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De Novo Synthesis of Phosphorylated Triblock Copolymers with Pathogen Virulence-Suppressing Properties That Prevent Infection-Related Mortality

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Abstract

Phosphate is a key and universal “cue” in response to which bacteria either enhance their virulence when local phosphate is scarce or downregulate it when phosphate is abundant. Phosphate becomes depleted in the mammalian gut following physiologic stress and serves as a major trigger for colonizing bacteria to express virulence. This process cannot be reversed with oral supplementation of inorganic phosphate because it is nearly completely absorbed in the proximal small intestine. In the present study, we describe the de novo synthesis of phosphorylated polyethylene glycol compounds with three defined ABA (hydrophilic/-phobic/-philic) structures, ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20, and linear polymer PEG20k-Pi20 absent of the hydrophobic block. The 10k, 16k, and 20k demonstrate the molecular weights of the poly(ethylene glycol) block, and Pi10, Pi14, and Pi20 represent the repeating units of

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Additional synthesis scheme and characterization results of PEG-Pis and ABA-PEG-Pis and effect of ABA-PEG-Pis on *P. aeruginosa* and *C. elegans* (PDF)

Notes

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phosphate. Polymers were tested for their efficacy against *Pseudomonas aeruginosa* virulence in vitro and in vivo by assessing the expression of the phosphate sensing protein PstS, the production of key virulence factor pyocyanin, and *Caenorhabditis elegans* killing assays. Results indicate that all phosphorylated polymers suppressed phosphate sensing, virulence expression, and lethality in *P. aeruginosa*. Among all of the phosphorylated polymers, ABA-PEG20k-Pi20 displayed the greatest degree of protection against *P. aeruginosa*. To define the role of the hydrophobic core in ABA-PEG20k-Pi20 in the above response, we synthesized PEG20k-Pi20 in which the hydrophobic core is absent. Results indicate that the hydrophobic core of ABA-PEG20k-Pi20 is a key structure in its protective effect against *P. aeruginosa*, in part due to its ability to coat the surface of bacteria. Taken together, the synthesis of novel polymers with defined structures and levels of phosphorylation may elucidate their antivirulence action against clinically important and lethal pathogens such as *P. aeruginosa*.

Graphical abstract

Keywords

phosphorylated triblock copolymer; antivirulence; *Pseudomonas aeruginosa*; PstS; pyocyanin; *C. elegans*

INTRODUCTION

The promiscuous use of antibiotics has led to the emergence of antibiotic resistance at an unprecedented pace and continues to place patients at risk for life-threatening infections following major surgery.¹ Many if not most of the pathogens that cause these infections use the intestinal tract as their primary site of colonization. Although surgeons routinely decontaminate the intestinal tract with antibiotics prior to surgery to prevent infection, this practice carries the unintended consequence of causing antibiotic resistance.² Furthermore, overuse of antibiotics can destroy the microbiome, which normally protects against high risk pathogens.³ A more evolutionarily stable strategy to this problem would be to develop compounds that can suppress pathogen virulence rather than kill bacteria.⁴ In this manner, bacterial pathogenicity could be contained and the colonization resistance of the normally protective microbiota preserved.

We have previously shown that polyethylene glycol (PEG)-related polymers can serve to suppress bacterial virulence without affecting bacterial growth. Initially, PEG molecules were identified to function as antifouling coating agents capable of preventing bacterial adhesion to surfaces and subsequent biofilm formation.⁵⁻⁸ Amphiphilic polymeric materials

were most promising in this regard due to their dual surface functionality.^{9,10} Our lab demonstrated that amphiphilic high molecular weight polyethylene glycol PEG15-20 can repel bacteria away from the epithelial surface of cultured monolayers by coating both bacterial and the epithelial surface, an effect that contributes to the prevention of sepsis.¹¹

The ability to attach diverse functional groups to PEG polymers has extended the medical applications of PEG derivative compounds, especially as drug delivery systems.^{12–14} We have previously hypothesized that the phosphate bonded to the PEG can enhance its protective capacity by the dual effect of PEG coating and phosphate concentrated at the bacterial surface.³

In our previous work, we prepared phosphorylated commercially available PEG15-20 (Sigma), Pi-PEG15-20, and demonstrated that it can function in the intestine as an antivirulence compound and prevent lethal sepsis due to several healthcare-acquired pathogens including *Pseudomonas aeruginosa*.³ The phosphate content of this compound was shown to be particularly important for its protective effect.³ The mechanism of this effect relies on phosphosensory/phosphoregulatory circuits that are a universal feature of most bacteria and play a key role in virulence.¹⁵ One obstacle of the previously used commercially available PEG15-20 (Sigma) is that PEG15-20 is not a pure triblock polymer but rather a mixture of polymers of varying molecular weights including ABA (hydrophilic/-phobic/-philic) triblock, AB diblock, and homopolymer poly(ethylene glycol) structures (Figure S1). This situation limits the ability to further interrogate the molecular mechanisms by which the polymers protect both in vitro and in vivo, and the limited content of phosphate inhibits the opportunity of further improving the efficacy of the polymers. Therefore, in the current work, we performed de novo synthesis of a family of block copolymers with defined ABA structure as well as a complementary linear phosphorylated polymer. In all compounds, we controlled the molecular weight and functional groups to more precisely define the amount of bound phosphorus. The antivirulence capacity of the synthesized polymers was verified using the well-established model opportunistic pathogen *P. aeruginosa*.

