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Polymyxin B Treatment Improves Bactofection Efficacy and Reduces Cytotoxicity

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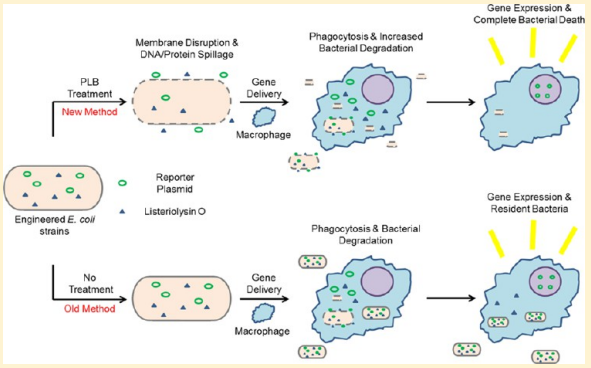
Supporting Information

ABSTRACT: Improvements to bacterial vectors have resulted in nonviral gene therapy vehicles that are easily prepared and can achieve high levels of transfection efficacy. However, these vectors are plagued by potential cytotoxicity and immunogenicity, prompting means of attenuation to reduce unwanted biological outcomes while maintaining transfection efficiency. In this study, listeriolysin O (LLO) producing *Escherichia coli* BL21(DE3) strains were pretreated with polymyxin B (PLB), a pore-forming antibiotic, and tested as a delivery vector for gene transfer to a murine RAW264.7 macrophage cell line using a 96-well high-throughput assay. PLB treatment resulted in statistically significant higher levels of gene delivery and lower cytotoxicity. The results suggest a fine balance between bacterial cellular damage, heightened gene and protein release, and increased mammalian cell gene delivery. Overall, the approach presented provides a simple and effective way to enhance bacterial gene delivery while simultaneously reducing unwanted outcomes as a function of using a biological vector.



KEYWORDS: *Escherichia coli, bactofection, gene delivery, gene therapy, polymyxin B*

INTRODUCTION

The development of gene therapy vectors that possess tunable loading, delivery, and release properties has become increasingly important due in part to limitations of viral vectors to address concerns of complex formulation, cytotoxicity, tumorigenicity, immunogenicity and low payload capacity.[1−3] Progress has been made by utilizing cationic polymers and cationic lipids that inherently contain increased delivery capacities and reduced immunogenicity through rational design and synthesis. Of these, cationic polymers, which are the easiest to design and manipulate, feature ready synthesis[4−6] and low cytotoxicity[7,8] but, as a class of vectors, are hindered by suboptimal in vivo delivery efficiency in comparison to viral-based vectors.[9] The in vivo delivery efficiency advantage of viral vectors is attributed to the evolution of serotypes that can successfully cross the numerous barriers associated with extra- and intracellular gene delivery. These potent delivery capabilities suggest the need for a novel class of vectors that can overcome the aforementioned drawbacks while possessing inherent biological properties that aid efficacious delivery of gene therapeutics.

Gene delivery utilizing bacteria, since first reported in the early 1980s,[10] has experienced continuous engineering improvements to overcome the barriers associated with in vitro and in vivo application. The realization that bacteria could be used for gene delivery resulted in a new class of study, bactofection, that is predicated on the use of bacterial strains (many of which were attenuated pathogens) possessing either native or engineered vector capabilities.[11] Of particular interest, *Escherichia coli* (*E. coli*), a rod-shaped Gram-negative bacteria that is approximately 2 μm in length and 0.5 μm in diameter, has been identified as an ideal gene carrier, as most strains are harmless and molecular biology manipulation techniques are highly developed. Despite lingering biocompatibility and toxicity concerns, *E. coli* has successfully been utilized for the safe delivery of plasmid[12−17] and larger DNA constructs[18] shRNA,[19] and immunogenic peptides.[17,20−22] Though not invasive or capable of intracellular localization in nature, *E. coli* has been engineered to express heterologous proteins, invasin (inv) from *Yersinia pseudotuberculosis* and LLO from *Listeria monocytogenes*, to allow penetration into nonprofessional phagocytic cells and subsequent endosomal escape, respectively, to aid gene delivery.[12−14,19,23,24]

Despite successful laboratory and clinical usage, bactofection still experiences significant resistance, as concerns of bacterial sepsis, immunogenicity, and reversion of pathogenicity cannot be ignored. To address these concerns and further improve the safety and efficacy of bactofection, we investigated the use of polymyxin B (PLB), a membrane-disrupting antibiotic (Scheme

