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De Novo Synthesis and Functional Analysis of Polyphosphate-Loaded Poly(Ethylene) Glycol Hydrogel Nanoparticles Targeting Pyocyanin and Pyoverdin Production in *Pseudomonas aeruginosa* as a Model Intestinal Pathogen

Yushu Yin¹, Georgia Papavasiliou², Olga Y. Zaborina³, John C. Alverdy³, and Fouad Teymour¹

¹Department of Chemical and Biological Engineering, Illinois Institute of Technology, Chicago, IL, USA

²Department of Biomedical Engineering, Illinois Institute of Technology, Chicago, IL, USA

³Department of Surgery, University of Chicago Medical Center, Chicago, IL, USA

Abstract

The human gastrointestinal tract is the primary site of colonization of multidrug resistant pathogens and the major source of life-threatening complications in critically ill and immunocompromised patients. Eradication measures using antibiotics carry further risk of antibiotic resistance. Furthermore, antibiotic treatment can adversely shift the intestinal microbiome toward domination by resistant pathogens. Therefore, approaches directed to prevent replacement of health promoting microbiota with resistant pathogens should be developed. The use of non-microbicidal drugs to create microenvironmental conditions that suppress virulence of pathogens is an attractive strategy to minimize the negative consequences of intestinal microbiome disruption. We have previously shown that phosphate is depleted in the intestinal tract following surgical injury, that this depletion is a major “cue” that triggers bacterial virulence, and that the maintenance of phosphate abundance prevents virulence expression. However, the use of inorganic phosphate may not be a suitable agent to deliver to the site of the host-pathogen interaction since it is readily adsorbed in small intestine. Here we propose a novel drug delivery approach that exploits the use of nanoparticles that allow for prolonged release of phosphates. We have synthesized phosphate (Pi) and polyphosphate (PPi) crosslinked poly (ethylene) glycol (PEG) hydrogel nanoparticles (NP-Pi and NP-PPi, respectively) that result in sustained delivery of Pi and PPi. NP-PPi demonstrated more prolonged release of PPi as compared to the release of Pi from NP-Pi. *In vitro* studies indicate that free PPi as well NP-PPi are effective compounds for suppressing pyoverdin and pyocyanin production, two global virulence systems of virulence of *P. aeruginosa*. These studies suggest that sustained release of polyphosphate from NP-PPi can be exploited as a target for virulence suppression of lethal pathogenic phenotypes in the gastrointestinal tract.

Address correspondence to John C. Alverdy, Department of Surgery, University of Chicago Medical Center, Chicago, IL, USA., jalverdy@surgery.bsdc.uchicago.edu, teymour@iit.edu.

Keywords

Gut-derived sepsis; Phosphate depletion; *P. aeruginosa*; Virulence; Polyphosphate-nanoparticles; Drug delivery

INTRODUCTION

Conditions such as critical illness, immunodeficiency, and medical interventions to treat human disease lead to destabilizing effects on the normal intestinal microbiota. In these situations, the gastrointestinal tract becomes a hostile and nutrient scarce environment which activates colonized hospital pathogens to express virulent phenotypes. To date, the most common approach to address this issue involves the use of oral antibiotics to eliminate pathogens in the gut.² This routine practice, however, often leads to further complications as normal intestinal microbiota are replaced by multi-drug resistant and potentially virulent health-care associated pathogens.^{3, 9, 12–14, 19, 21}

Among the various intestinal environmental cues that activate the virulence of intestinal pathogens is the local concentration of extracellular phosphate.¹⁰ Critical illness and host stress redistribute body stores of phosphate *via* phosphatonin^{6, 18} leading to increases in intestinal phosphate absorption and excretion in the urine. As a result of depletion of extracellular phosphate levels, pathogens must scavenge phosphate from host tissues leading to the disruption of intestinal epithelium and gut-derived sepsis. In previous *in vivo* studies involving mice subjected to surgical injury we have shown that intestinal mucus phosphate concentration becomes significantly depleted to levels that activate virulence in intestinal *P. aeruginosa*.¹⁰ Furthermore, significant mortality was observed in mice subjected to operative injury and intestinally inoculated with *P. aeruginosa*, while mortality was prevented in identically treated mice given oral supplementation of inorganic phosphate, but not when phosphate was administered systemically. Further *in vitro* studies using a *C. elegans* model identified three global virulence systems involved in the expression of *P. aeruginosa* virulence under phosphate depleted conditions such as pyocyanin, pyoverdine and biofilm formation, each of which was attenuated under phosphate abundant conditions.²³ This suggests that therapeutic strategies that allow for targeted delivery of phosphates to the intestinal epithelium, while allowing commensal bacterial to proliferate normally, have a major advantage in addressing bacterial virulence and its complications without the risk of the emergence of resistance and disruption of the intestinal microbiome. One of the disadvantages of oral administration of inorganic phosphate *in vivo* is due to its absorption in the small intestine. Due to this reason, a high concentration of orally administered phosphate is required to increase phosphate concentration in the colon; however, high concentrations of inorganic phosphate can affect kidney function. To overcome these issues we have focused on the synthesis of phosphate-delivering compounds. In our previous work, we have phosphorylated a commercially available high-molecular-weight polyethylene glycol block copolymer (PEG 15–20) which was shown to prevent the expression of virulence pathways in a manner similar to inorganic phosphate.^{5, 8, 17, 23, 24}