MATERIALS AND METHODS

Materials

Bisphenol A (BPA, 99%, Aldrich), naphthalene (99%, Aldrich), diphenylmethane (99%, Aldrich), and phosphorus oxychloride (POCl₃, 99%, Aldrich) were used as received. Ethylene oxide (EO, >99%, lecture bottle, Praxair) and ethoxyethyl glycidyl ether (EEGE, 97%, Synthonyx) were treated with di-*n*-butylmagnesium for 20 min and distilled into Schlenk flasks before use. Tetrahydrofuran (THF, HPLC, inhibitor-free, Aldrich) was purified with a solvent purification system (Mbraun SPS-800) and distilled from a sodium naphthalenide solution directly before use. Diphenylmethylpotassium (DPMK) was prepared as described. Initially, a potassium naphthalenide solution was prepared in dry THF with a 1:4 mol ratio of naphthalene to potassium. After stirring for 12 h, 0.66 mol equiv of diphenylmethane was introduced to the solution via a syringe, and the solution was allowed to stir at room temperature for at least 12 h prior to use.

Synthesis of Phosphorylated PEG-Based Block Copolymers with a Hydrophobic Core

Sequential Anionic Polymerization of ABA-PEG-PEEGE—A series of ABA-PEG-PEEGE were synthesized by the sequential anionic polymerization of EO and EEEG in a custom heavy-wall glass reaction flask on Schlenk line. In a typical reaction, BPA (251 mg, 1.1 mmol) was dissolved in 120 mL of anhydrous THF at 0 °C under a dry nitrogen atmosphere, titrated with DPMK to form the initiator, followed by addition of the first monomer EO (22.0 g, 500 mmol). After stirring for 1 h, the mixture was heated to 50 °C and reacted for 3 days to attain complete conversion of the EO monomer. Then, the second monomer EEEG (2.9 g, 19.8 mmol) was injected into the flask and allowed to react for another 3 days. The polymerization was terminated with methanol, and the polymer was recovered by precipitation in cold diethyl ether. Different chain lengths of EO and EEEG can be adjusted by the feed ratio of [EO]/[initiator] and [EEEG]/[initiator].

Hydrolysis of ABA-PEG-PEEGE—Hydrolysis of EEEG segments of block copolymer was carried out in THF with 4 wt % of HCl and stirred at room temperature for 30 min. The polymers were then purified by precipitating in cold hexane and finally dried under vacuum at 60 °C to obtain a yellow wax-like product ABA-PEG-PGly (PGly: polyglycerol). The disappearance of peaks at 4.70 ppm (q, 1H), 1.29 ppm (d, 3H), and 1.19 ppm (t, 3H) in ¹H NMR confirmed the success of deprotection.

Phosphorylation of ABA-PEG-PGly—ABA-PEG-Pis (Pi: polyphosphoric acid) were prepared by phosphorylation of ABA-PEG-PGly, which was performed in a flame-dried flask under a dry nitrogen atmosphere. ABA-PEG-PGly was dissolved in anhydrous THF at 50 °C, and a 10-fold equivalent molar amount of POCl₃ was added at once via gastight syringe. The solution was stirred under nitrogen pressure for 3 h and quenched by the addition of a small amount of water. After evaporation of THF and dialysis against Milli-Q water, the sample was lyophilized to give a white flocculent product. ³¹P NMR (D₂O): δ −0.18 ppm.

Synthesis of Phosphorylated PEG-Based Block Copolymers without a Hydrophobic Core (PEG-Pi)

The synthetic strategy of PEG-Pi is quite similar to that of ABA-PEG-Pi with the exception that the polymerization started with hydrophilic ethylene glycol instead of hydrophobic BPA, as shown in Scheme S1. First, PEG-PEEGE was prepared by starting with ethylene glycol. Next, polymerization through the sequential addition of EO and EEEG followed by the same hydrolysis (to obtain PEG-PGly) and phosphorylation processes resulted in PEG-Pi being obtained.

Characterization of Block Copolymers

¹H and ³¹P NMR spectra were obtained on a Bruker Ultrashield Plus 500 MHz spectrometer and referenced internally to solvent proton signal. Apparent molecular weights and dispersity () were characterized with a gel permeation chromatography (GPC) system equipped with a Waters 1515 pump, a Wyatt Optilab T-rEX differential refractive index (RI) detector, and a Waters 2998 photodiode array (PDA) detector. For ABA-PEG-PEEGEs, ABA-PEG-PGlys, PEG-PEEGEs, and PEG-PGlys, THF was used as elution at 35 °C with

an elution rate of 0.8 mL/min. Three Waters Styragel columns were used and calibrated by polystyrene standards (Aldrich). ABA-PEG-Pis and PEG-Pis were measured in 0.1 M NaNO₃ (aq) at 25 °C with an elution rate of 1.0 mL/min on the same setup, except three Waters Ultrahydrogel columns in series were used and calibrated by PEO standards (Aldrich).

Biological Tests

Bacterial Strains—*Pseudomonas aeruginosa* strains MPAO1-P1 and MPAO1-P2¹⁶ were used in all experiments. The MPAO1-P1 strain and its derivative mutant PvdD were used to create the reporter constructs MPAO1-P1/pstS-EGFP and PvdD/pstS-EGFP.

Construction of pSensor-PstS-EGFP—The promoter region of pstS gene (*P. aeruginosa* MPAO1) was cloned in a pSensor vector created in our laboratory. The pSensor consists of a pUCP24 vector backbone and Gateway C.1 cassette (Invitrogen) in frame with the EGFP reporter gene (derived from pBI-EGFP) cloned into SmaI and PstI/Hind III sites of the pUCP24 MCS region, respectively. The region upstream of pstS was amplified by PCR (Platinum PCR SuperMix (Invitrogen) using primers PstS_F: CACCTATCCCAAAACCCCTGGTCA and PstS_R: CAAACGCTTGAGTTTCATGCCTTG, and cloned into the Gateway entry vector (pCR8/GW/Topo kit (Invitrogen)). Nucleotide sequence and orientation of the inserts were confirmed by sequencing; inserts were transferred into a pSensor vector via LR reaction using Gateway LR Clonase II Enzyme Mix (Invitrogen). Throughout the study, vector constructs were propagated in One Shot TOP10 Chemically Competent E. coli cells. Gentamycin (100 µg/mL) selection was used for pUCP24, and pSensor and ampicillin (100 µg/mL) were used for pBI-EGFP vectors. The QIAGEN Plasmid Mini Kit (Qiagen) was used for plasmid DNA extraction.

