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Photodynamic Inactivation of Bacteria Using Polyethylenimine–Chlorin(e6) Conjugates: Effect of Polymer Molecular Weight, Substitution Ratio of Chlorin(e6) and pH

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Abstract

Background and Objectives—Antimicrobial photodynamic therapy (APDT) is a novel technique to treat local infections. Previously we reported that the attachment of chlorin(e6) to polyethylenimine (PEI) polymers to form PEI-ce6 conjugates is an effective way to improve ce6 PDT activity against bacteria. The aim of this work was to explore how the polymer molecular weight, substitution ratio (SR) of ce6 and pH value affect the PDT efficacy.

Study Design/Materials and Methods—We have synthesized PEI-ce6(10) (MW = 60,000, SR = 1) and PEI-ce6(11) (MW = 60,000, SR = 5) and compared these with the previous PEI-ce6(9) (MW = 10,000, SR = 1). We tested the PDT efficacy of these three conjugates against Gram-negative *E. coli* and Gram-positive bacteria (*S. aureus* and *E. fecalis*) at three different pH values (5.0, 7.4, 10.0) that may affect the charge on both the bacterial cells and on the conjugate (that has both basic and acidic groups).

Results—PEI-ce6(9) and PEI-ce6(10) were the most effective against these tested bacteria. The PDT effect of all three conjugates depended on pH values. The effective order was pH = 10.0 > pH = 7.4 > pH = 5.0 on *E. coli*. For *S. aureus* and *E. fecalis* the order was pH = 5.0 > pH = 10.0 > pH = 7.4. PEI-ce6(11) PDT activity was worse than PEI-ce6(10) activity which is probably connected to the fact that ce6 molecules are self-quenched within the PEI-ce6(11) molecule. Ce6 quenching within the PEI-ce6 molecules was proved by analyzing fluorescence spectra of PEI-ce6 conjugates at different pH values. There were no differences in bacterial uptake between different pH values in three PEI-ce6 conjugates.

Conclusion—We assume high pH (rather than low pH as was hypothesized) disaggregates the conjugates, so the higher pH was more effective than the lower pH against *E. coli*. But for Gram-positive bacteria, low pH was more effective possibly due to more overall positive charge on the conjugate. *Lasers Surg. Med.* 43:313–323, 2011.

Keywords

antimicrobial photodynamic therapy; polyethylenimine–chlorine(e6) conjugate; pH value; polymer molecular weight; substitution ratio; static and dynamic quenching

INTRODUCTION

Bacterial infections have been a constant threat to human health throughout history. There has been a relentless rise in antibiotic resistance that has taken place over many years in most regions of the world and in many different classes of microbial cells [1]. In recent times the phenomenon has become even more worrying, with concerns that hitherto fairly trivial infections could again become untreatable as in the days before antibiotics were discovered [2]. In fact the present time has been termed the “end of the antibiotic era” [3]. As a result of the continuing rise of drug-resistant bacteria, both in healthcare and in the community, there is a requirement for novel approaches to antimicrobial therapy.

Antimicrobial photodynamic therapy (APDT) has recently been used to efficiently destroy microorganisms. This technique combines a non-toxic photoactivatable dye or photosensitizer (PS) in combination with harmless visible light of the correct wavelength to excite the dye to its reactive triplet state that will lead to the formation of cytotoxic species (singlet oxygen and free radicals) that destroy those microorganisms. This technique has been shown to be effective in vitro against bacteria (including drug-resistant strains), yeasts, viruses, and protozoa [4,5], and has also demonstrated efficacy in vivo against localized infections in animal models [4,6,7].