In this work we propose an alternative drug delivery approach through the utilization of crosslinked poly(ethylene) glycol (PEG) hydrogel nanoparticles loaded with phosphates designed to enable their sustained release to replenish depleted phosphate levels in the intestinal epithelium. PEG-based materials have been extensively used for *in vivo* drug delivery and biomedical applications as they are hydrophilic, biocompatible and prevent non-specific protein adsorption and cellular adhesion. Furthermore, PEGylation of synthetic nanoparticles has been widely utilized to prevent non-specific cell and protein nanomaterial interactions as well as opsonization on nanomaterial surfaces.^{4, 22} The proposed hydrogel nanoparticles are entirely composed of a PEG crosslinked structure whose network crosslink density and swellability can be readily tuned to alter the release and duration of therapeutic factors. The nanoparticles are formed using an inverse phase miniemulsion polymerization process we have previously designed and optimized for nanoparticle synthesis.²⁰ Inverse miniemulsions are a specialized class of emulsions that are stabilized against coalescence by a surfactant and Ostwald ripening by a lipophobe (typically an ionic salt). In the present application, phosphate or polyphosphate was used as the lipophobe. This allowed for physical phosphate entrapment within and subsequent release from the hydrogel nanoparticles. The rationale for delivering polyphosphate, specifically sodium hexametaphosphate, is twofold: (1) similar to inorganic phosphate, it plays a key mechanistic role in survival, metabolism and virulence across a broad range of bacteria¹⁶ and (2) as the data demonstrate, due to its size and physical characteristics it allows for slower and prolonged phosphate release from the nanoparticles as compared to monophosphate. The goals of this study were to quantify the release kinetics of phosphate and polyphosphate from hydrogel nanoparticles and to determine the effectiveness of NP-Pi and NP-PPi in attenuating bacterial virulence *in vitro*. In this work *P. aeruginosa* was used as a model intestinal pathogen in order to assess the ability of phosphate and polyphosphate loaded nanoparticles to suppress two global virulent systems: pyocyanin and pyoverdine, both known to be suppressed by inorganic phosphate.

MATERIALS AND METHODS

Poly(ethylene glycol) diacrylate (PEGDA, MW = 575 Da), *N*-vinyl pyrrolidone (NVP), potassium monobasic phosphate (phosphate), sodium hexametaphosphate (polyphosphate, (NaPO₃)_n MW = 101.9618*n), potassium persulfate (KPS), sorbitan monooleate (SPAN80), PEG (20) sorbitan monolaurate (TWEEN20), cyclohexane, acetone, sodium citrate, ammonium sulfate, magnesium sulfate and tryptic soy broth (TSB) were purchased from Sigma-Aldrich. Agar was purchased from Fisher Scientific. Tryptone was purchased from EMD Millipore. Monomethyl ether hydroquinone (MEHQ) inhibitor (400–600 ppm) was removed from the PEGDA monomer by running the stock solution through a prepacked column (Sigma Aldrich). Sodium hydroxide inhibitor was removed from the NVP monomer by passing the stock solution through a 0.2 µm syringe filter. All other chemicals were used as received.

Synthesis of Phosphate and Polyphosphate PEG Hydrogel Nanoparticles by Inverse Phase Miniemulsion Polymerization

Hydrogel nanoparticles physically entrapped with phosphate (Pi) or polyphosphate (PPi) were formed using an inverse phase miniemulsion polymerization process we have previously developed and optimized for nanoparticle synthesis.²⁰ Two types of particles were prepared using this method and utilized for *in vitro* studies: nanoparticles containing monophosphate, potassium monobasic phosphate, (NP-Pi) and nanoparticles containing polyphosphate, sodium hexametaphosphate, (NP-PPi).