PstS Expression. *P. aeruginosa*—MPAO1-P1/pstS-EGFP or PvdD/pstS-EGFP were grown on tryptic soy agar plates supplemented with 100 µg/mL of gentamicin (Gm100) overnight. A few colonies from the overnight plates were used to inoculate liquid TSB + Gm100 for overnight growth. The overnight culture was used to inoculate fresh TSB + Gm100 at 1:100 dilution and grown to OD_{600 nm} = 0.5. Cells were pelleted by centrifugation at 3300g for 5 min and washed twice with defined citrate media (DCM: sodium citrate, 4.0 g/L (Sigma, S4641), (NH₄)₂SO₄, 1.0 g/L (Sigma, A4915), MgSO₄·7H₂O, 0.2 g/L (Fisher, M63-50). DCM medium is limited in both phosphate and iron. We used potassium phosphate buffer, pH 6.0 (PPB) for phosphate supplementation. The supplementation of DCM with 0.1 mM PPB was defined for phosphate limitation (DCM-Pi0.1) and with 25 mM PPB for phosphate abundance (DCM-Pi25). Washed cells were resuspended in DCM-Pi0.1 + Gm100 or DCM-Pi25 + Gm100, respectively, and grown overnight. In experiments carried out to test the phosphorylated polymers, bacterial cells were washed in DCM-Pi0.1 and resuspended in DCM-Pi0.1 + Gm100 supplemented with 2 mM ABA-PEG-Pis or ABA-PEG-PGlys and adjusted to pH 6.0 with KOH. After overnight growth, fluorescence (excitation 485/10, emission 528/20) and absorbance (600 nm) were measured with an FLx800 fluorescent reader (Biotek Instruments). Fluorescence readings

were normalized to absorbance. Culture conditions were 37 °C with shaking at 180 rpm (C25 Incubator Shaker, New Brunswick Scientific, Edison, NJ).

Pyocyanin Production during Low Phosphate Conditions—*P. aeruginosa* MPAO1-P2, which is known to produce higher amounts of pyocyanin than MPAO1-P1¹⁶, was used in this set of experiments. The design of the experiments was similar to the experiments described above for PstS expression except they were performed in the absence of Gm in the DCM media. First, 2 μM Fe^{3+} (1 μM $\text{Fe}_2(\text{SO}_4)_3$) was added to the media to enhance the production of pyocyanin. Pyocyanin was extracted by chloroform followed by re-extraction in 0.2 N HCl and measured at OD520 nm as previously described.¹⁷ Before extraction, cell density was measured by the absorbance at 600 nm, and pyocyanin values were normalized to bacterial cell density.

Pyocyanin Production Following Exposure Virulence-Activating Factor U-50,488, Kappa Opioids Agonist—

We have previously demonstrated that *P. aeruginosa* can be triggered to express enhanced virulence when exposed to kappa opioids, host factors known to be released into the gut during physiologic stress.¹⁷ *P. aeruginosa* MPAO1-P1, which is highly sensitive to U-50,488, was used in these experiments. MPAO1-P1 was grown on tryptic soy agar plates overnight, and a few colonies were used to inoculate liquid TSB for overnight growth. Overnight cultures were used to inoculate fresh TSB at 1:100 dilution followed by growth for 1 h. Next, 200 μM U-50,488 (Sigma) was added, and growth continued for 10 h. Pyocyanin was extracted and measured as described above.

Caenorhabditis elegans Killing Assays—*C. elegans* N2 nematodes provided by the *Caenorhabditis* Genetic Center (CGC), University of Minnesota, were used in these experiments. Synchronization and prefeeding of worms was performed by transferring them onto plain plates with kanamycin as previously described.³ *P. aeruginosa* MPAO1-P1 was grown overnight in tryptone/yeast extract medium (TY, tryptone, 10 g/L; yeast extract, 5 g/L) and diluted at 1:100 in 0.1 $\times$ TY (TY diluted 10-fold with water). Potassium phosphate buffer, pH 6.0, was included in the 0.1 $\times$ TY to a final concentration of 0.1 mM. After 1 h of growth, the kappa-opioid receptor agonist U-50,488 was added to a final concentration of 50 μM followed by 2 h growth as previously described.^{18,19} Two milliliters of the microbial culture was adjusted to room temperature and poured into 30 mm diameter dishes into which prefeeding nematodes (10 nematodes per plate) were transferred. *P. aeruginosa* grown overnight in TY was diluted at 1:100 in either 0.1 $\times$ TY or 0.1 $\times$ TY containing polymers at 2 mM (or 5% in selected experiments as indicated) final concentrations and adjusted to pH 5.2 with KOH. Plates were incubated at RT, without shaking, and mortality was defined if worms did not respond to the touch of a platinum picker.

In all experiments (unless specific ones, when 5 wt % concentration was used), the polymers were applied at 2 mM concentrations. We decided to use molar concentration (mM) to indicate polymer concentrations so that all polymers would be at the same concentrations for easy comparison. Obviously, displaying these units results in nonequal phosphate concentrations among the polymers such that higher molecular weight polymers deliver more phosphate. On the other hand, the use of weight concentration would result in equal phosphate concentrations among the polymers but would display differences in polymer

molar concentrations. Because all polymers at 2 mM contain high phosphate concentrations with the lowest of 20 mM in ABA-PEG10k-Pi10, we decided to use mM concentrations in our comparative studies. In pilot experiments, we performed dose–response runs and demonstrated that the 2 mM concentration was the most effective (data not shown), and therefore, this concentration was chosen for all experimental runs.