Due to the highly reactive nature of the radicals formed through this PDT process, their reactivity is confined to their immediate environment. Thus, activity is selective and dependent on the delivery of the PS to the target [8]. Several studies have demonstrated that Gram-positive bacteria are particularly susceptible to photodynamic inactivation (PDI) [9–11] but Gram-negative bacteria are significantly resistant to many PSs used commonly in PDT of tumors [12]. It was shown that PDI of Gram-negative bacteria could be effective if the PS used had a cationic charge [13,14], or a non-cationic PS could be used if the bacterial membrane was permeabilized using polymyxin nonapeptide [15] or Tris/EDTA [16].

The outer membrane (OM) of Gram-negative bacteria acts as a permeability barrier that is able to exclude macromolecules and hydrophilic substances, thereby being responsible for the intrinsic resistance of these bacteria to antimicrobial compounds [17–19]. In Gram-negative bacteria, the barrier function of the OM is mainly due to the presence and features of lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane, along with various multidrug efflux pumps that also contribute to the resistance of the cells [19–21].

Polyethylenimine (PEI) is a weakly basic aliphatic polymer, which is polycationic due to the presence of primary, secondary, and tertiary amino groups. PEI has been recognized as a permeabilizer of pathogenic Gram-negative bacteria acting by intercalating into the OM rather than by releasing LPS [22–24]. Lin et al. [25] reported that PEI can exert an antimicrobial effect when incorporated into surfaces and textiles. This phenomenon was explained by the fact that the alkyl chains were long enough to penetrate from the surface to the outer leaflet of the LPS molecules.

Chlorins (porphyrins with one reduced pyrrole ring) are receiving considerable interest as potential drugs for PDT. These molecules have strong absorption in the lower part of the therapeutic window (650–900 nm), high singlet oxygen quantum yield, low dark toxicity, and can have high tumor selectivity [26,27]. At present, chlorin(e6) (ce6) has been used as a second-generation photosensitizer [28]. The main advantages of ce6 are its low toxicity, easy synthesis and production, fast and sufficiently selective accumulation in target tissue, and high photosensitizing efficacy [29]. The ce6-sensitized photodamage to bacteria with various defects in their DNA repair systems has been examined [30]. Park et al. [31] pointed out although ce6-mediated PDT is very effective in inhibiting Gram-positive bacteria, it is further necessary to devise suitable strategies enhancing the permeability of the outer wall in order to make Gram-negative bacteria such as *E. coli* sensitive to the action of photodynamic processes. For these reasons, many researchers have made efforts to find more effective ways to kill both Gram-positive and Gram-negative bacteria. In 1997 our laboratory formed the hypothesis that by covalently conjugating a suitable PS such as ce6 to a poly-L-lysine (pL) chain, a bacterially targeted PS delivery vehicle could be constructed that would efficiently inactivate both Gram-positive and Gram-negative species [32]. We subsequently demonstrated the potency and selectivity of pL-ce6 conjugates and red light for treating actual infections by using the technology to successfully carry out PDT of infected wounds [33,34], and deep established soft-tissue infections [35] in mice. We recently also reported a novel set of polycationic conjugates formed from PEI (PEI-ce6) were capable of killing a panel of pathogenic Gram-positive and Gram-negative bacteria, and the yeast *Candida albicans* after exposure to low levels of red light [36,37]. We further demonstrated that PEI-ce6 mediated PDT was an effective new treatment for multidrug-resistant localized *Acinetobacter baumannii* infections [38].

We here report the preparation of a new set of second-generation PEI-ce6 conjugates with different molecular weights and substitution ratios of ce6 that allowed us to explore how polymer molecular weight, substitution ratio of ce6, and overall charge determined by pH value affect the PDT efficacy. It should be noted that the extreme pH values used (5.0 and 10.0) were not intended to be relevant to pH values found in infections, but were used to determine the effect of the different charges on the quenching and aggregation state of the conjugates (and possibly the charges on the microbial cells).

MATERIALS AND METHODS

Chemicals

Ce6 (Frontier Scientific, UT), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), polyethylenimine ($M_n = 10$ and 60 kDa), acetone, ethanol, acetic acid, 2,4,6-trinitrobenzene sulfonic acid, sodium dodecyl sulfate, triton-X-100 were all purchased from (Sigma-Aldrich, St Louis, MO).