In the case of synthesis of NP-PPi, the dispersed aqueous phase consisted of a prepolymer solution composed of PEGDA as the crosslinking agent (282 mM), NVP (121 mM), PPi (50 mM) which was used as the lipophobe and virulence suppressing agent, TWEEN 20 (water soluble surfactant), and KPS (thermal initiator). The total concentration of reactive double bonds in the precursor was fixed at 0.685 M. The initiator concentration used was based on a 1.5% molar ratio of KPS to total reactive double bonds. First, the monomer solution was prepared by dissolving sodium hexametaphosphate, in DI water in a glass tube under an ice bath for 10 min. Subsequently, PEGDA was dissolved in DI water followed by the addition of NVP and PPi. This solution was vortexed followed by the addition of TWEEN 20 and DI water. Initiator stock solution (0.113 M KPS) was prepared by dissolving KPS in DI water. Complete dissolution of KPS was achieved after 5 min of vortexing at high speed. Monomer and initiator solutions were mixed just prior to the emulsification step as described below. The organic phase consisted of cyclohexane and SPAN 80, the latter used as an oil-soluble co-surfactant to enhance colloidal stability. The total surfactant concentration was set to 31.9 mg/mL and the SPAN 80 and TWEEN 20 concentrations were fixed such that the hydrophilic-lipophilic balance (HLB) of the combination was 5.0. A volume of 50 mL cyclohexane was added to SPAN 80 (8 g) and the mixture was homogenized using high energy sonication (SONICS Vibracell VCX750) for 20 s at 90% amplitude. This solution was added to 200 mL cyclohexane to form the organic phase. The inverse miniemulsion was generated using a two-step homogenization process. The organic phase was placed in an ice bath and allowed to cool to 10 °C and then homogenized using a rotor–stator homogenizer with a 10 mm saw-tooth generator (IPRO 250, Pro-Scientific) at 20,000 rpm. The precursor (consisting of both monomer and initiator solutions) was added dropwise to the organic phase under homogenization. Homogenization was continued for an additional 15 min under controlled temperature conditions (14–16 °C) to create the emulsion. The homogenized emulsion was then cooled to 10 °C in an ice bath under gentle stirring followed by exposure to high energy sonication using an ultrasonic horn (SONICS Vibracell VCX750). Ultrasonic homogenization was performed at 90% amplitude and in pulsed mode: one minute ON when the temperature between 13 and 5 °C followed by 30–34 s OFF for cooling down to 10 °C. A total of five such cycles were performed to generate a stable inverse miniemulsion.

The inverse miniemulsion was transferred to a glass batch reactor set-up we have previously designed to carry out the polymerization reaction.²⁰ The reaction proceeded for 4.5 h at 56 °C under a nitrogen blanket until a stable polymer nanoparticle suspension in cyclohexane was obtained. After the reaction was complete, hydrogel nanoparticles were precipitated from the colloidal suspension in 70–80 mL of acetone (approximately 1/3 of the

final suspension volume). Agglomerated particles were allowed to settle overnight at 4 °C after which two distinct layers were formed: a white creamy bottom layer and a clear yellowish supernatant. The supernatant was removed by decantation, followed by the addition of 120 mL of fresh acetone to the settled nanoparticles. The mixture was sonicated at 90% amplitude until all agglomerates were broken and complete re-suspension of the nanoparticles in acetone was achieved. The suspension was centrifuged at 3220×g at 4 °C for 90 min, followed by removal of the supernatant and addition of acetone (30 mL). Residual material was mixed by shaking and vortexing followed by a second cycle of rinsing *via* sonication and separation of particulate material by centrifugation. Rinsed particulate material was dried under vacuum for 48 h. Fully dried agglomerates were transferred to a mortar and crushed with a pestle into a fine white powder. Nanoparticles were stored dry at room temperature until use.

Synthesis of phosphate loaded nanoparticles (NPPi) was achieved using the same procedure as described above but with a total concentration of reactive double bonds of 1.36 M, 85 mM potassium monobasic phosphate (Pi), and a total surfactant concentration of 55 g/mL.