Statistical Analyses

All data are from 3 or more replicates and presented as the mean with standard deviation presented as error bars. Statistical analysis was performed using SigmaPlot software. In *C. elegans* experiments, the log-rank (Mantel–Cox) test (GraphPad Prism 7) was used with significance accepted as a *p*-value < 0.05. In in vitro experiments, Student *t*-tests were used and significance was determined to be a *p*-value < 0.05.

Scanning Electron Microscopy (SEM)

P. aeruginosa MPAO1 was grown in tryptic soy broth (TSB) overnight. Overnight cultures (2 mL) were centrifuged at 6,000 rpm for 5 min at RT, and pellets were gently washed three times with DCM-Pi0.1 (see PstS expression section). Washed pellets were suspended in 1 mL of DCM-Pi0.1, 2 mM ABA-PEG20k-Pi20, or 2 mM PEG20k-Pi20. ABA-PEG20k-Pi20 and PEG20k-Pi20 solutions were prepared in DCM-Pi0.1, and pH was adjusted by KOH to DCM-Pi0.1. Bacteria were grown for 4 h, and then cells were pelleted by centrifugation at 6,000 rpm for 5 min at RT and gently washed three times with phosphate buffered saline (PBS). Bacterial cells were then dropped onto glass coverslips coated with poly-L-lysine. Cells were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.2, washed with 0.1 M phosphate buffer, and dehydrated in a graded ethanol solution in water (30% increased gradually to 100%; 20 min each). The samples were dried with a Leica CPD300 critical point dryer and coated with Pt(80)/Pd(20) of 2 nm thickness using a Cressington sputter coater, model 208HR. SEM images were obtained using a Zeiss Merlin FE-SEM with an accelerating voltage of 1 kV and a working distance of 3 mm.

RESULTS AND DISCUSSION

Design and Synthesis of Phosphorylated PEG-Based Block Copolymer with a Hydrophobic Core (ABA-PEG-Pi)

The purpose of this study was to develop phosphate-containing PEG-based block copolymers with a defined ABA structure and molecular weight and identify their effectiveness for suppress microbial virulence using biological tests. In our previous work,³ a phosphorylated product from commercially available polymer PEG15-20 (Pi-PEG15-20) was employed and proven to work effectively in preventing lethal gut-derived sepsis. In these studies, we learned that the ABA structure and phosphate were critical determinants for the biologic function of Pi-PEG15-20. However, molecular weight measurements (Figure S1) indicated that both Pi-PEG15-20 and its precursor PEG15-20 were polydisperse, i.e., PEG15-20 contained component A, 16% of block copolymer with ABA structure, and component B, 84% of block copolymer with an AB structure and a PEG homopolymer. The phosphorylated homopolymer failed to show a protective effect in biological tests,³ and separating it from the original polydisperse mixture to refine the active component proved

implausible because it has an almost identical molecular weight to that of the block copolymer with an AB structure and because they are both water soluble. As such, this complex composition presented challenges for determining the mechanism of protection of each component. Therefore, a rational design of an alternative PEG with uniform composition and similar structure to the active components in Pi-PEG15-20 was required to achieve both key features of the ABA structure and controllable phosphate content.

PEG chains contain only one or two terminal hydroxyl groups suitable for further functionalization. To incorporate more hydroxyl groups per polymer chain, sequential anionic copolymerization of ethylene oxide (EO) with a functional epoxide monomer, ethoxy ethyl glycidyl ether (EEGE), an ethoxyl ethylacetal-protected glycidol was used to acquire block copolymers with poly(ethylene oxide) as backbone along with controllable hydroxyl groups.^{20–22} As depicted in Scheme 1, the design of ABA-PEG-Pi involves the initial synthesis of symmetric block copolymer ABA-PEG-PEEGE from Bisphenol A followed by deprotection of the PEEGE block to recover the pendant hydroxyl groups, and the subsequent functionalization of all the hydroxyl groups of the block copolymer with phosphate.

This strategy allowed access to a series of block copolymers with defined ABA architecture, which consisted of three distinctive segments: (i) The B group represents the small yet very hydrophobic bis-phenol A moiety at the polymer center. (ii) PEG blocks adjacent to the biaromatic center formed the inert spacer and the inner part of hydrophilic A groups. As an integral part of the architecture, the chain length of the PEG block played a key role in the hydrophobicity/hydrophilicity balance of the whole polymer. (iii) Phosphorylated polyglycidol block acts as the outer part of hydrophilic A groups, offering biological functionality and defined phosphate content. Three ABA-PEG-Pis, ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20, were synthesized in this study. The 10k, 16k, and 20k correspond to the different molecular weights of the PEG block. By incorporating repeating units of phosphate accordingly (from 10 to 14 to 20, respectively), an almost identical molar concentration of phosphate can be maintained for each block copolymer (e.g., for 1 g of each block copolymer, the molar concentrations of phosphate were 0.78, 0.77, and 0.80 mmol, respectively, for ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20).

Initiated from potassium bis-phenoxide, the sequential anionic ring-opening polymerization of EO and EEGE was successful. This can be confirmed by the chemical shifts seen in ¹H NMR spectra (Figure 1A): a (δ = 1.60, 6H) and b (δ = 6.78, 7.09, 8H) are assigned to the dimethyl and aromatic groups of BPA, c ~ h (δ = 3.43–3.80) can be assigned to protons of the main chain and lateral chains; i (δ = 4.70, 1H), j (δ = 1.29, 3H), and k (δ = 1.19, 3H) are ascribed to the methyl protons of the EEGE moiety. Furthermore, the chain length of the PEG and PEEGE blocks could be varied by adjusting the feed ratio of EO and EEGE monomer to the initiator BPA, and the composition of the block copolymer can be determined by the integrals of specific signals from each block in the ¹H NMR spectra. The degree of polymerization of the PEEGE block (N_{EEGE}) can be calculated by the integration ratio

$$N_{\text{EEGE}} = 8 \frac{I_i}{I_b}$$

where I_i and I_b are the integration of peaks i and b in Figure 1, respectively.