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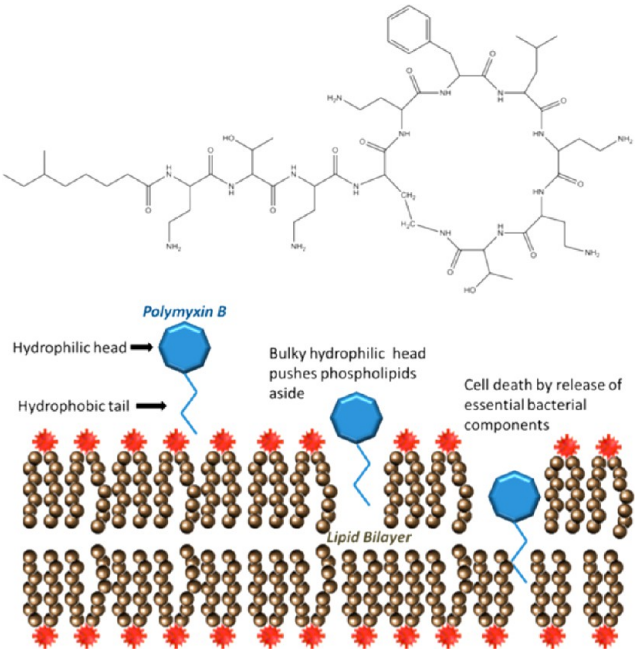
1), as a predelivery treatment applied to LLO-producing *E. coli*. Gene expression was reported through use of a cytomegalovirus promoter-driven luciferase expression plasmid. Upon treatment, target macrophage gene delivery and cytotoxicity were both improved, with results indicating that optimal performance requires a balance between cellular integrity and macromolecule release. The approach provides an option for addressing concerns about the future use of bacterial-based gene delivery therapeutics.

Scheme 1. Polymyxin B Structure and Mechanism



EXPERIMENTAL SECTION

Cell Lines and Strains. A murine RAW264.7 macrophage line was used for gene delivery assays and was kindly provided by Dr. Terry Connell (Department of Microbiology and Immunology, University at Buffalo, SUNY). The cell line was maintained in medium prepared as follows: 50 mL of fetal bovine serum (heat inactivated), 5 mL of 100 mM MEM sodium pyruvate, 5 mL of 1 M HEPES buffer, 5 mL of penicillin/streptomycin solution, and 1.25 g of D-(+)-glucose added to 500 mL of phenol red-containing RPMI-1640 and filter sterilized. Cells were housed in T75 flasks and cultured at 37 °C/5% CO_2.

The BL21(DE3) *E. coli* cell line (Novagen) was used as the parent strain for generation of all gene delivery bacterial vectors. Genetic manipulations were described previously.[12,13] Resulting strains are listed in Table 1 (with *hly* being the gene designation for LLO).

Growth Inhibition. To determine the minimum inhibitory concentration of polymyxin B (Krackeler Scientific), a glycerol stock of BL21(DE3) was used to inoculate an overnight starter culture at 37 °C/250 rpm in 2 mL of lysogeny broth (LB) medium. 10 mL LB cultures were inoculated with 2.5% (v/v) of the starter culture and allowed to incubate at 37 °C until an OD_{600} of 0.5 was obtained. Cultures were then centrifuged/pelleted and resuspended in an equal volume of phosphate buffered saline (PBS). Samples were split into eight Eppendorf tubes, and appropriate volumes of polymyxin B stock (50 mg/mL) were added to create concentrations ranging from 0 to 1 mg/mL. Every half-hour, 200 μL of each sample was centrifuged, washed twice, and resuspended with equal volume PBS. Subsequently, samples were plated on LB agar plates and allowed to incubate for 24 h before growth was quantified by counting colony forming units.

Table 1. *E. coli* Vectors Used for PLB Treatment and RAW264.7 Gene Delivery

strain	notes	reference
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS*(r_B- m_B-) *gal* λ(DE3)	25
BL21(DE3)/pCMV-Luc	BL21(DE3) carrying a high copy mammalian luciferase expression plasmid, driven by the CMV promoter	13
YWT7-*hly*/pCMV-Luc	strain contains an inducible T7-*hly* cassette in the BL21(DE3) chromosome	13
YWTet-*hly*/pCMV-Luc	strain contains a constitutively expressed Tet-*hly* cassette in the BL21(DE3) chromosome	13
BL21(DE3)/pCMV-Luc/pET29-*hly*	pET29-*hly* carries the *hly* gene under a *lac*-inducible T7 promoter	12, 13
BL21(DE3)/pCMV-Luc/pDP2615	pDP3615 carries the *hly* gene under a constitutively expressed Tet promoter	12, 13

Bacterial Cell Membrane Shear. BL21(DE3) cultures were generated and treated as described above, with use of only four PLB concentrations (0, 0.01, 0.025, 0.05 mg/mL). At every half-hour time point, 200 μL of treated bacteria was washed and resuspended in PBS, before being sonicated at 20% capacity for 5 s using a Branson 450D Sonifier (400 W, tapered microtip). Sonicated samples were then plated on LB agar plates and allowed to incubate for 24 h before growth was quantified by counting colony forming units.