Nanoparticle Characterization

Nanoparticles (NPs) were characterized in terms of their particle size distribution, surface charge characteristics (ζ -potential), and physical properties (swelling ratio). Particle size distribution was measured using a nanosizer (NanoSight LM10, Malvern, UK) equipped with nanoparticle tracking analysis software (NanoTracking v 3.0). Dilute samples of NPs in cyclohexane were sonicated immediately prior to testing. Three 60 s videos were recorded and pooled to determine particle size distributions and nanoparticle diameter. Hydrogel swelling ratio is inversely related to crosslink density which impacts the release of phosphate and polyphosphate. The mass swelling ratio of hydrogel nanoparticles was determined by gravimetric analysis. Dried nanoparticles (200–250 mg) were swollen in 12 mL DI water until equilibrium swelling was achieved for 48 h. This solution was then centrifuged at 2000×g for 10 min followed by removal of the supernatant (DI water). Swollen nanoparticles were then dried under vacuum for 48 h at 40 °C. The mass swelling ratio was calculated from the ratio of the swollen to dry nanoparticle weights. The ζ -potential of hydrated nanoparticles (in DIW) was obtained using a zetasizer (Malvern ZetaSizer NanoZS, Worcestershire, UK). Nanoparticle solutions (1 mg/mL) were briefly sonicated and passed through a 0.8 μ m filter prior to loading samples on the zetasizer.

Phosphate and Polyphosphate Release Kinetics from Hydrogel Nanoparticles

Release kinetics of phosphate and polyphosphate from phosphate and polyphosphate loaded nanoparticles (NP-Pi and NP-PPI, respectively) were quantified under perfect sink conditions. In both cases the nanoparticles were suspended in DI water at a concentration of 5% (w/vol) NP-Pi and 1.25% (w/vol) NP-PPI. At pre-determined times the solution was centrifuged at 2000×g and 4 °C for 10 min to allow for nanoparticle settling. At this point the supernatant was removed and used to quantify the phosphate concentration of the released sample. The nanoparticles were then re-suspended in DI water and left unperturbed until the next sampling time. A phosphate detection test kit (Aquarium Pharmaceuticals), based on the molybdenum blue method, was used to quantify phosphate and polyphosphate

release kinetics from nanoparticles (NP-Pi and NP-PPI, respectively). A volume of the collected supernatant was diluted and added to the phosphate kit as per manufacturer's instructions and the released phosphate and polyphosphate concentrations determined *via* absorbance (SpectraMax 190 Microplate Reader). In the case of phosphate loaded nanoparticles (NP-Pi) the concentration of released monophosphate was obtained by measuring absorbance at 690 nm. Hexametaphosphate is known to hydrolyze above room temperature.¹ Since the compound is exposed to 55 °C for ~4.5 h during nanoparticle synthesis, we hypothesized that this process would result in hexametaphosphate hydrolysis. Analysis of the absorbance spectra of heat treated vs. pristine hexametaphosphate confirmed that the compound hydrolyzes upon exposure to 55 °C (refer to "Results and Discussion" section, Fig. 1). Therefore, release kinetics of hexametaphosphate as well as its hydrolysis products (monophosphate (Pi)), from NP-PPI were quantified *via* absorbance at 430 and 690 nm, respectively.

Functional Analysis of Pi, PPI, NP-Pi and NP-PPI in *P. aeruginosa*

P. aeruginosa MPAO1 from frozen stock were plated on tryptic soy broth agarized medium. Plates were incubated overnight at 37 °C and a few colonies transferred to 2 mL of TY medium containing 10 g/L tryptone (peptone from casein, pancreatic) and 5 g/L yeast extract. Cultures in TY were grown in suspension for 24 h at 37 °C under shaking (180 rpm) after which experiments were performed to quantify virulence and bacterial survival. To determine the ability of phosphate and polyphosphate nanoparticles to suppress bacterial virulence, *P. aeruginosa* MPAO1 were grown in low-phosphate (0.1 mM)/low-iron defined citrate medium (DCM, composed of 4 mg/mL sodium citrate, 1 mg/mL ammonium sulfate and 0.2 mg/mL magnesium sulfate) and supplemented with 0.1 mM potassium monobasic phosphate (Pi) and with varying concentrations of the following groups: free phosphate (Pi), free polyphosphate (PPI), NP-Pi, and NP-PPI. Table 1 presents the experimental groups tested *in vitro* in terms of nanoparticle concentration and the corresponding free phosphate and polyphosphate concentrations. The free polyphosphate concentrations (PPI) of 4.5, 2.25 and 1.12 mM correspond to 27, 13.5 and 6.72 mM of equivalent monophosphate (Pi), respectively. Briefly, suspensions of the nanoparticle formulations in DCM were added to 20 µL of *P. aeruginosa* in TY media in 15 mL sterile tubes at a 1:100 dilution. After overnight incubation at 37 °C at 180 rpm, pyoverdine production (indicative of the transition to virulence) was measured at 400 ± 10/460 ± 40 excitation/emission in black, clear bottom, 96-well plates using a fluorescent plate reader (Synergy HT, BioTek Inc.). Pyocyanin formation was assayed as previously described.²⁵ *P. aeruginosa* MPAO1 virulence was also provoked by exposure to synthetic opioid U-50,488. Under these conditions, MPAO1 strains produce an abundant amount of pyocyanin. Following a 24-h incubation in TSB (positive control), TSB + U-50,488 (negative control) and TSB + U-50,488 supplemented with NP-Pi, PPI and NP-PPI, bacterial cultures were spun down by centrifugation and 1 mL of supernatant was extracted using 500 µL of chloroform, then re-extracted with 150 µL of 0.2 M HCl. Absorbance was measured at an optical density (OD) of 520 nm. Bacterial survival was assessed after overnight incubation in the presence of all formulations tested by measuring colony forming units (CFU) as well as OD. All *in vitro* experiments were performed in triplicate.

