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PEGylation of Polyethylenimine Lowers Acute Toxicity while Retaining Anti-Biofilm and β-Lactam Potentiation Properties against Antibiotic-Resistant Pathogens

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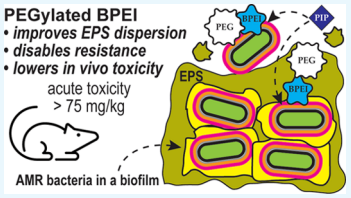
ABSTRACT: Bacterial biofilms, often impenetrable to antibiotic medications, are a leading cause of poor wound healing. The prognosis is worse for wounds with biofilms of antimicrobial-resistant (AMR) bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *S. epidermidis* (MRSE), and multi-drug resistant *Pseudomonas aeruginosa* (MDR-PA). Resistance hinders initial treatment of standard-of-care antibiotics. The persistence of MRSA, MRSE, and/or MDR-PA often allows acute infections to become chronic wound infections. The water-soluble hydrophilic properties of low-molecular-weight (600 Da) branched polyethylenimine (600 Da BPEI) enable easy drug delivery to directly attack AMR and biofilms in the wound environment as a topical agent for wound treatment. To mitigate toxicity issues, we have modified 600 Da BPEI with polyethylene glycol (PEG) in a straightforward one-step reaction. The PEG−BPEI molecules disable β-lactam resistance in MRSA, MRSE, and MDR-PA while also having the ability to dissolve established biofilms. PEG-BPEI accomplishes these tasks independently, resulting in a multifunction potentiation agent. We envision wound treatment with antibiotics given topically, orally, or intravenously in which external application of PEG−BPEIs disables biofilms and resistance mechanisms. In the absence of a robust pipeline of new drugs, existing drugs and regimens must be re-evaluated as combination(s) with potentiators. The PEGylation of 600 Da BPEI provides new opportunities to meet this goal with a single compound whose multifunction properties are retained while lowering acute toxicity.



INTRODUCTION

To counter the rise of antibiotic resistant infections, existing drugs and regimens are coupled with potentiators that overcome antimicrobial resistance (AMR) or biofilms.[1−3] In contrast, multipurpose potentiators offer therapeutic advantages by counteracting resistance and biofilms.[1,3−8] Low-molecular-weight (600 Da) branched polyethylenimine (600 Da BPEI) has the ability to overcome AMR in staphylococci and *Pseudomonas aeruginosa*, potentiating penicillins, carbapenems, cephalosporins, and macrolides.[4,9−14] Nevertheless, the presence of primary amines creates toxicity issues that are paramount. In vivo toxicity issues are mitigated by attaching a low-molecular-weight polyethylene glycol (PEG) group to 600 Da BPEI (PEG-BPEI). The need to couple potentiators with a standard-of-care antibiotic creates barriers to developing therapy for systemic bacteremia where matching the pharmacokinetic and pharmacodynamics of each component is essential. Instead, the most promising therapeutic opportunities exist with treating bacterial infections of acute and chronic wounds. As a topical agent for wound treatment, the water-soluble properties of the bioactive moiety, 600 Da BPEI, and the attached PEG group enable easy drug delivery to directly attack AMR and biofilms in the wound environment. The hydrophilic properties also mitigate problems with binding to proteins. We have shown that 600 Da BPEI does not suffer from detrimental protein binding using antibiotic potentiation assays in the presence of fetal bovine serum.[10] Additionally, 600 Da BPEI has low in vitro toxicity[10] that differentiates it from colistin[10] and polymyxins.[15] The advantages of using 600 Da BPEI as the active moiety arise from its hydrophilic nature that enables a potentiation mechanism of action (MOA) involving binding to the outer cellular envelope without disrupting the membrane bilayer.[4]

For chronic wound infections associated with AMR bacteria and their biofilms, treatment options are scarce. Bacterial biofilms, often impenetrable to antibiotic medications, are a leading cause of poor wound healing.[16] Patients afflicted with these chronic wounds suffer from physical pain, disabilities, psychological and emotional stresses, and poor quality of life. Current in-patient treatments include cleansing, debridement, maintaining a moist tissue environment, and, when possible, eliminating the underlying pathology or factors that contribute

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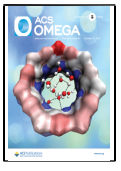

to poor wound healing.[17] In advanced cases, amputation may become necessary. Death, especially in elderly patients, may result from sepsis associated with chronic wounds. Survival is determined by patient age, comorbidities, severity of the acute infection, timely treatment, and effective treatment.[18] While the first two factors are beyond the control of pharmaceutical therapy, antibiotics can be used effectively against susceptible infections. For antibiotic-resistant infections, the best practices for effective in-patient intervention are strict sanitary guidelines and antibiotics, such as intravenous vancomycin plus piperacillin/tazobactam or IV treatment with new antibiotics of last resort.[17] Nevertheless, biofilms and antimicrobial resistance create substantial technological barriers to treating chronic wound infections. This presents a significant and critical need for new ways to counteract biofilms and antimicrobial resistance simultaneously. In the absence of a robust pipeline of new drugs, existing drugs and regimens must be re-evaluated as combination(s) with potentiators. Ideally, the potentiator should be a single compound with multi-function and broad-spectrum properties that disable biofilms and antibiotic resistance. We have discovered such a compound. PEG-BPEI is a potentiator that disables β-lactam resistance in MRSA, MRSE, and MDR-PA and disrupts their biofilms. We envision wound treatment with antibiotics given topically, orally, or intravenously in which external application of PEG-BPEIs disables biofilms and resistance mechanisms.