Synthesis of Conjugates

Ce6 (10 mg) was dissolved in 100 μ l of DMSO and added to 5 ml of distilled water, then EDC (17.6 mg) was quickly added. The mixture was stirred for 30 minutes at room temperature and added to 5 ml solution of polyethylenimine (10 or 60 kDa) in borate buffer (0.1 M, pH 8.5). This reaction mixture was stirred overnight at room temperature in the dark. Addition of cold acetone (-20°C , three volume to one) resulted in a green pellet or green precipitate. Precipitate was collected by centrifugation for 2 minutes at 2500g. The product was dried at normal pressure. Then product precipitate was dissolved in 10 ml of PBS and centrifuged 1.5 minutes at 2500g and the supernatant was dialyzed against distilled water for two days (MW cutoff 3.5 kDa or 10 kDa). The water was replaced three times:

after 2, 12, and 24 hours. Next, the dialyzed solution containing polymerchlorin e6 conjugate was lyophilized and kept sealed (hygroscopic) at -20°C .

Characterization: Thin Layer Chromatography and Gel Electrophoresis

The product was detected by thin layer chromatography (TLC) in the solvent system: ethanol/glacial acetic acid = 98:2. The R_f value measures how far up the TLC plate the compounds run with $R_f = 1$ being the solvent front. Positively charged and green colored conjugate interacts effectively with silica absorbent and have an R_f value of 0.05–0.1. Free ce6 has $R_f = 0.85$ in this system. Standard vertical 6% acrylamide (0.15 Tris-HCl, pH = 8, 10% SDS) and horizontal 3% agar (100 ml 1 × TBE) gels were prepared. Electrophoresis was performed at 100 V for both gels. Fluorescent products were imaged using a spectrally resolved fluorescence imaging system (Maestro, CRi Inc, Woburn, MA).

TNBS Assay

A standard 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay to quantify the number of amino groups was performed in 4% NaHCO_3 as described in Reference 39. The absorption wavelengths for ce6 and TNBS assays were 400 and 430 nm, respectively, to calculate the substitution ratio of ce6 to PEI.

Spectra

Absorbance spectra were measured in 0.1 M NaOH 5% SDS (or Triton-X100) unless otherwise mentioned. All PEI-ce6 conjugates fluorescence spectra were recorded in PBS adjusted to the three different pH values using a FluoroMax-3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ). PEI-ce6 was excited at 405 nm and emission was measured from 600 to 750 nm. All the measurements were carried out at room temperature.

Bacterial Strain and Culture Conditions

The bacteria used in this study were *Staphylococcus aureus* 8325-4, *Enterococcus faecalis* ATCC29212 and *Escherichia coli* K12. The bacteria were routinely grown in brain-heart infusion (BHI) broth with aeration in an orbital shaker at 37°C for 18 hours. An aliquot of this suspension was then refreshed in BHI to mid-log phase. Cell numbers were estimated by measuring the optical density [OD] at 600 nm (OD of 0.5 = 10^8 cells/ml).

