang, H., Wang, W., & Karl, M. (2020).*Patent No. WO/2020/243489* . The Johns Hopkins University.

Sunshine, J. C., Peng, D. Y., & Green, J. J. (2012). Uptake and Transfection with Polymeric Nanoparticles Are Dependent on Polymer End-Group Structure, but Largely Independent of Nanoparticle Physical and Chemical Properties. *Molecular Pharmaceutics* , *9* (11), 3375–3383. https://doi.org/10.1021/mp3004176

Wilson, D. R., Suprenant, M. P., Michel, J. H., Wang, E. B., Tzeng, S. Y., & Green, J. J. (2019). The role of assembly parameters on polyplex poly(beta-amino ester) nanoparticle transfections. *Biotechnology and Bioengineering* , *116* (5), 1220–1230. https://doi.org/10.1002/bit.26921

Yang, C., Li, H., Goh, S. H., & Li, J. (2007). Cationic star polymers consisting of α-cyclodextrin core and oligoethylenimine arms as nonviral gene delivery vectors. *Biomaterials* , *28* (21), 3245–3254. https://doi.org/10.1016/j.biomaterials.2007.03.033

Figure 1. Protein production workflow and particle characterization. (A) Schematic of transient transfection workflow for cytosolic and secreted proteins. Plasmid DNA and polymer were combined to allow for nanoparticle self-assembly. Cells were transfected with plasmid DNA encoding a fluorescent reporter or a secreted protein. (B) PBAE and PEI monomer structures. Backbone monomers B4 and B5, side chain monomers S3-S5, endcap monomers E6, E7, and E39 used to synthesize PBAE polymers, and PEI 25 kDa. (C) Structure of B4S4E6 (4-4-6) polymer. Additional polymer structures are shown in Figure S1. (D) Nanoparticle size determined via dynamic light scattering (DLS) in HEK media (blue) or CHO media (red). (E) Zeta potential of nanoparticles in HEK media (blue) or CHO media (red). Error bars represent SD.

Figure 2. Comparison of polymers for transient transfection of cytosolic mCherry in HEK and CHO cells. (A) HEK cells were transfected with 2 μg/mL mCherry-encoding DNA via PBAE or PEI nanoparticles*(n* =5). In HEK cells, PEI was used at a 2:1 polymer:DNA w/w ratio, whereas in CHO cells, PEI was used at a 3:1 polymer:DNA w/w ratio. All PBAEs were used at a 60:1 polymer:DNA w/w ratio in both cell lines. mCherry fluorescence was assessed via plate reader each day, and significance was calculated on day 5. (B) mCherry transfection efficiency was determined via flow cytometry 5 days following transient transfection of HEK cells with 4 μg mCherry-encoding DNA via PBAE or PEI nanoparticles *(n* =5). (C) CHO cells were transfected with 3 μg/mL mCherry-encoding DNA via PBAE or PEI nanoparticles *(n* =5).mCherry fluorescence was assessed via plate reader each day, and significance was calculated on day 5. (D) mCherry transfection efficiency determined by flow cytometry 5 days following transient transfection of CHO cells with 6 μg mCherry-encoding DNA via PBAE and PEI nanoparticles (n =5). (E) Representative fluorescence microscopy images of HEK and CHO cells 5 days following transient transfection of mCherry-encoding DNA (4 μg for HEK cells; 6 μg for CHO cells) with 4-4-6 or PEI nanoparticles. Scale bars are 200 μm. For all panels, error bars represent SD. Significance on day 5 was calculated using one-way ANOVA with Dunnett post-test, comparing all conditions to treatment with PEI. Increases relative to PEI are designated: **p $<$ 0.01, ***p $<$ 0.001, ****p $<$ 0.0001.

Figure 3. Comparison of 4-4-6 and PEI for transient transfection and secretion of two recombinant antibodies at various scales in HEK cells. All transfections used 1 μg/mL DNA at a 60:1 polymer:DNA w/w ratio for 4-4-6 or a 2:1 polymer:DNA w/w ratio for PEI. (A) Comparative yields from scaled transfections of the

10H2 monoclonal antibody utilizing 4-4-6 or PEI nanoparticles. (B) Comparative yields from transient transfections of 10H2 antibody utilizing 4-4-6 or PEI nanoparticles at 2 L scale. (C) Comparative yields from transient transfections of the BS2 bispecific antibody utilizing 4-4-6 or PEI nanoparticles at 2 L scale. Significance was determined by unpaired Student's t -test (**p < 0.01). Error bars represent SD.