RESULTS AND DISCUSSION

Even if a drug is effective, toxicity can preclude its clinical use. According to Wiegand et al., high-molecular-weight BPEIs (over 25,000 Da) are toxic; however, 600 Da BPEI has high biocompatibility and a low likelihood for mutagenesis.[19] This report demonstrated BPEI's safety, biocompatibility, and antimicrobial properties, but it did not evaluate BPEI's synergy with antibiotics against MDR bacteria and their biofilms.[19] We believe it is possible to increase drug safety while retaining potentiation by reacting the epoxy group of a polyethylene glycol monoglycidyl epoxide with one of the amine groups on 600 Da BPEI in a straightforward one-step reaction under mild conditions (Figure 1). PEGylation has a strong foundation in

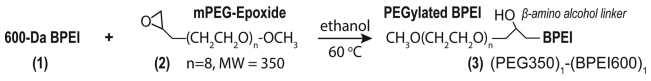

Figure 1. PEGylation reaction of 600 Da BPEI and PEG to produce $(PEG350)_1$-$(BPEI600)_1$.

medicinal chemistry,[20−22] and data show that PEGylation of cationic amine polymers reduces toxicity.[23,24] The PEG-BPEIs are not prodrugs, they are stable entities that balance cationic properties for binding to anionic species (teichoic acids, lipopolysaccharides, and biofilm extracellular polymeric substance) with hydrophilic properties to promote faster antibiotic diffusion and uptake.

The synthesis of PEGylated BPEI involves a simple bimolecular substitution (SN2) epoxide ring-opening reaction of a monofunctionalized PEG epoxide (2, Figure 1). PEG epoxides are available in different molecular weights. Here, we used 350 MW PEG. The reaction mechanism generally involves nucleophilic attack by a primary amine on BPEI (1, Figure 1). Using a one-to-one stoichiometry, the product (3) is generated and denoted $(PEG350)_1$-$(BPEI600)_1$.

Nuclear magnetic resonance (NMR) spectroscopy was used to follow the PEGylation reaction. The ^{1}H NMR spectrum of 600 Da BPEI (1, Figure 2A) is composed of signals between

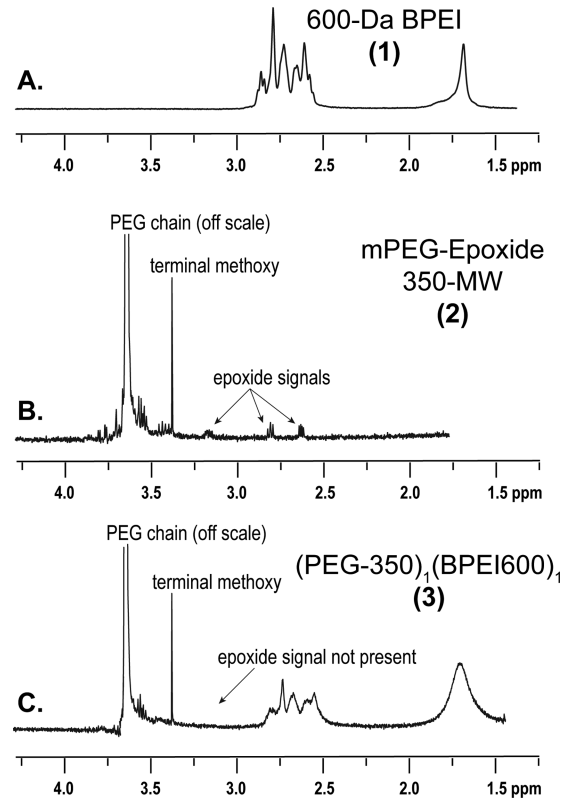

Figure 2. ^{1}H NMR spectra of (A) 600 Da BPEI, (B) mPEG-epoxide, and (C) $(PEG350)_1$-$(BPEI600)_1$ dissolved in $CDCl_3$. In panel (C), the signals for the epoxide group is absent, indicating that the reaction is complete.

2.5 and 3 ppm assigned to backbone CH_2 groups, while the broad signal at ∼1.65 ppm is assigned to backbone amine protons. Interaction between BPEI and water leads to proton exchange processes that cause exchange-averaged broadening of NMR signals, and $CDCl_3$ NMR solvents are known to have small amounts of residual water.[25] These observations are consistent with previously reported data for BPEI.[26] The spectrum for mPEG-epoxide (2, Figure 2B) has a very strong signal for backbone CH_2 groups at 3.65 ppm, while the terminal methoxy group gives a ^{1}H signal at 3.3 ppm. Characteristic epoxide signals are observed at 2.6, 2.8, and 3.2 ppm. These signals are absent in the spectrum of $(PEG350)_1$-$(BPEI600)_1$ (3, Figure 2C), indicating that the reaction is complete. A similar conclusion was reached with mPEG-epoxide reacting with a cellulose substrate.[27] PEG has also been grafted onto BPEI using an amide-linking approach with the samples analyzed as aqueous solutions using NMR spectroscopy.[28,29]