The degree of polymerization of the PEG block (N_{EG}) was given by

$$N_{\text{EG}} = 2 \frac{I_{c \sim h} - 7N_{\text{EEGE}}I_i}{I_b}$$

where $I_{c \sim h}$ is the integration of peak $c \sim h$ in Figure 1. Detailed molecular weight characterization results for all of the samples are summarized in Table 1.

EEGE was chosen to be the outer block due to the advantages that (i) it has a similar main chain with PEG and can be copolymerized with EO through an anionic mechanism, (ii) this structural similarity also suggests that PEG-PEEGE should be nontoxic and safe, which is important when further developing ABA-PEG-PEEGE for biomedical applications, and (iii) the protective ethoxy ethylacetal groups can be easily removed by acidic hydrolysis, yielding a pendant hydroxyl group in each repeating unit, offering perfect functionalization sites for phosphorylation. Complete hydrolysis could be verified by the disappearance of specific EEGE signals i , j , and k comparing Figure 1A and B. Finally, phosphorylation was performed by the reaction between ABA-PEG-PGly samples with phosphorus oxychloride, which was shown to be highly effective in our previous work.³ The existence of phosphate in ABA-PEG-Pi samples can be verified by the chemical shift $\delta = -0.18$ ppm in the ^{31}P NMR spectrum (Figure 1D). The number-average molecular weights of ABA-PEG-Pi measured by GPC for ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20 were 12.9k, 18.7k, and 25.8k, respectively, and corresponded well with those determined from NMR results also shown in Table 1 (12.8k, 18.2k, and 25.0k, respectively), which implied that the degree of functionalization of the available hydroxyl groups was complete.

To further identify the degree of phosphorylation, phosphoric acid titration experiments were performed to identify the average number of phosphate groups per polymer chain. Briefly, 0.1 M of sodium hydroxide (NaOH) solution was titrated into the ABA-PEG-Pi/PEG-Pi solution, and the pH changes were monitored using a pH meter with automatic temperature compensation. Figure 1E shows a typical titration curve: the pH value of the solution increased with the gradual addition of NaOH (left axis), and two buffer regions (gray column areas) were observed; after simply taking the first derivation (right axis), two equivalence points were clearly visualized. It shows the characteristic behavior of a diprotic acid, and the relatively broader peaks during the buffer region implied the behavior of poly(phosphoric acid) as well. These results are in accordance with the structure of phosphoric acid units on the polymer chain. The average number of phosphate groups per polymer chain $N_{\text{phosphate}}$ can be calculated by the equation

$$N_{\text{phosphate}} = \frac{[\text{NaOH}]V_1}{m/M_n} \text{ or } N_{\text{phosphate}} = \frac{[\text{NaOH}]V_2}{2m/M_n}$$

where [NaOH] is the concentration of sodium hydroxide solution, V_1 and V_2 are the volumes of sodium hydroxide solution consumed at first titration end and second titration end, respectively, m is the mass of ABA-PEG-Pi polymer used in the titration, and M_n is the number-average molecular weight of the ABA-PEG-Pi polymer. Theoretically, the volume of NaOH solution consumed at second titration end (V_2) should be twice that at the first titration end (V_1); for our case, V_2 is a little bit lower than $2V_1$, which may be due to the dissociation constant difference between the phosphoric acid units at the chain ends and those far from the chain ends.

Through this method, the average numbers of phosphate groups per polymer chain $N_{\text{phosphate}}$ for ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20 are 9.8 ± 0.2 , 13.0 ± 0.8 , and 19.5 ± 0.5 , respectively. These results confirm our assumption that the degree of phosphorylation of the available hydroxyl groups was complete.

Synthesis of Phosphorylated PEG-Based Block Co-polymer without Hydrophobic Core (PEG-Pi)

For the structural importance of the hydrophobic moiety to be demonstrated, another phosphate-containing PEG-based block copolymer without a hydrophobic core, namely PEG-Pi, was also synthesized for comparison. The only structural difference between PEG-Pi and ABA-PEG-Pi is the center moiety (Figure 2): ethylene glycol for PEG-Pi and BPA for ABA-PEG-Pi. As depicted in Scheme S1, the synthesis of PEG-Pi started from ethylene glycol through the same sequential anionic ring-opening polymerization of EO and EEGL, hydrolysis and phosphorylation, produced the final product PEG-Pi. Only PEG20k-Pi20 was synthesized in this study. As with ABA-PEG20k-Pi20, 20k corresponded to the designed molecular weight of the PEG block, and 20 was the designed repeating units of phosphate incorporated on each chain. GPC elution curve analyses demonstrated the uniform composition in the synthesized polymers (Figure S2). The polymerization, hydrolysis, and phosphorylation were similarly monitored by NMR spectroscopy. The spectra are shown in Figure S3. a ~ g ($\delta = 3.43\text{--}3.80$) can be ascribed to protons of the main chain and lateral chains; h ($\delta = 4.70$, 1H), i ($\delta = 1.29$, 3H), and j ($\delta = 1.19$, 3H) are assigned to the methyl protons of the EEGL moiety. The disappearance of signals h, i, and j confirmed the completion of hydrolysis, and the chemical shift $\delta = -0.17$ ppm in the ^{31}P NMR spectrum verified the existence of phosphate in PEG20k-Pi20. The average number of phosphate groups per polymer chain $N_{\text{phosphate}}$ for PEG20k-Pi20 calculated from the phosphoric acid titration experiment is 19.8 ± 0.3 , indicating nearly 100% phosphorylation. The number-average molecular weights measured by GPC for PEG20k-E18, PEG20k-G20, and PEG20k-Pi20 were 36.5k, 31.6k, and 26.2k, respectively, which are very close to those values of ABA-PEG20k-Pi20, making PEG20k-Pi20 an excellent analogue to ABA-PEG20k-Pi20. Detailed molecular weight characterization results, GPC elution curves, and NMR spectra are summarized in Table S1 and Figures S2 and S3.