Protein and DNA Release. To quantify the amount of protein and DNA released from the supernatant of PLB-treated bacterial samples (from Table 1), 10 mL LB cultures were inoculated with 2.5% (v/v) of an overnight starter culture, incubated at 37 °C, induced with 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD_{600} of 0.3, and incubated at 30 °C for an additional hour. Each strain was then standardized to 0.5 OD_{600} and incubated with various concentrations of PLB (0, 0.01, 0.025, 0.05 mg/mL) in PBS. Every half-hour (1.5 h in total), 200 μL of treated bacteria cells were pelleted and supernatants were measured for absorbance of 260 nm for DNA quantification and 280 nm for protein quantification.

Hemolysis Assay. To determine the relative amount of LLO induced hemolysis, supernatant from each sample of the protein and DNA release quantification studies was used to assay sheep red blood cell lysis. 100 μL of treated supernatant was mixed with 900 μL of 5% sheep red blood cells (HemoStat Laboratories) in assay buffer,[12] and another 100 μL was mixed with 900 μL of PBS (pH 7.4), followed by incubating for one hour at 36 °C. LLO activity was then measured by recording absorbance at 541 nm with results depicted as percentage of total blood lysis (interpolated from a blood lysis standard plot).

Gene Delivery. For gene delivery experiments, the RAW264.7 cell line was seeded into two different types of 96-well plates at 3×10^4 cells/well in 100 μL of antibiotic-free medium and incubated for 24 h to allow attachment. A tissue culture-treated, flat-bottom, sterile, white, polystyrene 96-well plate was used for luciferase assays, whereas bicinchoninic acid assay (BCA) assays were conducted in tissue culture-treated,

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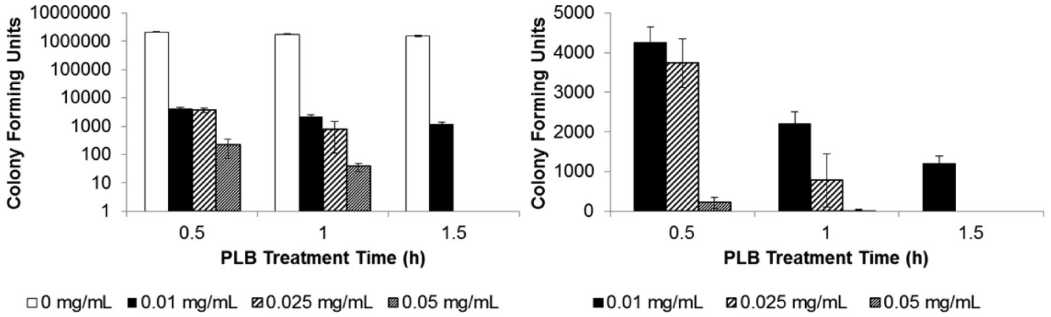

Figure 1. Membrane shear studies of PLB-treated BL21(DE3) strains. Samples were treated with various concentrations of PLB over time prior to brief sonication and viability analysis. Both graphs presented are the same with the control removed (right) to better visualize sample differences.

sterile, clear, polystyrene 96-well plates. Each plate was carried out as a duplicate of the other.

Bacterial cultures were completed as described above for the protein and DNA release quantification studies. Subsequently, all strains were washed and standardized to 0.5 OD_{600} in PBS to allow strain comparison. Four PLB concentrations were prepared per strain (0.0, 0.01, 0.025, and 0.05 mg/mL) by addition of PLB stock to washed cultures. Every half-hour, 20 μL of each concentration and strain was subsequently diluted in antibiotic free RPMI-1640 to create a bacteria-to-macrophage ratio of 10:1. Following attachment, macrophage medium was replaced with 50 μL of each respective bacterial sample and allowed to incubate for an hour. After incubation, 50 μL of gentamicin-containing RPMI-1640 was added to each well to eliminate external/nonphagocytized bacteria. After a second 24 h incubation (48 h after initial seeding), plates were analyzed for luciferase expression using the Bright Glo assay (Promega) and protein content using the Micro BCA Protein Assay Kit (Pierce) according to each manufacturer's instructions. Gene delivery was calculated by normalizing luciferase expression by protein content for each well/plate.

Supernatant Gene Delivery. To determine if gene delivery was the result of bacterial lysis and subsequent plasmid and protein uptake, assays using the treated supernatant were completed. Macrophage seeding, bacterial vector culture, and PLB treatment were conducted as described above. At each half-hour time point, all bacterial samples were pelleted and 20 μL of supernatant was diluted into RPMI-1640 and used in subsequent gene delivery experiments as described above.

Cellular Toxicity. Cytotoxicity resulting from PLB-treated *E. coli* vectors was determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide) colorimetric assay. RAW264.7 cells were seeded in 96-well polystyrene plates at a density of 50×10^4 cells/well. After 24 h incubation, medium was removed and cells combined with bacterial vectors were prepared as described above except that the delivery volume was 100 μL (as compared to 50 μL) and gentamicin treatment was excluded. Following another 24 h incubation, cells were assayed with MTT solution (5 mg/mL), added at 10% v/v, for 3 h at 37 °C/5% CO_2. Medium plus MTT solution then was aspirated, and the formazan reaction products were dissolved in DMSO and shaken for 1 h. The optical density of the formazan solution was analyzed using a Synergy 4 Multi-Mode microplate reader (BioTek Instruments, Inc.) at 570 nm with 630 nm serving as the reference wavelength. Results are presented as a percentage of untreated cells (100% viability).