PEGylation addresses the weakness that 600 Da BPEI may not be clinically viable because of its toxicity. The ability of some cationic species to permeate the membranes of eukaryotic cells causes toxicity that hinders drug development. Toxicity is mitigated by lowering the number of primary amines, such as Spero Therapeutic Inc.'s SPR741, a cyclic peptide with three primary amines.[30] This paradigm has been verified for PEG-BPEI. PEGylating 600 Da BPEI lowers single-

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Table 1. Minimum Inhibitory Concentrations (MIC) and Fractional Inhibitory Concentration Indices (FICI) of 600-Da BPEI and $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ as Potentiators of β-Lactam Activity against MRSA, MRSE, and *P. aeruginosa*[e]

	MIC μg/mL (μM)						
strain	600 Da BPEI	OXA[a]	OXA[a]	+	600 Da BPEI	FICI	outcome
MRSE 35984	8 (13.3)	32	8	+	2 (3.3)	0.5	synergy
MRSA USA300	32 (53.3)	32	4	+	8 (13.3)	0.38	synergy
MRSA MW2	>64 (>106.7)	32	2	+	16 (26.7)	0.19	synergy
	MIC μg/mL (μM)						
strain	PEG-BPEI[b]	OXA[a]	OXA[a]	+	PEG-BPEI[b]	FICI	outcome
MRSE 35984	64 (67.4)	64	8	+	16 (16.7)	0.38	synergy
MRSA USA300	64 (67.4)	16	2	+	16 (16.8)	0.38	synergy
MRSA MW2	64 (67.4)	16	4	+	16 (16.7)	0.5	synergy
	MIC μg/mL (μM)						
strain	600 Da BPEI	PIP[c]	PIP[c,d]	+	600 Da BPEI	FICI	outcome
PA 27583	16 (26.7)	4	0.25	+	4 (6.7)	0.31	synergy
PA OU1	16 (26.7)	64	4	+	2 (3.3)	0.31	synergy
	MIC μg/mL (μM)						
strain	PEG-BPEI[b]	PIP[c]	PIP[c,d]	+	PEG-BPEI[b]	FICI	outcome
PA 27583	64 (67)	4	0.5	+	16 (16.8)	0.31	synergy
PA OU1	256 (268)	64	4	+	32 (33.6)	0.19	synergy

[a]Oxacillin (OXA) susceptibility breakpoints are resistant at ≥4 μg/mL and susceptible at <4 μg/mL. [b]PEG-BPEI = $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$. [c]Piperacillin (PIP) susceptibility breakpoints are resistant at ≥32 μg/mL and susceptible at <16 μg/mL. [d]Piperacillin only, no tazobactam added. [e]Concentrations are listed in units of μg/mL, and the corresponding μM values are in parentheses for comparison between 600 Da BPEI and PEG-BPEI.

dose acute toxicity. Toxicity data was collected by TransPharm Preclinical Solutions Inc., a contract research lab. The acute toxicity LD_0, or maximum tolerable dose (MTD), was evaluated over 3 days using female ICR mice with daily subcutaneous dosing (Figure S1). For 600 Da BPEI, the MTD is 25 mg/kg. Adding a single 350 MW PEG group to 600 Da BPEI, $(PEG\text{-}350)_1(BPEI\text{-}600)_1$ increases its MTD to 75 mg/kg. These data show that subcutaneous PEG-BPEI, for instance applied to a wound with exposed tissue layers, has lower acute toxicity and is safer to use than 600 Da BPEI. Additional safety studies to evaluate off-target effects will be needed in the future. The strategy of disabling resistance with cationic potentiators and reducing toxicity by reducing the number of primary amines has strong precedence. Spero Therapeutics, Inc. has a drug based on a polymyxin B derivative that binds to lipopolysaccharides (LPSs) and increases the potency of rifampicin, clarithromycin, and azithromycin.[30,31] Unmodified polymyxin B has five primary amines, while the Spero compound, SPR741, is a peptide with three primary amines. Phase 1a and 1b clinical trials demonstrate its tolerability and pharmacokinetic profile.[32,33] SPR741 has been proven useful against Gram-negative pathogens such *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. Our approach has advantages because, as described below, $(PEG350)_1$-$(BPEI600)_1$ is a multifunctional broad-spectrum antibiotic potentiator that also disrupts biofilms. Second, our potentiators are neither cationic peptides nor peptide mimetics that can disable biofilms but lack in vivo activity due to rapid proteolytic degradation and/or protein binding in wounds.[34,35] The PEG-BPEI synthesis requires a single straightforward reaction using low-cost starting materials.