Figure S1. PBAE synthesis and resulting polymer structures. (A) Synthesis of 4-4-6 PBAE. Acrylate and amine monomers react for 24h at 85C, followed by an end-capping reaction, resulting in a linear capped polymer. (B) Resulting structures of 4-4-6, 4-5-7, 4-5-39, and 5-3-6 polymers. (C) Dynamic light scattering measurements and electrophoretic mobility for PBAE and PEI nanoparticles in HEK or CHO media. Measurements represent mean values from *n=3* individually prepared replicates.

Figure S2. 4-4-6 and PEI DNA dose optimization in HEK and CHO cells. In HEK cells, PEI was used at a 2:1 polymer:DNA w/w ratio, whereas in CHO cells, PEI was used at a 3:1 polymer:DNA w/w ratio. 4-4-6 was used at a 60:1 polymer:DNA w/w ratio in both cell lines. (A) DNA dose optimization in HEK and CHO cells over 5-day time courses. Cells were transfected with varying amounts of mCherry DNA and fluorescence was assessed via plate reader on each day (n =1). (B) Viability was assessed via MTS assay 24 h following transfection with 2 μg/mL mCherry DNA for HEK cells or 4 μg/mL mCherry DNA for CHO cells using 4-4-6 or PEI nanoparticles *(*n =5). Error bars represent SD. (C) mCherry transfection efficiency determined via flow cytometry 5 days following transfection with 2 μg/mL mCherry DNA for HEK cells or 4 μg/mL mCherry DNA for CHO cells using PBAE or PEI nanoparticles. mCherry$^+$ cells were gated on live cells. Representative plots are presented (n =5). (D) Representative fluorescence microscopy images (n =5) of HEK and CHO cells 5 days following transfection with 2 μg/mL mCherry DNA for HEK cells or 4μg/mL mCherry DNA for CHO cells using PBAE or PEI nanoparticles. Scale bars are 200 μm.

Figure S3. DNA dose optimization for transient transfection of secreted recombinant antibodies. In HEK cells, PEI was used at a 2:1 polymer:DNA w/w ratio, whereas in CHO cells, PEI was used at a 3:1 polymer:DNA w/w ratio. 4-4-6 was used at a 60:1 polymer:DNA w/w ratio in both cell lines. (A) Reducing SDS-PAGE analysis showing expression of the 10H2 monoclonal antibody following transient transfection of HEK cells with the indicated doses of DNA encapsulated in PEI ($^+$) or 4-4-6 nanoparticles. (B) Quantification of 10H2 expression from (A), presented as fold improvement over PEI. (C) Reducing SDS-PAGE analysis showing expression of the BS2 bispecific antibody following transient transfection of HEK cells with the indicated doses of DNA encapsulated in PEI ($^+$) or 4-4-6 nanoparticles. (D) Quantification of BS2 expression from (C), presented as fold improvement over PEI. (E) Reducing SDS-PAGE analysis showing expression of the 602 monoclonal antibody following transient transfection of CHO cells with the indicated doses of DNA encapsulated in PEI ($^+$) or 4-4-6 nanoparticles. (F) Quantification of 602 expression from (E), presented as fold improvement over PEI. (G) Comparative yield (pre-FPLC) from transient transfection of CHO cells with the 602 monoclonal antibody utilizing 4-4-6 or PEI DNA-containing nanoparticles at 50 mL scale (4 μg/mL DNA dose). HC, heavy chain; LC, light chain.