Statistical Evaluation. Data presented were generated from three independent experiments. Error bars represent standard deviation values. Where indicated, statistical significance (95% confidence) was calculated using mean values from select experimental samples compared with respective control means.

RESULTS AND DISCUSSION

Bacterial Vector Design. Vectors for bactofection were engineered to deliver a mammalian expression reporter gene (pCMV-Luc) and to test three gene delivery parameters associated with LLO: gene dosage, promoter strength, and gene expression regulation. Two promoters were selected in this study to accommodate expression parameters. The *lac*-inducible bacteriophage T7 promoter was selected on the basis of its tight regulation and high expression profile.[25] Alternatively, a weaker (compared to T7), constitutively expressed Tet promoter was selected. Both LLO expression cassettes were either inserted into the chromosome or carried by plasmid.

Growth Inhibition and Cell Membrane Weakening. Polymyxin B was initially identified as a potential treatment strategy for improved bactofection based upon previous studies where PLB was utilized to permeabilize *E. coli* for controlled release of toxins to kill cancerous cells.[26] Bacterial destabilization of the outer membrane is driven by electrostatic interactions between the cationic amino groups of PLB (in the cyclic peptide moiety) and negatively charged membrane content including lipopolysaccharide and ready interaction with the PLB aliphatic component (Scheme 1).[27,28] Subsequently, growth studies were carried out (data not shown) to determine concentrations required to inhibit and/or kill BL21(DE3) *E. coli* strains. A critical concentration of 0.05 mg/mL was identified as the point where any additional PLB resulted in complete bacterial lysis after 0.5 h. Four concentrations were then selected for future studies: 0, 0.01, 0.025, 0.05 mg/mL.

Upon identification of lethal dosages of PLB, we next sought to evaluate bacterial membrane integrity post PLB treatment. BL21(DE3) was incubated with four concentrations of PLB with samples collected every 0.5 h, for a total of 1.5 h (3 samples). Following brief sonication, samples were then compared for viability. Figure 1 indicates that the number of viable bacteria is negatively correlated with both PLB concentration and treatment time. The critical PLB concentration (0.05 mg/mL) resulted in the highest level of bacterial membrane weakening, with less than 38 colony forming units after 1 h treatment. Interestingly, with exception of 0.01 mg/mL, all concentrations facilitated complete bacterial lysis after 1.5 h treatment. Successful confirmation of PLB concentration

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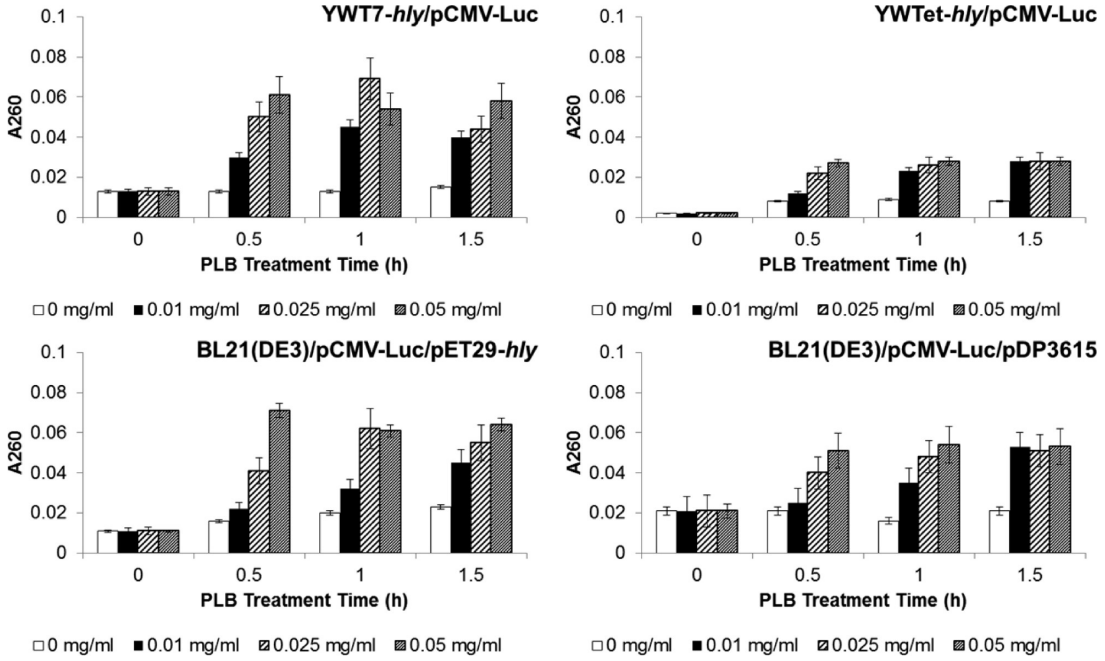

Figure 2. DNA release studies of PLB-treated bacterial vector strains.