PDI Studies

Suspensions of bacteria (10^8 /ml) were incubated in the dark at room temperature for 30 minutes with various concentrations of ce6 equivalent of these three PEI-ce6 conjugates in three different pH values phosphate-buffered saline (PBS). 1 ml aliquots was transferred to a 24-well plate and illuminated from below in the dark at room temperature with a red light source (660 ± 15 nm band pass filter, Lumacare, Newport Beach, CA) to deliver $10 \text{ J}/\text{cm}^2$ at an irradiance of $100 \text{ mW}/\text{cm}^2$ as measured with a power meter (model DMM 199 with 201 standard head; Coherent, Santa Clara, CA). The wells were illuminated in groups of four that were equally covered by a single spot of diameter 3 cm. No adjacent wells contained bacterial suspensions. Cells treated with PEI-ce6 conjugates in the dark were incubated, covered with aluminum foil for the same time as the PDT groups (30 minutes). At the completion of illumination (or dark incubation), aliquots (100 μl) were taken from each well to determine colony-forming unit (CFU). Care was taken to ensure that the contents of the wells were mixed thoroughly before sampling, as bacteria can settle at the bottom. The aliquots were serially diluted 10-fold in PBS to give dilutions of 10^{-1} – 10^{-5} times in addition to the original concentration and 10 μl aliquots of each of the dilutions were streaked horizontally on square BHI agar plates as described by Jett et al. [40]. Plates were

streaked in triplicate and incubated for 12–18 hours at 37°C in the dark to allow colony formation.

A control group of cells treated with light alone (no PEI-ce6 conjugates added) showed the same number of CFU as absolute control (data not shown). Survival fractions were routinely expressed as ratios of CFU of microbial cells treated with light and PEI-ce6 conjugates (or PEI-ce6 conjugates in the absence of light) to CFU of microbes treated with neither.

Uptake of Conjugates by Microbial Cells

Suspensions of microorganisms (10^8 /ml) were incubated in the dark at room temperature for 30 minutes with various concentrations of PEI-ce6 conjugates measured as μ M ce6 equivalent (final concentration in three different pH values PBS). The cell suspensions were centrifuged, the PS solution was aspirated and bacteria were washed twice by resuspending the cell pellet in 1 ml of the same pH value PBS and centrifuged. Finally, the cell pellet was dissolved by digesting it in 200 μ l of SOLVABLE solution (PerkinElmer Inc., Waltham, MA) at 37°C in an incubator for 2 hours to give a homogeneous solution. The fluorescence of cell lysate was measured using excitation at 405 nm and detection at 670 nm with a fluorescence plate reader (Molecular Devices, Sunnyvale CA). Following the fluorescence measurement, the total protein concentration was determined with a bicinchoninic acid protein assay kit (Sigma) using bovine serum albumin to prepare protein calibration curves. Fluorescence calibration curves were prepared for known concentrations of each of the three PEI-ce6 compounds in SOLVABLE that allowed results to be expressed as nmol ce6/mg cell protein.

Statistics

Values are means of three separate experiments, and bars presented in the graphs are standard errors of the means (SEM).

RESULTS

Preparation and Characterization of Conjugates

The chemical structure of the conjugate (PEI-ce6) is partly shown in Figure 1A and their molecular characteristics are shown in Table 1. The three conjugates were completely water soluble. TLC was used as a routine method to monitor progression of the reaction as shown in the example in Figure 1B where it can be seen that the conjugate remains at the baseline and the unconjugated free ce6 runs up the TLC plate. Purity and characterization were obtained using either polyacrylamide gel electrophoresis (PAGE, Fig. 1C) or agar gel electrophoresis (Fig. 1D). Both methods appeared to work equally well.

PDI Studies Against Gram-Positive *S. Aureus*

The best way to compare the phototoxicity of a group of PS with very different potencies is to vary the concentration of the PS over several orders of magnitude and determine the survival fraction with and without (dark toxicity) a single light dose. In the present case we varied the concentration of the ce6 equivalent so that the concentration of PEI present was lower in the case of PEI-ce6 10 because of the lower ratio of molecular weight of ce6 to molecular weight of PEI. In the case of conjugates PEI-ce6 9 and PEI-ce6 11, the ratio of ce6 to PEI was approximately equal.