Here, 600 Da BPEI restores susceptibility of MRSE[12,13] and MRSA[9−11] to β-lactam antibiotics by inhibiting the PBP2a/4 functionality. Here, checkerboard assays were conducted to examine the potentiation activity of $(PEG350)_1$-$(BPEI600)_1$ when combined with oxacillin against MRSE and MRSA (Table 1 and Figure S2). The minimum inhibitory concentrations (MICs) of $(PEG350)_1$-$(BPEI600)_1$ and oxacillin for all three tested strains are tabulated in Table 1. The $(PEG350)_1$-$(BPEI600)_1$ MICs for MRSE 35984, MRSA MW2, and MRSA USA300 are each 64 μg/mL. The oxacillin MICs are 64 μg/mL for MRSE 35984, 32 μg/mL for MRSA MW2, and 32 μg/mL for MRSA USA300. According to standard EUCAST guidelines,[36] these values denote oxacillin resistance, the breakpoint MIC for resistance is ≥4 μg/mL, while values <2 μg/mL denote oxacillin susceptibility. The checkerboard assay data show that growth inhibition is possible with different combinations of 600 Da BPEI and oxacillin or $(PEG350)_1$-$(BPEI600)_1$ and oxacillin (Figure S2). Synergistic effects are indicated when the fractional inhibitory concentration index (FICI) is ≤0.5,[36] which was found for all three strains tested. Non-PEGylated BPEI was slightly more effective than PEGylated 600 Da BPEI at overcoming oxacillin resistance. Achieving an oxacillin MIC of 2 μg/mL against MRSE 35984 required 3.3 μM 600 Da BPEI versus 33.7 μM $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$, 13.33 μM 600 Da BPEI versus 16.8 μM $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ for MRSA USA300, and 26.67 μM 600 Da BPEI versus 33.7 μM $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ for MRSA MW2. The ability to increase antibiotic efficacy can be described by a fourfold minimum potentiating concentration (MPC_4).[37] The MPC_4-OXA for 600 Da BPEI was 3.33 μM for MRSE 35984, 6.67 μM for MRSA USA300, and 13.33 μM for MRSA MW2. For $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$, the MPC_4-OXA was 16.8, 4.2, and 8.4 μM for these three species, respectively. The differences between PEGylated and non-PEGylated 600 Da BPEI are likely caused by reducing the number of primary amines in 600 Da BPEI by PEGylation and/or steric effects of the PEG group. The methicillin resistance gene *mecA* is responsible for synthesis of PBP2a, a 78 kDa transmembrane protein that can block all bindings to β-lactams, enabling MRSA/MRSE to survive in the presence of these antibiotics. Wall teichoic acid (WTA) is known to be PBP2a's cofactor,

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which localizes PBP2a to where to function.[38−40] As with 600 Da BPEI, $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ bears positive charges from the amine groups at physiological pH, allowing it to electrostatically bind the negatively charged phosphodiester backbone of WTA. Therefore, $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ likely inhibits proper localization of PBP2a/4, disabling this resistance factor and restoring susceptibility of MRSA and MRSE to β-lactams.

Potentiation of piperacillin against *P. aeruginosa* is also affected when 350 MW PEG is attached to 600 Da BPEI. The strain *P. aeruginosa* 27853 is piperacillin susceptible (MIC ≤16 μg/mL), and the MPC_4-PIP is 6.67 μM for 600 Da BPEI and 16.8 μM for $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ (Table 1). Against the *P. aeruginosa* clinical isolate OU1, which is multidrug resistant,[4] the MPC_4-PIP of 600 Da BPEI is 1.67 μM, while 3.33 μM lowers the piperacillin MIC to 8 μg/mL, which indicates antibiotic susceptibility (Figure S3).[36] However, $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ is less effective as the MPC_4-PIP is 8.4 μM, and it takes 16.8 μM PEG-BPEI to lower the piperacillin MIC to levels considered antibiotic susceptible (Figure S3). The MOA for β-lactam potentiation involves 600 Da BPEI binding to the anionic LPS in the outer membrane of *P. aeruginosa*.[4] The phosphate and carboxylate groups of LPS are located on the lipid A and core oligosaccharides, approximately 1−2 nm away from the acyl chains.[41−43] These anionic sites allow for the chelation of metals that stabilize the LPS layer and provide targets for 600 Da BPEI binding. Cationic polymyxin B and colistin also bind to these sites, but their hydrophobic alkyl tails penetrate the LPS acyl chain region to disrupt membrane integrity and cause widespread catastrophic disruption. The MIC for polymyxins is low, 1−3 μg/mL.[44] In contrast, 600 Da BPEI has weaker antimicrobial action (MIC >26 μM, 16 μg/mL) because, without hydrophobic regions, it does not disrupt the membrane.[4] Instead, 600 Da BPEI increases the ability of β-lactams to traverse the O-antigen and core oligosaccharides of LPS and reach porin transporters. It is likely that $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ shares this MOA. The higher MIC and slightly weaker potentiation property suggest that interactions between LPS and PEG-BPEI are reduced by PEGylation.

Isothermal titration calorimetry (ITC) directly measures the enthalpy of molecular binding interactions. We used ITC to confirm interactions between 600 Da BPEI and LPS.[4] A recent report describes SPR741's MOA as LPS binding.[45] Likewise, we posit a LPS-binding MOA for PEG-BPEIs. The isotherm obtained from a titration of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ with *P. aeruginosa* LPS (Sigma #L8643) is shown in Figure 3. The negative ΔH values indicate exothermic binding. This is due to electrostatic attractions between the cationic bioactive moiety, 600 BPEI, and the anionic lipid A, inner-core and outer-core oligosaccharide chains that chelate Mg^{2+} ions.[46] However, when compared to the isotherm for 600 Da BPEI (Figure 3A), PEG-BPEI has a less exothermic interaction with *P. aeruginosa* LPS (Figure 3B). Likewise, the molar ratio of PEG-BPEI to LPS is approximately lower than that observed with 600 Da BPEI. These data demonstrate that $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ does bind with LPS but that PEGylation reduces binding energetics and the ability of a single 600 Da BPEI molecule to bind with multiple LPS molecules. This is not surprising as the PEG group would form a large steric barrier to shield some cationic amines from their anionic targets while allowing other amines to bind with LPS. This weakening of LPS binding may explain why PEGylation of 600 Da BPEI reduces antibiotic potentiation (Figure 3C). More $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$, 17 μM, than 600 Da BPEI (3.3 μM) is needed to potentiate piperacillin against MDR-PA (Figure S3). This weakness is mitigated by considering that $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ has lower in vivo toxicity (MTD = 75 mg/kg) than 600 Da BPEI (MTD = 25 mg/kg), and as discussed below, this does not cause β-lactam ring hydrolysis but does possess superior anti-biofilm properties. While studies are underway to further elucidate the MOA for PEG-BPEI, it is possible that the PEG group prevents the active moiety, 600 Da BPEI, from reaching