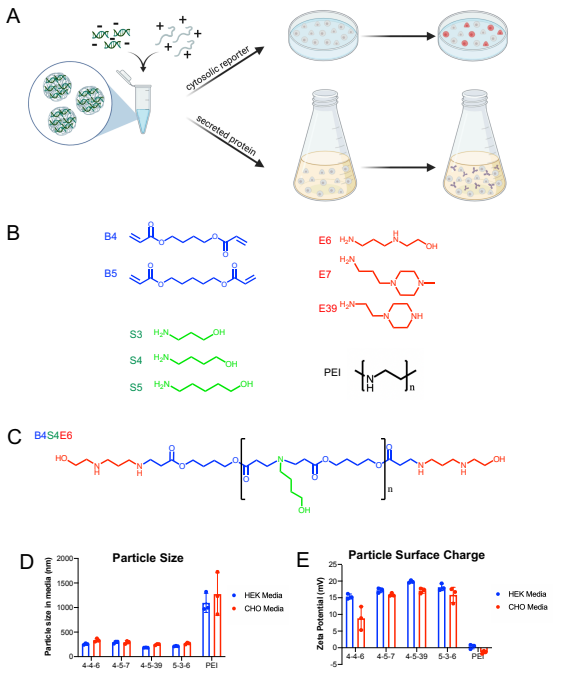

Figure 1. Protein production workflow and particle characterization. (A) Schematic of transient transfection workflow for cytosolic and secreted proteins. Plasmid DNA and polymer were combined to allow for nanoparticle self-assembly. Cells were transfected with plasmid DNA encoding a fluorescent reporter or a secreted protein. (B) PBAE and PEI monomer structures. Backbone monomers B4 and B5, side chain monomers S3-S5, endcap monomers E6, E7, and E39 used to synthesize PBAE polymers, and PEI 25 kDa. (C) Structure of B4S4E6 (4-4-6) polymer. Additional polymer structures are shown in Figure S1. (D) Nanoparticle size determined via dynamic light scattering (DLS) in HEK media (blue) or CHO media (red). (E) Zeta potential of nanoparticles in HEK media (blue) or CHO media (red). Error bars represent SD.

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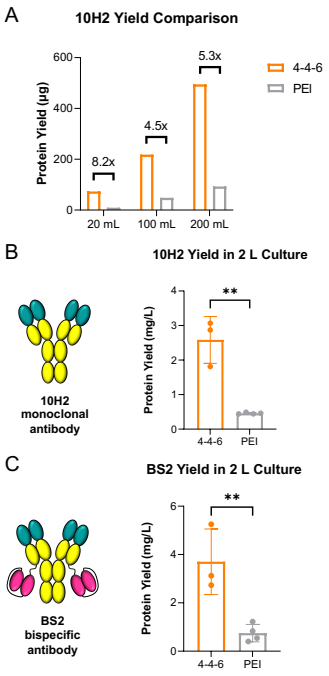
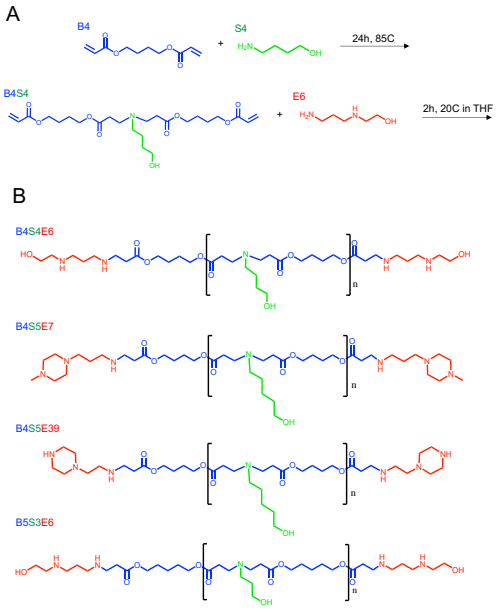


Figure 3. Comparison of 4-4-6 and PEI for transient transfection and secretion of two recombinant antibodies at various scales in HEK cells. All transfections used 1 µg/mL DNA at a 60:1 polymer:DNA w/w ratio for 4-4-6 or a 2:1 polymer:DNA w/w ratio for PEI. (A) Comparative yields from scaled transfections of the 10H2 monoclonal antibody utilizing 4-4-6 or PEI nanoparticles. (B) Comparative yields from transient transfections of 10H2 antibody utilizing 4-4-6 or PEI nanoparticles at 2 L scale. (C) Comparative yields from transient transfections of the BS2 bispecific antibody utilizing 4-4-6 or PEI nanoparticles at 2 L scale. Significance was determined by unpaired Student's *t*-test (**p < 0.01). Error bars represent SD.