Statistical Analyses

All data are from three replicates and presented as the mean with standard deviation presented as error bars. In the case of pairwise multiple comparisons ANOVA followed by a Holm-Sidak post-test was used. Statistical significance was considered when $p < 0.05$.

RESULTS AND DISCUSSION

During host injury, *P. aeruginosa* can be cued to express a lethal phenotype within the intestinal tract reservoir as a result of depletion of inorganic phosphate. In this study we investigated the effects of a polyphosphate compound, sodium hexametaphosphate, on *P. aeruginosa* virulence and survival *in vitro*. Polyphosphate is known to play an essential role on the growth of cells, cell response to stress and virulence of pathogens, and a critical role in bacterial metabolism as it is highly conserved among a variety of bacterial species.¹⁶ While several forms of polyphosphates exist, sodium hexametaphosphate has been widely used as a food additive and has been previously shown to exhibit bactericidal activity through a number of mechanisms.¹⁵ When applied in combination with ionic silver, sodium hexametaphosphate leads to putative antimicrobial synergy of *P. aeruginosa* and *S. aureus*.⁷ Polyphosphate compounds have also been shown to inhibit cellular septation in *Bacillus cereus* through interaction with the divalent cation-dependent GTPase activity of the cell division protein FtsZ.¹¹

We synthesized phosphate as well as polyphosphate loaded PEG hydrogel nanoparticles (NP-Pi and NP-PPi, respectively) using inverse phase miniemulsion polymerization to enable prolonged phosphate release. The nanoparticles were first characterized in terms of particle size and distribution, ζ -potential and swelling ratio (Table 2). Phosphate and polyphosphate nanoparticles were found to be of similar size with an average nanoparticle diameter ranging from 181 ± 52.2 and 179.6 ± 71.8 , respectively. No statistical significant differences in diameter were noted for the different nanoparticle formulations. Zeta potential is an indicator of surface charge characteristics and is known to dictate cell-nanoparticle interactions. The ζ -potential of the nanoparticle formulations in DI water was found to be 217.92 ± 1.05 and 218.34 ± 1.44 for NP-Pi and NP-PPi, respectively. Statistical significant differences in ζ -potential were not noted between NP-Pi and NP-PPi. Negative ζ -potential is known to minimize cellular nanoparticle uptake which is desired in the present application as the goal is to deliver the nanoparticles to the intestinal mucus to fully replenish depleted phosphate levels at this target site. Differences in the observed ζ -potentials are attributed to the presence of phosphate and or polyphosphate within the nanoparticles which may contribute to the ionic charge within and near the NP surface. The nanoparticles were also characterized in terms of their swelling ratio which is an indicator of their ability to imbibe aqueous solution. Swelling ratio is also inversely correlated to hydrogel network crosslink density, the latter influencing diffusive phosphate release. The swelling ratio of NP-PPi was found to be significantly higher than that of NP-Pi ($p < 0.05$) This is attributed to the fact that a more dilute aqueous precursor solution (total reactive double bond concentration of 0.754 M) was used in the synthesis of NP-PPi as compared to NP-Pi (total reactive double bond concentration of 1.508 M). A more dilute aqueous precursor will lead to decreases in network crosslink density and increases in hydrogel swelling ratio. The lower double bond

concentration was required for the synthesis of NP-PPi to avoid phase separation above certain polyphosphate concentrations.