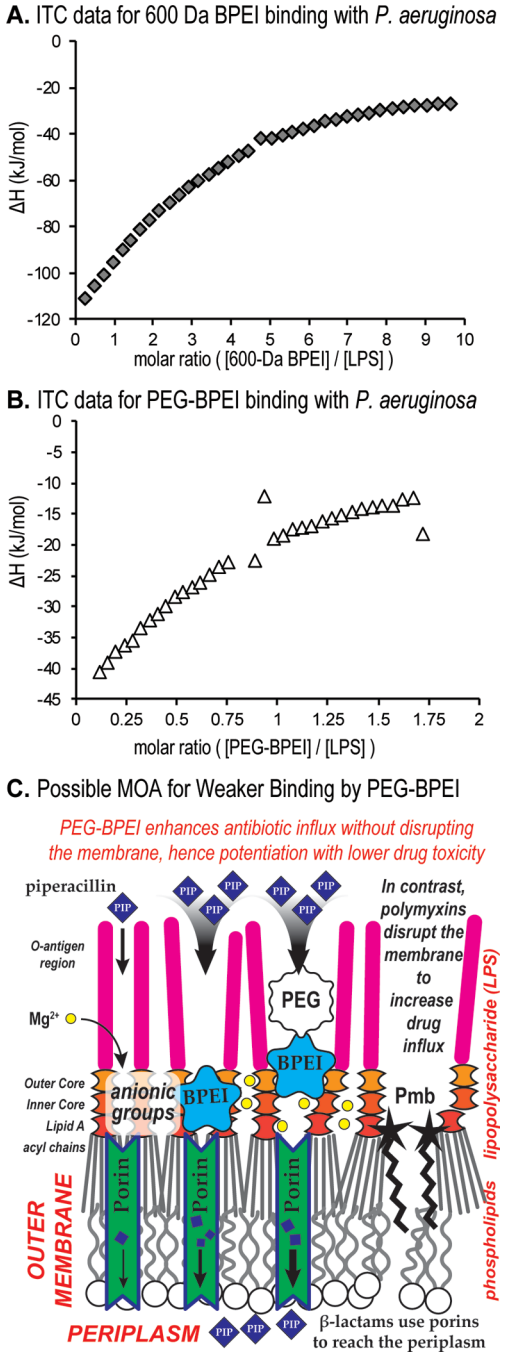

Figure 3. Isothermal titration calorimetry data demonstrate that *P. aeruginosa* LPS binds with (A) 600 Da BPEI and (B) $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$. The differences in binding energetics and molar ratio are attributed to steric hindrance from the 350 MW PEG group attached to 600 Da BPEI. This effect would also explain why PEGylation reduces piperacillin potentiation, a paradigm illustrated in panel (C).

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the phosphates of lipid A at the acyl chain interface. This scenario may also explain why PEGylation increases drug safety, perhaps by preventing PEG-BPEI from disrupting eukaryotic membranes.

The ability of PEGylation to increase safety and lower the acute toxicity is strong benefits that outweigh any reduction in potentiation efficacy. Because the most likely use of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ would be as a topical application to acute and chronic wounds containing MRSA, MRSE, and/or MDR-PA bacteria, higher drug concentrations can be directly applied to the wound. As noted above, PEG-BPEI exposure to subcutaneous tissue does not cause adverse toxicity. Furthermore, the benefits of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ extend beyond disabling β-lactam resistance It is important to consider that primary amino groups could disrupt the β-lactam ring of the antibiotics. A colorimetric assay of β-lactam hydrolysis was performed with nitrocefin, a chromogenic cephalosporin whose β-lactam ring, which is susceptible to β-lactamase, mediated hydrolysis.[47] Once hydrolyzed, the degraded nitrocefin compound rapidly changes color from yellow to red. As shown in Figure S4, the unmodified 600 Da BPEI causes slight hydrolysis at a molar ratio of 0.017:0.005 (3.4:1), whereas $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ has a similar effect at a molar ratio of 0.168:0.005 (33.6:1). Thus, PEGylation of BPEI leads to a 100× reduction in hydrolytic activity of the constrained β-lactam ring of nitrocefin. Bacterial biofilms play a vital role in the ability of AMR pathogens to withstand antibiotic therapy. They deploy a protective layer of extracellular polymeric substances (EPSs) composed of polysaccharides, extracellular DNA, and proteins. These biomacromolecules are cross-linked and encase bacteria. The resulting matrix hinders the diffusion and accessibility of antibiotics and host immune agents. Treating wound biofilms often involves antibiotic therapy plus mechanical debridement and irrigation with saline that may contain detergents. The presence of MRSA, MRSE, and/or MDR-PA renders many standard-of-care antibiotics useless. Bacterial cells that remain after cleansing survive antibiotic therapy, quickly populate the wound bed, and regenerate the biofilm matrix. An advantage of 600 Da BPEI is its ability to disrupt biofilms of staphylococci[12,13,48] and *P. aeruginosa*.[4] PEGylated 600 Da BPEI retains these anti-biofilm properties and is a superior anti-biofilm agent compared to non-PEGylated 600 Da BPEI.