It is also important to note that, due to the use of a living anionic polymerization technique, the dispersity () of all these PEG-based block copolymers were kept narrow (<1.10), and significant broadening of the corresponding GPC traces was not observed even after deprotection and phosphorylation (Figure S2), indicating excellent control over molecular weight, architecture, and the number of phosphate units that were desired for biological tests.

ABA-PEG-Pis Inhibit Phosphate Signaling in *P. aeruginosa* under Phosphate Limited Conditions

Multiple biological tests were performed to assess the functionality of the synthesized polymers as antivirulence compounds. Expression of the phosphate transport protein PstS in *P. aeruginosa* was used as a biomarker to determine phosphate availability of the various polymers. If PstS expression was increased, it served as a proxy, indicating that extracellular phosphate was depleted and unavailable within the phosphorylated compound. On the other hand, if PstS was observed to be decreased, it indicated that *P. aeruginosa* detected sufficient phosphate availability in the test compound. PstS is the phosphate-binding component of the ABC-type transporter complex pstSACB involved in phosphate transport into the bacterial cytoplasm. PstS is known to be induced by phosphate limitations and suppressed in a phosphate-rich extracellular environment. To track the expression of PstS, we first constructed the pSensor-PstS-EGFP plasmid (see Material and Methods) that was electroporated in the *P. aeruginosa* MPAO1-P1 strain to obtain the MPAO1-P1/pstS-EGFP reporter strain. The expression of PstS was detected by fluorescence (excitation 485/10, emission 528/20) normalized to cell density measured by the absorbance at 600 nm. As a control, PstS expression in *P. aeruginosa* grown in low phosphate and high phosphate defined citrated media (DCM) was used. Data indicated, as expected, that PstS expression was increased in low phosphate medium and was nearly completely suppressed in medium containing 25 mM of inorganic phosphate. All three phosphorylated polymers (ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20, 2 mM) (Figure 3A) suppressed PstS expression, suggesting that Pi was available for bacteria. In contrast, the nonphosphorylated parent polymers ABA-PEG10k-G10, ABA-PEG16k-G14, and ABA-PEG20k-G20 did not suppress PstS expression, demonstrating that there was no effect of the nascent ABA structure on the PstS expression via some type of nonspecific interaction (Figure 3A). We then performed reiterative experiments in which we used the PvdD/pstS-EGFP strain, a pyoverdine-deficient mutant derivative of MPAO1-P1 harboring pSensor-PstS-EGFP. By using this mutant, we verified that the decrease in fluorescence observed with ABA-PEG-Pis was indeed attributable to decreased PstS expression and not to the production of pyoverdine, a fluorescent compound that is also produced in this medium.²³ The pattern of PstS expression in PvdD/pstS-EGFP was similar to that observed with the MPAO1-P1/pstS-EGFP (Figure 3B). These data demonstrate that phosphorylated polymers suppress the main signal indicating phosphate limitation, i.e., PstS expression. Phosphorylated polymers did not inhibit bacterial growth (Figure S4), which is in agreement with our previous data.³

ABA-PEG-Pis Significantly Decrease Pyocyanin Production by *P. aeruginosa* under Phosphate Limited Conditions and during Exposure to Opioids

One of the most distinguishing features of strains of *P. aeruginosa* is their production of pyocyanin, a water-soluble blue-green phenazine compound. Pyocyanin is one of the major toxins of *P. aeruginosa* that induces rapid apoptosis of human neutrophils and thus defines the virulence of this highly lethal opportunistic pathogen. The production of pyocyanin is controlled by the quorum sensing system (QS), a central virulence circuit in *P. aeruginosa* and other pathogens. The PstS-PhoB phosphate regulon, a two component membrane regulator, is activated during phosphate limitation and is involved in the transcriptional activation of QS. Thus, enrichment of media with phosphate leads to suppression of pyocyanin production.^{23,24} Therefore, we next tested if ABA-PEG-Pi can suppress pyocyanin production in *P. aeruginosa* in phosphate-limited medium using DCM-Pi0.1. In this set of experiments, we used an MPAO1-P2 strain that produces a higher amount of pyocyanin compared to that of the MPAO1-P1 strain.¹⁶ In our preliminary experiments, we found that supplementation of media with iron increases pyocyanin production in this nutrient-limited DCM media. Therefore, we supplemented DCM with 2 μM Fe^{3+} (1 μM $\text{Fe}_2(\text{SO}_4)_3$). Results demonstrated that both ABA-PEG10-Pi10 and ABA-PEG20-Pi20, 2 mM, significantly decreased pyocyanin production in *P. aeruginosa* MPAO1-P2 (Figure 4A). The effect of non-phosphorylated compounds was significantly lower.

Previous work from our lab demonstrated that endogenous opioid compounds are released into the intestine during physiologic stress and induce pyocyanin production via the quorum sensing (QS) system of virulence activation.^{17,25} We established that the MPAO1-P1 strain is highly responsive to the synthetic kappa-opioid U-50,488 in terms of pyocyanin production.¹⁷ Consistent with our previous results, we again demonstrated that pyocyanin production was significantly increased in MPAO1-P1 when exposed to 200 μM of the kappa-opioid receptor agonist U-50,488 (Figure 4B). All three ABA-PEG-Pis polymers at 2 mM reduced pyocyanin at the expected background level with ABA-PEG20k-Pi20 being the most effective. The paired molecular weight nonphosphorylated polymers were less effective in these experiments, again suggesting that the polymers phosphate content is important for its suppressive effect on pyocyanin production.