Hexametaphosphate compounds can exist as cyclic or linear structures, both observed to undergo hydrolysis above room temperature or under alkaline conditions.¹ Hydrolysis may still occur at neutral pH and room temperature, albeit at a much slower rate. The hydrolysis products are usually associated with trimetaphosphate and monophosphate compounds. Since the polymerization process used to form the nanoparticles is carried out at 55 °C for 4.5 h, we investigated whether exposure of sodium hexametaphosphate to this temperature would lead to hydrolysis of hexametaphosphate. It must also be noted that polyphosphates in general, as purchased by the manufacturer, are polydisperse and composed of a mixture of polymeric phosphates. Absorption spectra of hexametaphosphate compared both at room temperature and after heat treatment (exposure of the compound to 55 °C for 4.5 h, conditions used to carry out the polymerization) are shown in Fig. 1. Absorption spectra of the unheated hexametaphosphate–molybdenum complex display one prominent absorbance peak at 417 nm, associated with hexametaphosphate and two slight shoulders at higher wavelengths; one in the same vicinity as that observed for the pristine monophosphate compound, potassium monobasic phosphate (~690 nm). This confirms that the hexametaphosphate, as received by the manufacturer is polydisperse. On the other hand, absorbance spectra of the heated polyphosphate demonstrate considerable changes in the height of the three peaks indicating that the relative amounts of the three components they represent have changed upon heating. This is consistent with the fact that polyphosphate hydrolyzes at elevated temperatures and that the three peaks of the heated compound represent the hydrolysis products. Generally, the peaks appear in similar locations after heat treatment with a slight shift of the main peak observed at ~424 nm and a second peak at ~575 nm, the latter associated with a compound other than monophosphate. Studies to identify this compound were not conducted, but it is hypothesized that it is associated with a polyphosphate trimer (trimetaphosphate) previously reported to exist with hexametaphosphate.¹ A third peak was also observed at ~690 nm, representative of the monophosphate compound. Since the relative amounts of all three components change upon heating the relative peak ratios of the hexamer and the monophosphate were used to quantify the state of polyphosphate within as well as released from the nanoparticles.

Release kinetics of phosphate and polyphosphate from NP-Pi and NP-PPi were quantified under perfect sink conditions. In the case of NP-Pi, phosphate is released at a high rate in the first 30 min after which the rate of release significantly decreases and approaches zero after 45 min (Fig. 2). The phosphate released is approximately 20% of the total amount of phosphate, suggesting that phosphate is lost post encapsulation, during polymerization and after the acetone washes from the nanoparticles. In the case of NP-PPi, the release kinetics of hexametaphosphate and its hydrolysis product, monophosphate, were quantified. Figure 3 indicates that both polyphosphate and monophosphate are released from NP-PPi and that the release of the hexamer is higher than that of monophosphate with release approaching zero in ~1.25 h. While our reported release duration may seem relatively short, it must be noted that the release kinetics were quantified under perfect sink conditions and are not representative of the actual duration of release when the nanoparticles are to be applied to the intestinal epithelium *in vivo*. It is anticipated that the *in vivo* release of phosphates from

the nanoparticles will be prolonged to enable sustained phosphate replenishment at the treatment site.