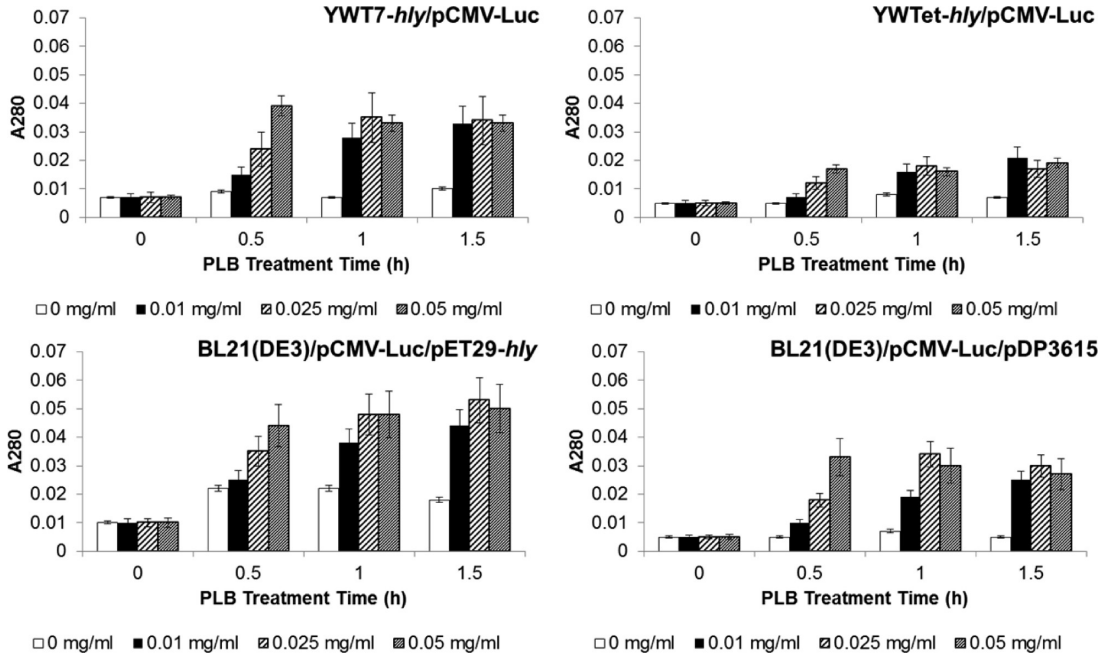

Figure 3. Protein release studies of PLB-treated bacterial vector strains.

ranges and membrane weakening was the basis for further gene delivery and cytotoxicity studies.

DNA and Protein Release. With bacterial membrane weakening confirmed, different BL21(DE3) strains expressing LLO constitutively (Tet promoter) or inducibly (T7 promoter) and either chromosomally or by plasmid were evaluated for the amount of DNA and protein released post PLB treatment. Supernatant absorbance was measured at both 260 (Figure 2) and 280 nm (Figure 3) to allow DNA and protein quantification, respectively. For all strains, a positive correlation is observed for each A_{260} and A_{280} measurement and both PLB concentration and treatment time.

Strain-dependent release profiles were observed, with increasing levels of release correlated to intracellular production of LLO (trends established previously).[13] Protein release profiles (A_{280}) relative to promoter expression capability were expected; however, we anticipated that DNA release (A_{260}) may have been similar between strains given the consistent copy number shared between the expression plasmids used in this study. Instead, the DNA release trend is similar to strain-dependent protein release curves. We hypothesize that a synergistic interaction between PLB treatment and LLO production is occurring, resulting in increased perforations and subsequent cell leakage. This notion was supported by repeat experiments of growth and shear studies with LLO-