ABA-PEG-Pis Attenuate Animal Mortality Caused by *P. aeruginosa* Exposed to Opioids

In our previous work, we developed two small animal models (i.e., *Caenorhabditis elegans* and mice) to create local phosphate depletion at sites of colonization of *P. aeruginosa* and validated the fidelity between these models.^{23,26} Therefore, in current work we have used the *C. elegans* model in which the opioid-induced lethality of *P. aeruginosa* can be suppressed by the delivery of inorganic phosphate.¹⁸ To test the in vivo efficacy of the de novo synthesis of Pi-PEG compounds, we created conditions of both opioid exposure and phosphate limitation. Results indicated that all three ABA-PEG-Pi polymers, at equal concentrations of 2 mM, effectively decreased *C. elegans* mortality (Figure 5) with the ABA-PEG20k-Pi20 displaying the greatest degree of protection. Because ABA-PEG20k-Pi20 carries the highest phosphate at equal molarity, to verify that the protective effect is not dependent on phosphate concentration, we performed reiterative experiments comparing ABA-PEG10k-Pi10 to ABA-PEG20k-Pi20 at a concentration of 5 wt %. At the same weight

concentration, ABA-PEG20k-Pi20 and ABA-PEG10k-Pi10 contain nearly equal quantities of phosphate. Results demonstrated that ABA-PEG20k-Pi20 still exhibited a significantly higher protective effect compared to that of ABA-PEG10k-Pi10 (Figure S5), suggesting that the higher molecular weight may influence the greater protective effect of ABA-PEG20k-Pi20.

The Hydrophobic Core BPA in ABA-PEG20k-Pi20 Significantly Contributes to Bacterial Coating and Its in Vivo Protection against Lethality

To confirm our hypothesis, that it is the unique ABA structure of ABA-PEG20k-Pi20 that plays a significant role in its protective capacity, we synthesized PEG20k-Pi20. This polymer has a similar structure to ABA-PEG20k-Pi20 but lacks the hydrophobic core (Figure 2). Dynamic light scattering (DLS) measurements of PEG20k-Pi20 and ABA-PEG20k-Pi20 in water solution showed that the mean hydrodynamic radius (R_h) of PEG20k-Pi20 is 7.2 nm, indicating a single-chain conformation in water, although ABA-PEG20k-Pi20 was able to form aggregates of ~50 nm (Figure S6).

Analysis from biological tests (Figure 6A) showed that both phosphorylated polymers suppressed PstS expression to the same degree, demonstrating that both can serve as phosphate delivery molecules. However, in *C. elegans* experiments, the protective effect of amphiphilic ABA-PEG20k-Pi20 is significantly greater compared to that of the hydrophilic PEG20k-Pi20 molecule (Figure 6B). Because we have previously demonstrated that ABA-PEGs may adhere to and shield the bacterial surface, we next tested whether the coating capacities of ABA-PEG20k-Pi20 and PEG20k-Pi20 are different. This was performed using scanning electron microscopy (SEM) on *P. aeruginosa*. Bacteria were cultured in different media for several hours; then immediately after washing with buffer solution, they were dried in a critical point dryer and coated with Pt/Pd, and then images were taken. Panels C–E in Figure 6 display images of *P. aeruginosa* cultured in phosphate-limited (DCM Pi-0.1 mM) media only, cultured in DCM Pi-0.1 mM containing 2 mM PEG20k-Pi20 and cultured in DCM Pi-0.1 mM containing 2 mM ABA-PEG20k-Pi20, respectively. SEM images showed pili-like filaments in Figure 6C and D (shown by arrows), and in Figure 6E, pili-like filaments disappeared when bacteria were coincubated in the presence of ABA-PEG20k-Pi20. These findings suggest that motility appendages, key structures involved in virulence, are influenced by the composition of the two compounds.²⁷ Intriguingly, in the presence of ABA-PEG20k-Pi20, the surface of bacterial cells displayed a distinct rugged appearance. Although speculative, it is possible that the hydrophobic linkage BPA acts as an anchor inserting itself into the alkyl chain region of the bacterial membrane, thus firmly attaching the ABA-PEG20k-Pi20 polymer to the bacterial cell surface. In this way, amphiphilic block copolymers like ABA-PEG20k-Pi20 may be more advantageous as bacterial surface coating agents and hence more protective in vivo. Coating by ABA-PEG20k-Pi20 will create an envelope enriched by the phosphate-conjugated polymer. Additional work is needed to clarify whether the release of inorganic phosphate by bacterial phosphatases from the polymer or the phosphate-conjugated polymer itself can suppress low phosphate signaling. Further biophysical experiments are in progress to detail the interactions between the phosphorylated block copolymer and bacterial membranes.

CONCLUSIONS

Linear phosphorylated block copolymers with a defined ABA structure were synthesized de novo, and their antivirulence activity was verified by biological analyses using *P. aeruginosa* as a test pathogen. Results indicated that all phosphorylated polymers prevented phosphate signaling in *P. aeruginosa*, confirming that they can serve as phosphate-delivering molecules. In vivo, using the *C. elegans* killing assay, ABA-PEG20k-Pi20 appeared to be the most protective compound when compared to ABA-PEG-Pis with a lower molecular weight and PEG-Pi polymers without the hydrophobic core. Scanning electron microscopy analysis demonstrated that, in the presence of the ABA-PEG20k-Pi20 structure, bacterial cell surfaces displayed distinct characteristics, a finding that may explain its enhanced activity in vivo. Through the ability to vary the hydrophobic moiety and the length of the PEG spacer while controlling the functionalization of the outer block, synthesis of this ABA triblock copolymer could represent a versatile platform for antivirulence applications of this design against highly lethal and drug-resistant pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

¹H NMR spectra of (A) ABA-PEG20k-E18, (B) ABA-PEG20k-G20, and (C) ABA-PEG20k-Pi20; (D) ³¹P NMR spectrum of ABA-PEG20k-Pi20, and (E) titration curve of ABA-PEG20k-Pi20 with NaOH solution. 20k is the designed molecular weight of the PEG block; E18 means the designed repeating units of the EEGE block is 18. G20 means the designed repeating units of glycerol is 20; Pi20 means the designed repeating units of the phosphorylated glycerol block is 20.