Our data reveal that sodium hexametaphosphate is hydrolyzed at high temperatures and specifically at 55 °C which was the temperature required to carry out the polymerization during nanoparticle synthesis (Fig. 1). We hypothesize that the release of PPi occurs *via* a two-step process involving the hydrolysis of the hexamer into a trimer, and eventually monophosphate. In this situation the smaller species diffuse more quickly from NP-PPi while the larger species diffuse slower and result in virulence suppression. This explains the ability of NP-PPi to sustain release for very long durations further supporting our hypothesis that this formulation will result in a superior phosphate replenishment effect when locally administered *in vivo*. Release kinetics of the hexamer and the monophosphate from NP-PPi confirm this phenomenon (Fig. 3). While the data in Fig. 3 indicate that the release of monophosphate (Pi) and hexametaphosphate (PPi) from polyphosphate loaded nanoparticles (NP-PPi) approaches very small levels after 1–2 h there is some level of release that is sustained for prolonged times. This is especially true for hexametaphosphate. This is evident when considering the molar ratio of released hexametaphosphate to monophosphate from NP-PPi (Fig. 4) which indicates that the release of phosphates from NP-PPi is sustained for prolonged times. While the released concentrations of both monophosphate and the hexametaphosphate are small, the levels of the latter are considerably higher indicating that some therapeutic effect could be sustained for up to 100 h (Fig. 4). Interestingly, this ratio remains fairly constant for the first few hours at a value close to the peak ratio calculated from the spectrum for heated polyphosphate samples (Fig. 1b). The release of hexametaphosphate extends to much longer times than that of monophosphate as evident from the increase of the molar ratio after 3 h (Fig. 4). This further confirms that the polyphosphate in the nanoparticles exists in the hydrolyzed state. In the case of NP-PPi, polyphosphate is released at a significant concentration after 96 h (data not shown) while the released monophosphate concentration approaches zero. It should be noted that the targeted concentration of inorganic phosphate in suppressing virulence is 25 mM which corresponds to 4.17 mM hexametaphosphate. According to the NP-Pi release data shown in Fig. 2, the maximum concentration of phosphate released from the NP-Pi is ~2.25 mM which does not reach this target. This can be attributed to the fact that a significant amount of monophosphate is lost after post-processing of the nanoparticles to extract the surfactants and cyclohexane during the acetone washes and centrifugation steps. In the case of NP-PPi, the maximum concentration of released hexametaphosphate is ~9.1 mM (corresponding to 54.6 mM Pi) which exceeds the targeted phosphate saturation level and works effectively in virulence suppression. In future studies, the concentration of phosphate and polyphosphate encapsulated within the nanoparticles can be adjusted during the inverse phase miniemulsion polymerization process in order to more precisely target desired therapeutic levels.

We also tested the efficacy of NP-Pi, NP-PPi, and free PPi on suppressing two global virulence systems, pyoverdinin and pyocyanin, as well as on their ability to preserve *P. aeruginosa* growth. The effectiveness of NP-Pi and NP-PPi in preventing bacterial transition to virulence *via* the pyoverdinin-mediated iron acquisition system was assessed with *P. aeruginosa* MPAO1 grown in low-phosphate (0.1 mM)/low-iron DCM, with varying amounts of NP-Pi and NP-PPi and varying concentrations of free phosphate (Pi) and

polyphosphate (PPi). After overnight incubation, pyoverdine production (indicative of the transition to virulence) was quantified. Figure 5 shows the normalized pyoverdine signal measured for the formulations tested compared to that measured in control samples. Polyphosphate formulations were the most effective in suppressing virulence, as indicated by the greatest attenuation of pyoverdine, with statistical significant reductions ($p < 0.05$) in pyoverdine expression noted as compared to phosphate formulations (Pi and NP-PPi) for fixed equivalent phosphate concentrations (6 and 12.9 mM). The virulence suppression effect is dose-dependent and increases with increasing concentration (represented in terms of equivalent phosphate concentration). It should be noted, that NP-PPi would allow for prolonged phosphate release *in vivo* as compared to free PPi which would be instantly absorbed.

The goal of the presented therapeutic approach is to suppress bacterial transition to virulence while maintaining bacterial survival as normal flora protect against overgrowth and invasion of pathogens by suppressing key virulence traits. We therefore, quantified *P. aeruginosa* survival (by counting CFUs) in the absence and presence of free Pi, PPi, and NP-Pi and NP-PPi. The data in Fig. 6 indicate that Pi and NP-Pi promote *P. aeruginosa* growth. Statistically significant increases in survival (expressed in log CFU) were noted for NP-Pi as compared to free Pi at nanoparticle concentrations equivalent to 1.25% w/vol (equivalent phosphate concentration = 3.1 mM). At higher nanoparticle concentrations (nanoparticle concentration = 5% w/vol; equivalent phosphate concentration = 3.1 mM) statistically higher CFU levels were noted in the case of free Pi as compared to NP-Pi. In the case of polyphosphate formulations, it was noted that PPi and NP-PPi result in a bactericidal effect above certain concentrations with NP-PPi suppressing growth to a lesser extent than free PPi. Statistically higher CFU levels were noted in the case of NP-PPi for nanoparticle concentrations of 0.625% w/vol and 1.25% w/vol (equivalent phosphate concentrations of 13.5 and 27 mM, respectively) as compared to free PPi. Previous studies have also indicated that polyphosphates exhibit a bactericidal effect at certain concentrations.^{7, 11} Our *in vitro* data (Fig. 7) indicate that *P. aeruginosa* virulence suppression and survival (quantified using OD measurements) can be achieved in the range of 0.6–1.4 mM PPi (or 3.6–8.4 mM equivalent Pi). In future studies we will target delivery of these therapeutic levels from nanoparticles and test their efficacy at attenuating virulence *in vitro* and *in vivo*. Additional *in vitro* studies of bacterial survival quantified *via* OD measurements indicate that both *P. aeruginosa* pyoverdine virulence suppression and survival can be achieved over a certain range of PPi concentration (Fig. 7).