Data from a crystal violet biofilm assay are shown in Figure 4. MRSE 35984 produces strong and consistent biofilms. Biofilms were stained with crystal violet and treated with $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$, 600 Da BPEI, water, and acetic acid. The supernatant was carefully transferred into a new plate for the OD_{550} measurement, which corresponds to the amount of dissolved biofilm (Figure 4B). The biofilm is dissolved with 214 μM (128 μg/mL) 600 Da BPEI, consistent with a previous report where 214 μM 600 Da BPEI dissolved twice as much MRSE 35984 biofilm as 53 (32 μg/mL) and 106 μM (64 μg/mL) 600 Da BPEI.[13] Adding 350 MW PEG to 600 Da BPEI improves its anti-biofilm properties. The MRSE 35984 biofilm is completely dispersed by 67.4 μM $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$, a concentration that is 3.6 times lower than the 214 μM 600 Da BPEI required to give the same results. This highlights the biofilm-disrupting potential of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ and that PEGylation improves disruption. The biofilm EPS of staphylococci contains a large component of poly-*N*-acetyl glucosamine (PNAG) and anionic extracellular teichoic acid and eDNA.[49−51] These components facilitate and stabilize

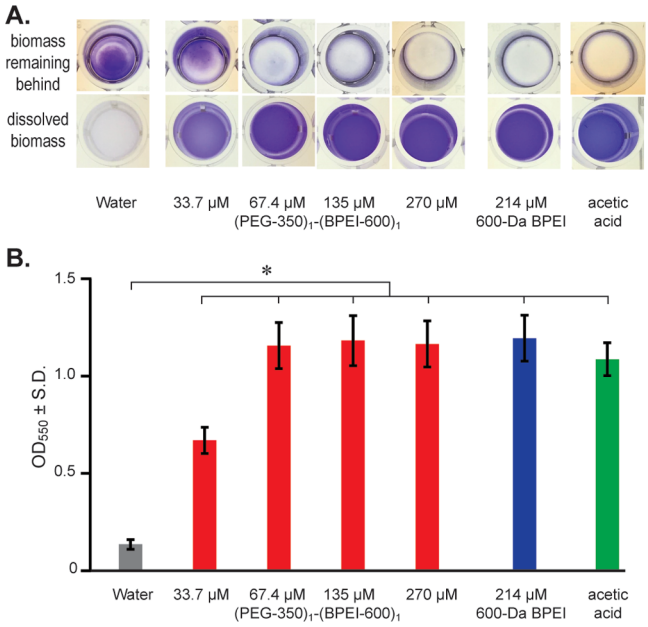

Figure 4. Biofilm disruption assays using crystal violet to stain the biomass. Preformed MRSE 35984 biofilms stained with crystal violet and washed prior to treatment with different concentrations of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ or 600 Da BPEI, in addition to treatment with of water only and acetic acid. Photographs of the stained biomass dissolved by the test agent were transferred into a new plate, and the biomass remaining in the original plate is in panel (A). The absorbance of the dissolved biomass at 550 nm was measured and is reported in panel (B). Error bars denote standard deviation (n = 6). An asterisk indicates a significant difference between the treatments and the negative control of water (t-test, p-value <0.01).

biofilm formation. The primary amines of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ bind with anionic EPS moieties to disrupt biofilm integrity and stability. The hydrophilic nature of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ increases the ability of antibiotics to penetrate the biofilm matrix while simultaneously causing the biofilm to disperse. The staphylococci cells become vulnerable to β-lactam antibiotics when additional $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ molecules bind to the planktonic cells and disable PBP2a/4 resistance mechanisms.

Importantly, biofilms can be eradicated without dissolving the EPS. For methicillin-resistant *S. epidermidis* (MRSE), we can overcome oxacillin resistance in planktonic cells where the MIC drops from 32 to 4 μg/mL with 6.67 μM 600 Da BPEI[12] and 33.37 μM $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$. Eradication of MRSE biofilms requires a higher amount of oxacillin, MBEC = 512 μg/mL, because of barriers imposed by the biofilm EPS.[13] However, 600 Da BPEI can weaken the EPS to increase oxacillin activity without dissolving the EPS. The oxacillin MBEC drops to 16 μg/mL in the presence of 13 μM 600 Da BPEI.[13] However, this 13 μM 600 Da BPEI does not dissolve the biofilm according to the crystal violet assay. Rather, 214 μM 600 Da BPEI are required to disperse the biofilm EPS into the solution.[13] In the MBEC assay using the Calgary biofilm device, biofilms are grown on polystyrene prongs on the lid of a 96-well plate. After biofilms are established on the prongs, they are transferred to a 96-well plate for treatment before transferring to a third plate of media only, where sonication is used to dislodge the biofilms from the prongs. Biofilms that remain attached to the prongs during the treatment phase are weakened by the treatment solution. In this case, 13 μM 600

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Da BPEI was able to weaken the MRSE biofilm, allowing 16 μg/mL oxacillin to kill the cells in the biofilm EPS that remained attached to the prong.