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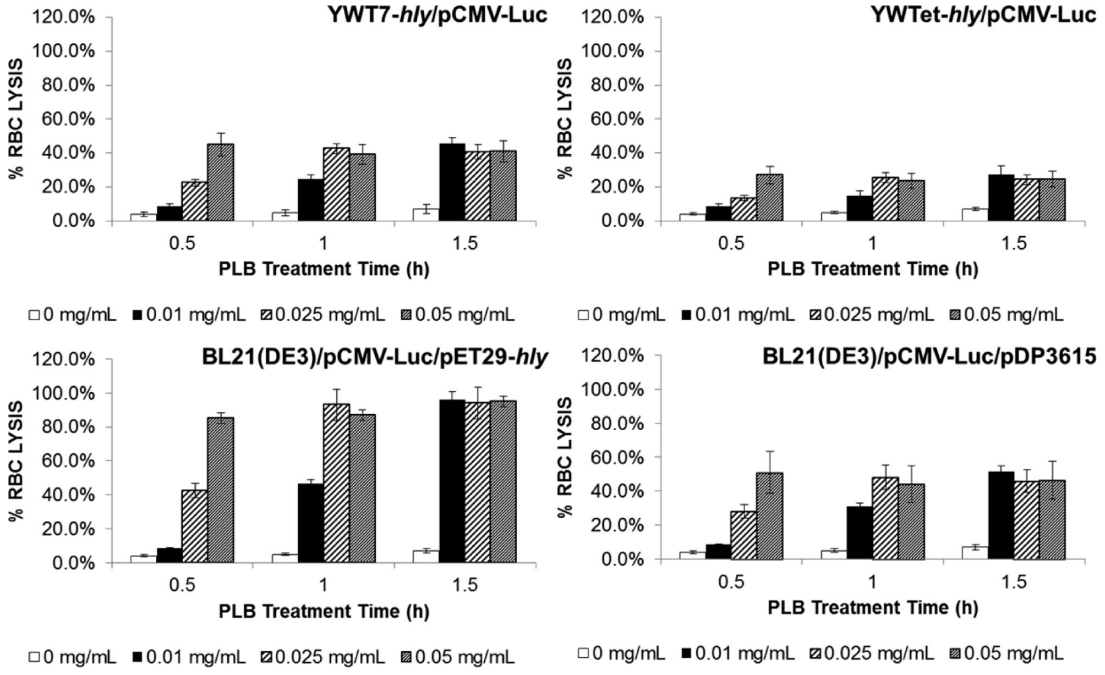

Figure 4. Hemolysis (at pH 5.5) of red blood cells (RBCs) upon incubation with PLB-treated bacterial vector supernatants.

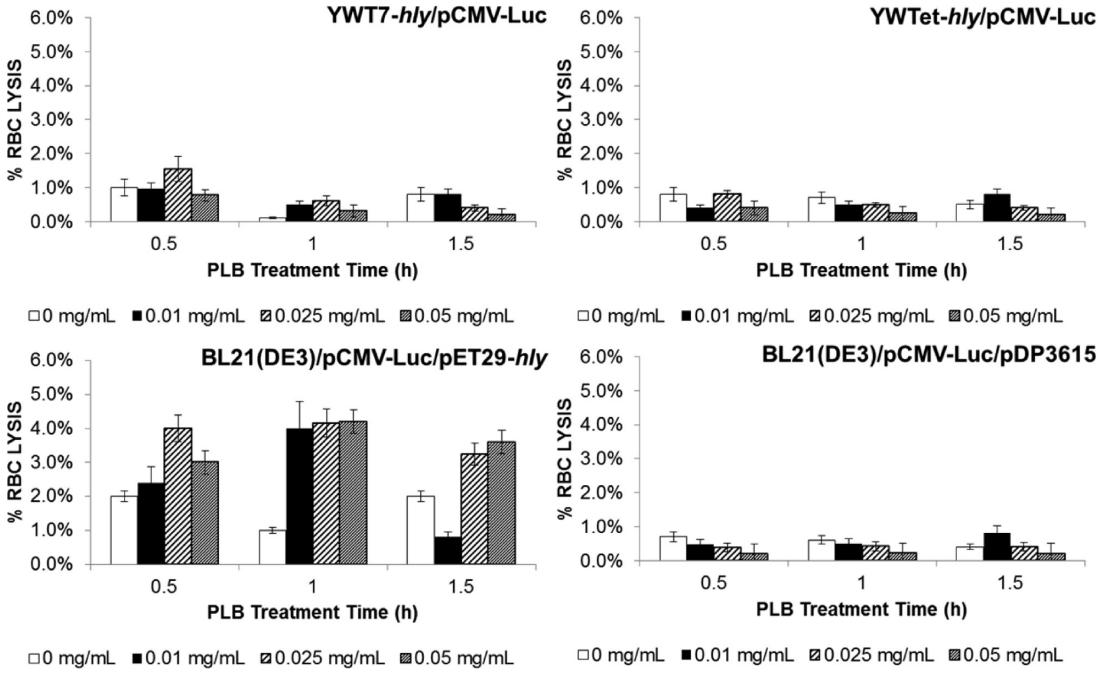

Figure 5. Hemolysis (in pH 7.4) of red blood cells (RBCs) upon incubation with PLB-treated bacterial vector supernatants.

expressing strains (data not shown) in which increased LLO expression also resulting in increased cell death.

Released Hemolytic Activity. Previously, the different strains (Table 1) were tested for intracellular LLO production and activity.[13] However, having established protein release curves, the hemolytic activity of released LLO was evaluated by measuring percent of red blood cell lysis after incubation with the supernatant of PLB-treated strains (prepared as described in DNA and Protein Release) at different pHs. Similar to protein release curves, hemolysis at pH 5.5 was positively correlated with PLB concentration and treatment time (Figure 4). Additionally, strain-dependent profiles were observed with the greatest hemolytic activity (100%) associated with PLB-treated BL21(DE3)/pCMV-Luc/pET29-*hly* (the strain capable of highest LLO gene expression). Each strain treated with PLB demonstrated a hemolysis upper limit, a trend that matches the results obtained previously in which cells were mechanically disrupted by sonication prior to hemolytic assessment.[13] Interestingly, a low level of hemolysis (<10%) was observed for all nontreated strains while negligible levels were observed for BL21(DE3) (data not shown), further indicating that LLO causes endogenous lysis.