An additional key virulence factor known to be involved in intestinal epithelial barrier disruption and gut-derived sepsis is the expression of pyocyanin, a redox active pigment that causes epithelial cell oxidative stress and induces neutrophil apoptosis.²⁵ Figure 8 shows that the pyocyanin signal normalized to *P. aeruginosa* survival (CFU) is significantly suppressed in the presence of PPi as well as NP-Pi and NP-PPi. The data also indicate that NP-PPi result in statistically significant suppression of relative pyocyanin levels as compared to the NP-Pi formulation (Fig. 9). Combined, the *in vitro* studies suggest that the NP-PPi formulation is effective for suppressing both pyoverdine and pyocyanin virulence systems.

CONCLUSIONS AND FUTURE OUTLOOK

In summary, the utilization of polyphosphate loaded nanoparticles shows significant promise as a drug delivery strategy for suppressing virulence in the gastrointestinal tract of the stressed host. Our data indicate that NP-Pi and NP-PPI loaded nanoparticles allow for prolonged release of phosphates and that polyphosphate as compared to phosphate loaded nanoparticles are the most effective in suppressing virulence, as indicated by the greatest attenuation of pyoverdine and pyocyanin. Future studies will explore the role of delivering NP-PPI dispersed in the high molecular weight PEG 15–20 copolymer, the latter shown to adhere to the intestinal epithelium with the goal being local and sustained phosphate delivery at the target site.

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FIGURE 1. Absorbance spectra of the hexametaphosphate-molybdenum complex before and after heat treatment.

FIGURE 2.
Phosphate release kinetics from phosphate loaded nanoparticles (5% w/v NP-Pi suspension).

FIGURE 3.
Monophosphate and polyphosphate release from polyphosphate-loaded nanoparticles
(1.25% (w/v) NP-PPI suspension).

FIGURE 4.
Release ratio of polyphosphate to monophosphate from polyphosphate-loaded nanoparticles.

FIGURE 5.

Effect of Pi and PPI formulations on attenuation of *P. aeruginosa* virulence. Data present mean $\pm$ standard deviation ($n = 3$). Normalized pyoverdinin levels statistically different at a given equivalent phosphate concentration with $p < 0.05$ (ANOVA followed Holm-Sidak post-test) by unless denoted with #, #Statistically not different. All other group comparisons at fixed equivalent phosphate concentration are statistically significant.

FIGURE 6.

Effect of (a) Pi and (b) PPi formulations on *P. aeruginosa* growth as a function of nanoparticle concentration; CFU colony function unit. Data present mean 6 standard deviation ($n = 3$). CFU values statistically different at a given equivalent phosphate concentration with $p < 0.05$ (t test); *Statistical difference.

FIGURE 7.

Pseudomonas aeruginosa survival and virulence as a function of free PPI concentration. Data presented in terms of equivalent monophosphate concentration.

FIGURE 8.

Effect of PPi, NP-PPi and NP-Pi on pyocyanin *P. aeruginosa* production. CFU colony function unit. Data present mean $\pm$ standard deviation ($n = 3$). All group statistically different as compared to positive (TSB) and negative (TSB + Opioid) controls with $p < 0.05$ (ANOVA followed Holm-Sidak post-test); *Statistical difference.

FIGURE 9. Effects of NP-Pi and NP-PPi on relative pyocyanin levels. Data present mean 6 standard deviation ($n = 3$). Normalized pyocyanin levels statistically different with $p < 0.05$ (t test); *Statistical difference.

TABLE 1

Experimental groups used for *in vitro* studies.

NP-PPi (% w/v)	NP-Pi (% w/v)	PPi (mM)	Pi (mM)
1.25	5.0	4.5	12.4
0.625	2.5	2.25	6.2
0.313	1.25	1.12	3.1

TABLE 2

Characterization of phosphate and polyphosphate nanoparticles.

Nanoparticle type	Diameter (nm)	ζ -potential (mV)	Swelling ratio
NP-Pi	181.7 ± 52.2	-17.92 ± 1.05	2.8 ± 0.08
NP-PPi	179.6 ± 71.8	-18.34 ± 1.44	4.9 ± 0.71