	Z Average Mean Size (nm)		PDI		Zeta Potential (mV)	
	HEK Media	CHO Media	HEK Media	CHO Media	HEK Media	CHO Media
4-4-6	256	335	0.09	0.12	15.4	8.8
4-5-7	291	293	0.15	0.14	17.2	15.9
4-5-39	184	248	0.07	0.13	19.8	17.1
5-3-6	214	268	0.14	0.15	18.2	15.9
PEI	1095	1274	0.33	0.71	0.4	-1.3

Figure S1. PBAE synthesis and resulting polymer structures. (A) Synthesis of 4-4-6 PBAE. Acrylate and amine monomers react for 24h at 85C, followed by an end-capping reaction, resulting in a linear capped polymer. (B) Resulting structures of 4-4-6, 4-5-7, 4-5-39, and 5-3-6 polymers. (C) Dynamic light scattering measurements and electrophoretic mobility for PBAE and PEI nanoparticles in HEK or CHO media. Measurements represent mean values from *n=3* individually prepared replicates.

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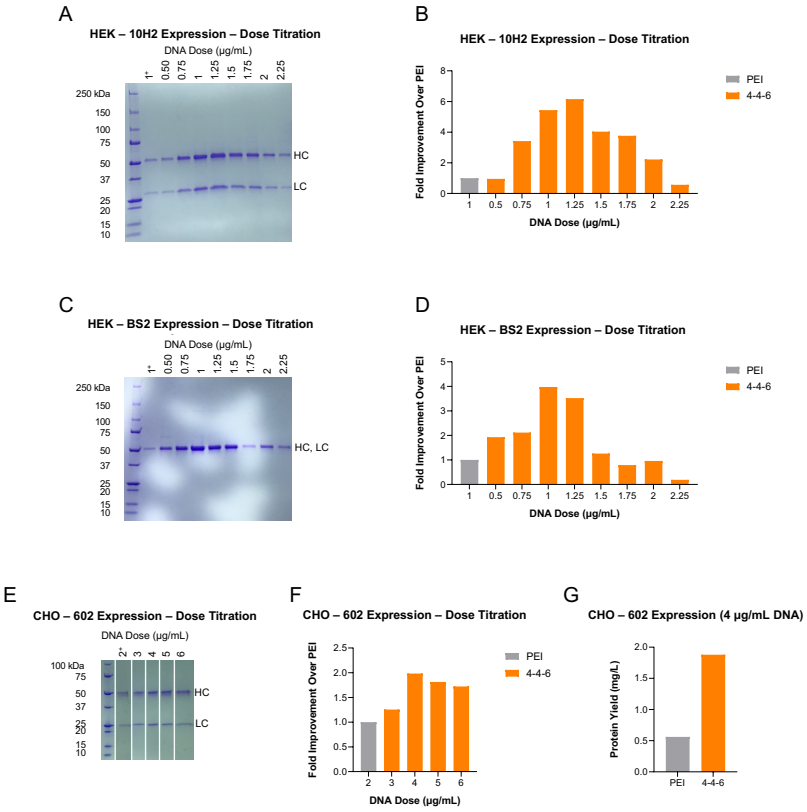

Figure S3. DNA dose optimization for transient transfection of secreted recombinant antibodies. In HEK cells, PEI was used at a 2:1 polymer:DNA w/w ratio, whereas in CHO cells, PEI was used at a 3:1 polymer:DNA w/w ratio. 4-4-6 was used at a 60:1 polymer:DNA w/w ratio in both cell lines. (A) Reducing SDS-PAGE analysis showing expression of the 10H2 monoclonal antibody following transient transfection of HEK cells with the indicated doses of DNA encapsulated in PEI (*) or 4-4-6 nanoparticles. (B) Quantification of 10H2 expression from (A), presented as fold improvement over PEI. (C) Reducing SDS-PAGE analysis showing expression of the BS2 bispecific antibody following transient transfection of HEK cells with the indicated doses of DNA encapsulated in PEI (*) or 4-4-6 nanoparticles. (D) Quantification of BS2 expression from (C), presented as fold improvement over PEI. (E) Reducing SDS-PAGE analysis showing expression of the 602 monoclonal antibody following transient transfection of CHO cells with the indicated doses of DNA encapsulated in PEI (*) or 4-4-6 nanoparticles. (F) Quantification of 602 expression from (E), presented as fold improvement over PEI. (G) Comparative yield (pre-FPLC) from transient transfection of CHO cells with the 602 monoclonal antibody utilizing 4-4-6 or PEI DNA-containing nanoparticles at 50 mL scale (4 µg/mL DNA dose). HC, heavy chain; LC, light chain.