Released LLO can potentially limit future clinical usage if hemolysis is observed in physiologically relevant pH. Thus,

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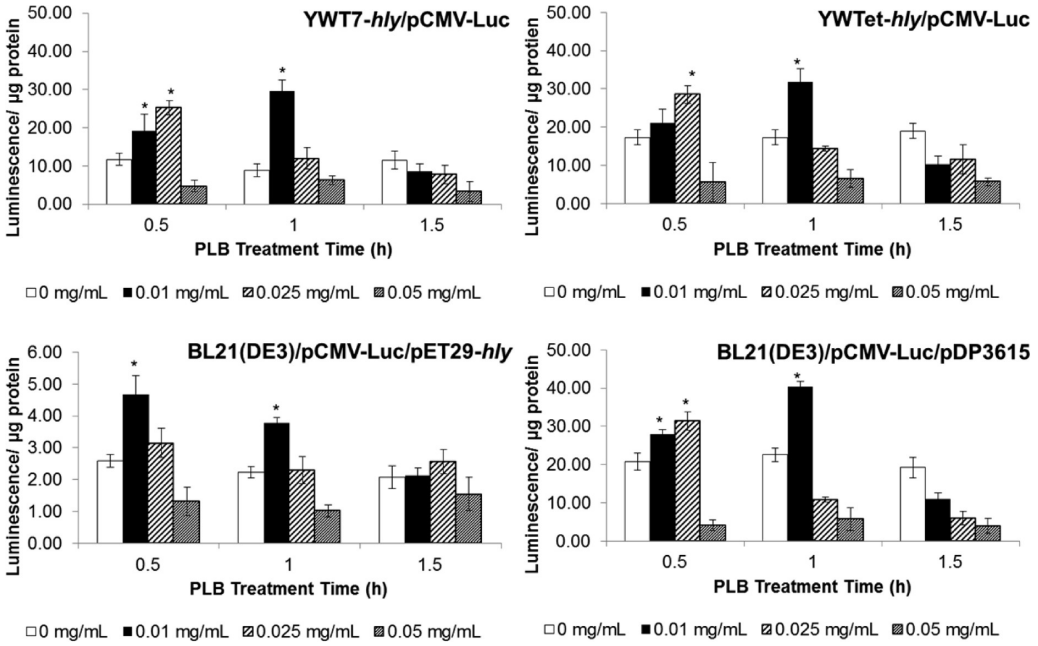

Figure 6. Gene delivery resulting from PLB-treated bacterial vector strains incubated with RAW264.7 cells. The *y*-axis for the BL21(DE3)/pCMV-Luc/pET29-*hly* strain is reduced relative to the other graph *y*-axes to better illustrate the differences in values associated with this data set. Delivery is measured in luminescence units normalized by total protein (luminescence/ μg protein, *y*-axis). *Statistically significant (95% confidence) when compared to respective untreated PLB control.