Figure 2A–C display the survival fraction curves obtained against the Gram-positive bacterium *S. aureus* incubated for 30 minutes with the three PEI-ce6 conjugates with and without illumination with 10 J/cm^2 $660 \pm 15 \text{ nm}$ red light at three different pH values. None of the conjugates displayed any appreciable dark toxicity. From these graphs we can see that

the shape of the killing curves with all three PEI-ce6 conjugates was different at three different pH values and moreover the shape depended on conjugate concentration. The overall order of effectiveness at different pH values was pH 5 > pH 10 > pH 7.4. Overall PEI-ce6 9 and 10 were more effective than PEI-ce6 11 against *S. aureus*. Closer examination showed that the shape of the concentration curves was very different depending on both conjugate structure and on pH value. At pH 5, PEI-ce6 9 was most effective at 100 nM but the relative increase in phototoxicity was less than expected at 1000 nM (Fig. 2A). In contrast conjugate PEI-ce6 10 had little phototoxicity at 100 nM but showed a large increase at 1000 nM (Fig. 2B). PEI-ce6 11 had concentration curves with similar shapes (comparing 100 nM to 1000 nM) at all three pH values (Fig. 2C).

PDI Studies Against Gram-Positive *E. fecalis*

We sought to ascertain whether the surprising results we obtained with *S. aureus* could be generalized to other Gram-positive bacteria by testing another relatively similar species, *E. fecalis*. Figure 2D–F display the results. It can be seen that many of the results obtained with *S. aureus* (Figs. 2A–3C) were duplicated. PEI-ce6 9 showed similar behavior at pH 5, a high degree of killing at 100 nM and relative lack of proportionate killing on going from 100 to 1000 nM (Fig. 2D). PEI-ce6 10 at pH 7.4 showed no killing at 100 nM but an increase at 1000 nM, while at pH 10 there was a bigger increase at 1000 nM (Fig. 2E). A similar set of killing curves was found for PEI-ce6 10 as for PEI-ce6 9 at the different pH values. Again (similar to *S. aureus*) the killing curves attributed to PEI-ce6 11 were of similar shapes against *E. fecalis* at all three pH values (Fig. 2F).

PDI Studies Against Gram-Negative *E. coli*

The results obtained against *E. coli* were very different from those found for the two Gram-positive species. First of all the Gram-negative *E. coli* was less sensitive to PDT with all three conjugates compared to the Gram-positives and a ten-fold higher concentration of ce6 equivalent (up to 10,000 nM) had to be used to obtain good killing (Fig. 2G–I). This was an expected result as many papers show that typical Gram-negatives are about ten times more resistant than Gram-positives [41,42]. However there were also important differences between the different conjugates and between the different pH values. For every conjugate pH 10 gave more killing than pH 5, while for the Gram-positives the opposite applied (pH 5 gave more killing than pH 10). For PEI-ce6 9 the killing curves had a similar shape with a relative lack of additional killing on going from 1000 to 10,000 nM (Fig. 2G). For PEI-ce6 10 it was only pH 10 that showed a relative lack of killing on going from 1000 to 10,000 nM while both pH 7.4 and pH 5 showed a marked increase (Fig. 2H). For PEI-ce6 11 pH values 10 and 7.4 showed an increase of killing at 10,000 nM while for pH 5 there was very little killing even at 10,000 nM (Fig. 2I).

Cellular Uptake of PEI-ce6

One obvious explanation for the differences in killing found for the various parameters tested (bacterial species, conjugate structure, and pH value) is that the bacterial cell uptake of ce6 was different in different cases. Therefore we measured cell uptake by fluorescence extraction from cell pellets and plotted the uptake values (in nmol of ce6 per mg of cell protein) versus concentration of the three PEI-ce6 conjugates at three different pH values.

Uptake values in *S. aureus* were somewhat higher at pH 10 for PEI-ce6 9 and PEI-ce6 10 than at other pH values (Fig. 3A and B) but not in the case of PEI-ce6 11 where there was no difference (Fig. 3C). Uptake of PEI-ce6 11 was markedly higher than PEI-ce6 10 at all pH values and that in turn was somewhat higher than the uptake of PEI-ce6 9 at all pH values. For *E. fecalis* uptake (Fig. 3D–F) the results were almost identical to those obtained for *S.*

aureus. The uptake results for *E. coli* (Fig. 3G–I) showed no difference regardless of the conjugate structure or the different pH values.