CONCLUSIONS

PEGylated BPEI is a multifunction potentiator. It disrupts biofilms that are otherwise impenetrable to antibiotics and counteracts β-lactam resistance mechanisms. These events, biofilm dispersal and β-lactam potentiation, occur through independent mechanisms. Overcoming β-lactam resistance in staphylococci involves binding with anionic cell wall teichoic acids that prevent the function and localization of PBP4 enzymes.[9−13,48] Potentiation against *P. aeruginosa* occurs when the active moiety, 600 Da BPEI, binds to anionic LPS in the cell envelope. This increases drug influx by facilitating access to porin transporters without membrane disruption that occurs with polymyxin B and colistin.[4] Biofilm dispersal is possible because the bioactive moiety, 600 Da BPEI, can bind to anionic species in the EPS matrix. Compared to 600 Da BPEI, $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ has superior biofilm dispersal properties and lower acute toxicity, although the β-lactam potentiation activity is slightly reduced. These data suggest that $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ is likely to be more favorable for therapeutic opportunities than 600 Da BPEI.

Skin or soft-tissue infections (SSTIs) arise from abrasions, nonsurgical wounds, burns, or chronic health problems.[52] For example, diabetic foot ulcers are the most common cause of nontraumatic lower leg amputation in the United States. Despite efforts to prevent and treat foot ulcers, each year about 108,000 Americans with diabetes will lose part of their lower extremities because a foot ulcer becomes infected and does not heal.[53] Diabetic wounds often become chronic because they stall in a suboptimal inflammatory phase of healing that is perpetuated by a microbial infection with biofilm-forming pathogens, resulting in an accumulation of microorganisms embedded in a polysaccharide matrix.[54,55] Employing $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ as a means to treat chronic wounds afflicted with biofilms of AMR bacteria not only attacks the underlying pathology, but topical use mitigates drawbacks of matching its pharmacokinetics (PK) and pharmacodynamics (PD) with those of antibiotics. There is a strong likelihood of developing a topical agent for wound treatment because the water-soluble hydrophilic properties of PEGylated BPEI appear to be perfectly suited for wound treatment where straightforward drug delivery directly attacks AMR and biofilms in the wound environment. We also recognize that it is important to evaluate the full safety profile of PEGylated BPEI and its impact on wound healing. These studies are underway. The conceptual origin of PEG-BPEIs as antibiotic potentiators is based on recognizing that cationic properties allow binding to anionic targets, such as WTA, LPS, and EPS. Identifying anionic targets for BPEI derives from our work describing the equilibrium binding behavior of Ca^{2+} and Mg^{2+} with WTA,[56,57] and the WTA metal-binding model has been independently verified.[58] Previously, we found that 600 Da BPEI had low in vitro cytotoxicity with an IC_{50} of 1090 and 690 μg/mL (1817 and 1150 μM, respectively) on human HeLa cells and HEK293, respectively. Additionally, lactate dehydrogenase (LDH) assays showed that 600 Da BPEI has a low nephrotoxicity of 3.5% at 63 μg/mL (106 μM), much lower than that of colistin, which was >20% nephrotoxicity at the same concentration tested.[10] However, the in vivo toxicity of 600 Da BPEI was concerning (MTD = 25 mg/kg), and thus PEGylation was pursued to improve drug safety (MTD = 75 mg/kg).

A strength of PEG-BPEI is addressing the unmet medical need for new therapies to treat wound infection caused by prominent antibiotic-resistant Gram-positive and Gram-negative pathogens with a compound whose PEGylation reduces toxicity, reduces β-lactam hydrolysis activity, and improves anti-biofilm properties. However, we also recognize that the utility PEG-BPEI may be restricted to topical treatment. However, this does not imply limited impact on patient health. For example, each year, virulent MDR-PA contributes to 4−6 million chronic infections that arise from 100 million skin and soft-tissue infections (SSTIs).[16,52,59−61] Chronic wounds exacerbated by *P. aeruginosa* infection often result in amputation.[62,63] This outcome can be avoided by addressing (1) antimicrobial resistance (AMR) against standard-of-care antibiotics and (2) biofilms that stifle host defense and antimicrobial activity. PEG-BPEI provides this opportunity.

METHODS

Materials. In this study, methicillin-resistant *S. epidermidis* 35984, methicillin-resistant *S. aureus* USA300 (BAA-1717), and *P. aeruginosa* 2785 bacteria were purchased from the American Type Culture Collection. Additionally, MDR-PA OU1 was obtained from clinical isolates from the University of Oklahoma Health Sciences Center using appropriate IRB protocols and procedures. MRSA MW2 (referenced in Campbell et al., 2011[64]) was a generous gift from Dr. Suzanne Walker. Chemicals and antibiotics were purchased from Sigma-Aldrich. Then, 600 Da BPEI was purchased from Polysciences, Inc. Monofunctionalized PEG epoxide was obtained from Nanocs, Inc.