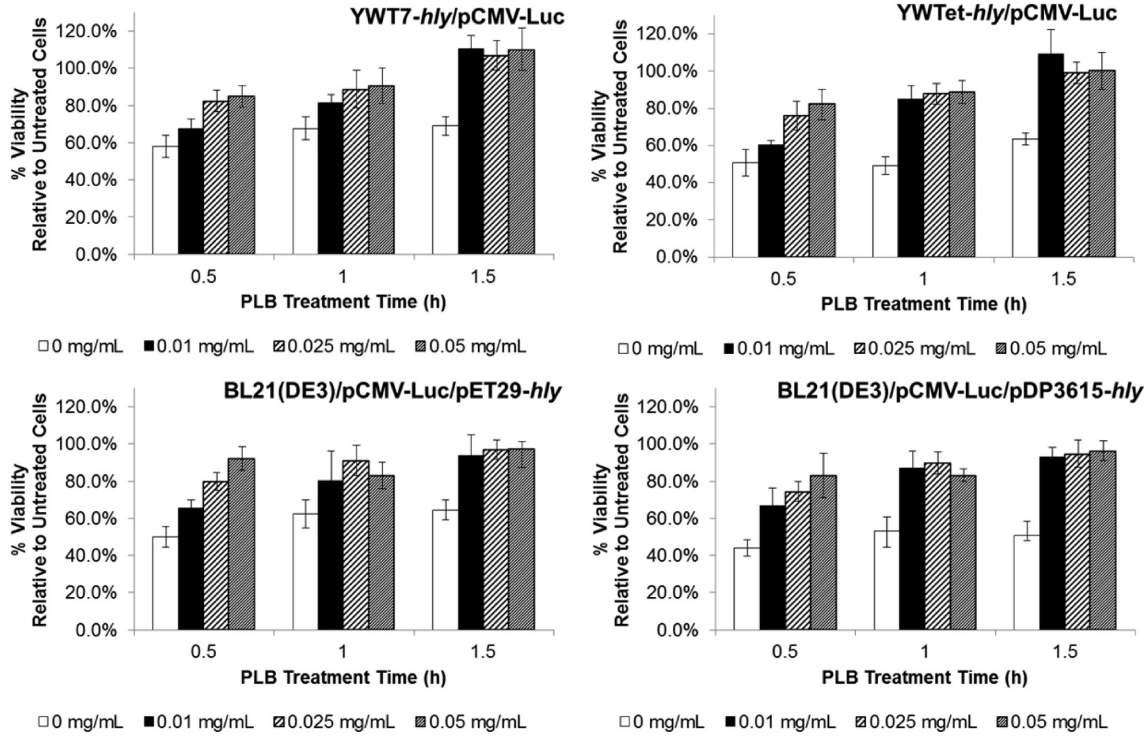

Figure 7. MTT assay with RAW264.7 cells incubated with PLB-treated bacterial vector strains.

samples were prepared as described above and subjected to the hemolysis assay conducted at pH 7.4 (Figure 5). All strains demonstrated <5% hemolysis, with all but strain BL21(DE3)/pCMV-Luc/pET29-*hly* demonstrating levels comparable to untreated samples. Observed hemolysis under these conditions is also similar to that for uninduced strains measured at pH 5.5 (data not shown), with BL21(DE3)/pCMV-Luc/pET29-*hly* being the only exception. These observations support previous studies that indicate LLO is only active in low pH solutions.[29]

Gene Delivery Studies. PLB-treated bacterial vectors were evaluated for gene delivery efficacy in the context of a high-throughput 96-well assay. Our high-throughput assay confers the ability to test all vectors in all conditions at once to facilitate rapid identification of relative success. The particular assay used here was adapted and improved upon from our previous

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bactofection studies.[13,14] Observed gene delivery of PLB-treated bacterial strains (Figure 6) showed highest delivery related to lower LLO expression profiles and the lowest delivery related to the highest LLO expression profile (BL21(DE3)/pCMV-Luc/pET29-*hly*).

We initially expected that higher concentrations of PLB and lengthened treatment time would result in increased gene delivery; however, at the highest concentration (0.05 mg/mL) and treatment time (1.5 h), gene delivery is the lowest for all strains. Optimal gene delivery occurs at 0.025 mg/mL at 0.5 h and 0.01 mg/mL at 1 h for all strains. At these two points, membrane weakening, DNA and protein release, and hemolysis data are comparable for each individual strain, suggesting that an optimal level of weakening and external DNA and protein release enhance gene delivery.

To eliminate the possibility that improvements in PLB-treated bacterial-mediated gene delivery are caused by complete rupture of the bacteria followed by delivery of released plasmid DNA, we prepared and tested supernatant from each sample and time point. Observed gene delivery from the supernatant of PLB-treated bacterial strains demonstrated low delivery and was comparable to results when using the supernatant of non-PLB-treated bacterial strains (Figure S1 in the Supporting Information).

Cellular Toxicity. A major hindrance of bactofection is the possibility that use of bacterial vectors will cause sepsis and/or immunogenicity, thus, evaluation of cellular cytotoxicity is a critical parameter for determining future biomedical and clinical applications. A modified MTT assay was carried out on RAW264.7 to assess cytotoxicity. Modifications of the assay from our prior studies[13,14] were made by removing the step of adding RPMI-1640 containing gentamicin to eliminate non-phagocytosed bacteria, which would normally lead to drastic macrophage cell death. Here, however, the assay was designed to test whether PLB pretreatment could minimize the deleterious effects previously observed upon incubating bacteria with the macrophage cell line. Samples were prepared in the same manner as described during gene delivery studies, and it was observed that cytotoxicity decreased upon PLB treatment (Figure 7). Improvements upon cytotoxicity can be attributed, at least in part, to PLB's ability to bind and inactivate endotoxin.[28] Additionally low to no cytotoxicity was observed for optimal gene delivery conditions, 0.025 mg/mL at 0.5 h and 0.01 mg/mL at 1 h.

CONCLUSION

Bactofection has emerged as a promising tool for gene therapy by synergistically combining the ease and flexibility of designing and engineering bacterial vectors with inherent properties that promote improved delivery. However, bacterial vectors still experience significant resistance clinically. We investigated the use of polymyxin B as a predelivery treatment for engineered *E. coli* gene delivery strains. Results indicate that PLB pretreatment significantly improves both gene delivery and target cell viability. Additionally, the data suggest that gene delivery can be enhanced/optimized by balancing cellular integrity and the degree of DNA and protein leakage. These studies present an easy route to improve bactofection delivery efficacy while reducing cytotoxicity and strengthen the case for future applied usage of bacterial vectors.

ASSOCIATED CONTENT

Supporting Information

Figure depicting gene delivery resulting from the supernatant of PLB-treated bacterial vector strains incubated with RAW264.7 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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