Figure 2. Comparison of the chemical structure of phosphate-containing PEG-based block copolymers: ABA-PEG-Pi with the hydrophobic core BPA and PEG-Pi without BPA.

Figure 3.

ABA-PEG-Pis significantly decrease PstS expression in *P. aeruginosa*. PstS expression in MPAO1/pstS-EGFP (A) and Δ PvdD/pstS-EGFP (B). $n = 3$ per group, $*p < 0.01$. Columns represent average values, and error bars represent standard deviations. DCM-Pi0.1 indicates citrate medium containing limited quantities (0.1 mM) of inorganic phosphate; DCM-Pi25 indicates citrate medium containing sufficient quantities (25 mM) of inorganic phosphate. All polymers were used in 2 mM concentration that provides 20, 28, and 40 mM phosphate on ABA-PEG10k-Pi10, ABA-PEG16k-Pi14, and ABA-PEG20k-Pi20, respectively.

Figure 4.

ABA-PEG-Pis significantly decrease pyocyanin production in *P. aeruginosa*. (A) Production of pyocyanin in *P. aeruginosa* MAPO1-P2 grown in phosphate/iron-limited medium DCM-Pi0.1, phosphate-limited medium DCM-Pi0.1 + Fe³⁺, 2 μM, and phosphate-limited/iron-enriched media supplemented with 2 mM phosphorylated and nonphosphorylated polymers. (B) Production of pyocyanin in MPAO1-P1 in TSB supplemented with 0.2 mM U-50,488 in the presence or absence of phosphorylated and nonphosphorylated polymers. *n* = 3 per group, **p* < 0.01. Columns represent average values, and error bars represent standard deviations.

Figure 5.

Effect of each of the three phosphorylated ABA polymers on *C. elegans* survival. Experiments are performed on *C. elegans* nematodes feeding on *P. aeruginosa* in low nutrient media (0.1× TY) and exposed to opioids (U50,488) as a provocative agent known to enhance *P. aeruginosa* virulence. Kaplan–Meyer survival curves demonstrate a statistically significant ($p < 0.05$) protective effect of all three polymers at 2 mM concentration when compared to that of the no treatment group. Results indicate that ABA-PEG20k-Pi20 confers a superior protective effect compared to those of the remaining polymers ($n = 10$ worms/plate (treatment group) with 3 independent runs per group).

Figure 6.

The hydrophobic core BPA in ABA-PEG20k-Pi20 significantly contributes to the bacterial coating and its in vivo protection against lethality. (A) PstS expression. $n = 3/\text{group}$, $*p < 0.001$. (B) *C. elegans* survival. $n = 60/\text{group}$, $p < 0.0001$ between groups (log-rank (Mantel–Cox) test). (C–E) Scanning electron microscopy images of *P. aeruginosa* cultured in different media. The bacteria were first cultured in different media for several hours; then immediately after washing with buffer solution, they were dried in a critical point dryer and coated with Pt/Pd, and images were taken. Arrows indicate pili-like filaments (C) cultured in phosphate-limited (DCM Pi-0.1 mM) media only, (D) cultured in DCM Pi-0.1 mM containing 2 mM PEG20k-Pi20, and (E) cultured in DCM Pi-0.1 mM containing 2 mM ABA-PEG20k-Pi20.

Scheme 1.
Synthetic Strategy of ABA-PEG-Pi Block Copolymers

Table 1

Molecular Characterization of the PEG-Based Block Copolymer with a Hydrophobic Core^a

	M_n^b (kDa) GPC	M_n (kDa) NMR	c	N_{EEGE}^d	$N_{hydroxyl}^e$	$N_{phosphate}^f$
ABA-PEG10k-E8	26.1	12.4	1.05	8.0		
ABA-PEG-PEEGE						
ABA-PEG16k-E12	31.7	17.8	1.07	11.6		
ABA-PEG20k-E 18	35.9	24.6	1.04	17.5		
ABA-PEG10k-G10	23.2	11.9	1.06		10.0	
ABA-PEG-PEGly						
ABA-PEG16k-G14	27.8	17.1	1.06		13.6	
ABA-PEG20k-G20	31.1	23.3	1.05		19.5	
ABA-PEG10k-Pi10	12.9	12.8	1.10			9.8 ± 0.2
ABA-PEG16k-Pi14	18.7	18.2	1.08			13.0 ± 0.8
ABA-PEG20k-Pi20	25.8	25.0	1.07			19.5 ± 0.5

^aNomenclature of the polymers: Take ABA-PEG10k-E8/ABA-PEG10k-G10/ABA-PEG10k-Pi10 as examples, 10k is the designed molecular weight of the PEG block, E8 means the designed repeating units of the EEGE block is 8, and G10 means the designed repeating units of glycerol is 10. Because hydrolysis of the EEGE block releases eight alcohol groups plus two primary alcohol groups at the chain ends, making it 10 repeating units for glycerol; thus, Pi10 indicates that the designed repeating units of the phosphorylated glycerol block is 10. Other polymers can be deduced in the same manner.

^b ABA-PEG-PEEGE and ABA-PEG-PEGly samples were measured in THF against PS standards; ABA-PEG-Pi samples were measured in 0.1 M NaNO₃ against PEO standards.

^c Measured by GPC.

^d Calculated from NMR.

^e $N_{hydroxyl} = N_{EEGE} + \text{two primary alcohol groups at chain ends}$; NMR confirmed the completion of the deprotection.

^f $N_{phosphate}$ of ABA-PEG-Pi samples was determined by phosphoric acid titration experiments.