Synthesis and Characterization of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$. Approximately 200 mg of 600 Da BPEI was added to a small glass vial and dried overnight under high vacuum. The vial was reweighed to determine the final mass of the dry BPEI. This value was used to determine the amount of mPEG-epoxide (350 MW) required to react with 600 Da BPEI in a one-to-one stoichiometric ratio. The 600 Da BPEI was dissolved in 3 mL of 100% ethanol with stirring. Afterward, a solution of mPEG-epoxide dissolved in 3 mL of 100% ethanol was added dropwise. The mixture was stirred at 60 °C for 24 h. Afterward, the mixture was cooled, and the solvent was removed under high vacuum for 72 h. A 1-D ^1H NMR spectrum was collected by dissolving a portion of the dry reaction product in $CDCl_3$ followed by transfer to a 3 mm NMR tube. All NMR experiments were performed using a 28-shim Agilent VNMRS-300 MHz equipped with a triple-resonance PFG probe. Pulse sequences for each experiment were supplied by Agilent. Data acquisition and processing were completed using VNMRJ 2.2C software on the Red Hat Linux 4.03 operating system. MestreNova software was used to analyze the spectra.

Checkerboard Assays. Checkerboard assays followed the methods of Lam et al.[12] to determine the synergistic effect between $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ and antibiotics against drug-resistant strains growing in cation-adjusted Mueller−Hinton broth (CAMHB). Bacterial growth used CAMHB media augmented with various amounts in serial dilutions of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ and/or antibiotic (oxacillin or piperacillin) inoculated with bacterial cells from an overnight culture (5×10^5 CFU/mL). Cells were grown at 37 °C. The change in OD_{600} (optical density at 600 nm) was measured

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and recorded after 24 h of treatment. Each checkerboard trial was done in triplicate using sterile Greiner CellStar flat bottom polystyrene plates, catalog #655180.

In Vivo Toxicity Studies. Experiments to determine the acute toxicity of 600 Da BPEI and $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ were performed by a contract research organization (TransPharm Preclinical Solutions, Jackson, MI). Fully immunocompetent, uninfected, ICR mice (4−6 weeks old, 18−20 g each, Envigo, Inc.) were treated once a day for 3 days via subcutaneous injection with low concentrations of 600 Da BPEI or $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ and closely monitored for adverse reactions. Adverse events and mortality were tracked through study day 4. Mice were administered 6.25, 12.5, 25, 50, 75, and 100 mg/kg of 600 Da BPEI or $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ once daily on day 0, 1, and 2 in a volume of 0.2 mL via subcutaneous (sc) injection, beginning with the lowest dose concentration before dosing the next highest concentration. Mice in each group were closely observed for 15 min following dose administration for adverse events prior to dosing the next highest dose concentration. Both 600 Da BPEI and $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ are very soluble in water, which was formulated in phosphate-buffered saline (PBS) at 20 mg/mL solution and handled in a manner to minimize endotoxin and bacterial contamination. The solutions were sterilized by filter sterilization prior to the initial dose. The mice could tolerate 25 mg/kg 600 Da BPEI and 75 mg/kg $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ with no visible toxicity. Mice injected with 50 mg/kg 600 Da BPEI and 100 mg/kg $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ succumbed to death within 5 min of treatment.

Biofilm Disrupting Assay. Overnight cultures of MRSE 35984 were used to inoculate a tissue culture treated in a 96-well plate (100 μL of tryptic soy broth or TSB/well) with an inoculation size of 1 μL/well ($\sim 5 \times 10^5$ CFU/mL). The plate was incubated at 35 °C for 48 h to allow the bacteria to form a biofilm. It was then washed with water to remove planktonic bacteria and stained with 100 μL of crystal violet solution (0.1%) per well for 15 min. The stained plate was washed excessively with water five times to remove any unbound stain and air dried overnight. After washing to remove the crystal violet, various concentrations of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)_1$ or 600 Da BPEI were added to the stained-biofilm plate with a total volume of 100 μL/well. Water only and 30% acetic acid were also used for treatment. After 20 h, without touching the biofilm layer in the bottom of the plate, a solubilized solution containing dissolved, stained biomass in each treated well was carefully transferred to a new 96-well plate for an OD_{550} measurement, which represents the corresponding amount of biomass disrupted by each treatment.

Isothermal Titration Calorimetry (ITC). Isothermal titration calorimetry (MicroCal PEAQ-ITC, Malvern Inc., Malvern, U.K.) was utilized to test the interactions between *P. aeruginosa*-isolated LPS and PEGylated BPEI following the methods of Lam et al.[4] Briefly, solutions of $(PEG\text{-}350)_1$-$(BPEI\text{-}600)$ (1 mg/mL) and *P. aeruginosa* LPS (Sigma product L8643, 5 mg/mL) prepared in 50 mM Tris−HCl (pH 7) were titrated using injections of 2 μL lasting 4 s and separated by 150 s time intervals. Controls were performed, and the experiment was done in duplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04111.

Tabulated data from the in vivo MTD study, checkerboard assay data, and data showing that BPEI and PEGylated BPEI do not hydrolyze the lactam ring (PDF)

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Notes

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■ ABBREVIATIONS

AMR, antimicrobial resistance; MDR, multidrug resistant; BPEI, branched polyethylenimine; PEG, polyethylene glycol; LPS, lipopolysaccharide; WTA, wall teichoic acid; EPS, extracellular polymeric substances; PBS, phosphate-buffered saline; MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index; NMR, nuclear magnetic resonance; ITC, isothermal calorimetry; OXA, oxacillin; PIP, piperacillin; MTD, maximum tolerable dose; sc, subcutaneous; MPC_4, minimum potentiating concentration; PNAG, poly-*N*-acetyl glucosamine; SSTI, skin or soft-tissue infections; OD_{600}, optical density at 600 nm; CAMHB, cation-adjusted Muller–Hinton broth; TSB, tryptic soy broth; Da, Dalton

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