Fluorescence Intensity Determination of Static and Dynamic Quenching

We formed the hypothesis that one of the factors governing the different PDT efficacies of the three conjugates at the three different pH values was the balance between intramolecular (static) and intermolecular (dynamic) quenching of the ce6 molecules in the conjugates. To test this hypothesis we measured the fluorescence of the ce6 contained in the conjugate at the three different pH values using a series of twofold dilutions. The graphs are shown in Figure 4. It can be seen that for PEI-ce6 9 at all pH values (Fig. 4A–C) there is a significant amount of dynamic quenching as the highest fluorescence intensity is obtained at ce6 concentrations lower than 10 μ M. The dynamic quenching is lower for PEI-ce6 10 (Fig. 4D–F) and lowest for PEI-ce6 11 (Fig. 4G–I). Static quenching was higher for PEI-ce6 11 than it was for PEI-ce6 10 as can be seen by comparing the absolute heights of the emission peaks at both pH 5.0 (Fig. 4D, G) and pH 7.4 (Fig. 4E, H). Conjugates PEI-ce6 9 and PEI-ce6 10 cannot undergo static quenching as they have a substitution ratio of only one ce6 per PEI chain. As the pH is raised from 5.0 through pH 7.4 to pH 10, the heights of the emission peaks from all conjugates increase. This shows that raising the pH reduces both kinds of quenching showing a large progressive change in the case of PEI-ce6 9 (dynamic quenching), and a large change in PEI-ce6 11 (static quenching).

These data were confirmed by using fluorescent probes for the generation of reactive oxygen species (ROS) in solution. We used singlet oxygen sensor green to measure generation of singlet oxygen and hydroxyphenyl-fluorescein to measure generation of hydroxyl radicals [43]. Both probes showed greater generation of both species of ROS at pH = 10.0 than was seen at pH = 5.0 and 7.4 (data not shown). This confirmed the findings of decrease in dynamic quenching with higher pH. In addition relatively higher generation of ROS was seen for conjugates PEI-ce6 9 and PEI-ce6 10 compared to that seen for PEI-ce6 11 confirming the presence of static quenching in conjugate 11. Much higher levels of singlet oxygen generation were seen compared to the levels of hydroxyl radicals suggesting that these conjugates behaved as type 2 PS as expected.

DISCUSSION

We report interesting and unexpected findings concerning the role of conjugate size, charge, and substitution ratio on the effectiveness of APDT. We originally supposed that the amount of cationic charge born by the conjugates would be the most important determinant of effectiveness and that this cationic charge would be maximized at low pH values when each amino group would be more likely to be protonated. Moreover we supposed that the effect of protonation and consequent cationic charge would be most pronounced in the case of Gram-negative bacteria where it is known that the outer membrane permeability barrier is susceptible to being overcome by cationic molecules [44].

When the experiments were completed we found some surprising results. In many cases the conjugates were more effective at high pH 10 than they were at neutral pH 7.4 and low pH 5. This finding can be rationalized by considering that there are three types of functionality whose charge can change with pH. Two of these functionalities are on the conjugate, amino groups on PEI can be protonated and carboxyl groups on the ce6 macro-cycle can be deprotonated. The third ionizable functionality comprises the acidic groups present on the bacterial cells, which are largely composed of carboxylate and phosphate groups. At pH 5.0 amino groups of the PEI would tend to be positively charged while ce6 and bacteria are neutral. At pH 7.4 both PEI-ce6 and cells are neutral. The acidic groups on ce6 bear negative charges at pH 10.0. For bacteria cells there are different charges between different pH values

also. At pH 5.0 amino groups have positive charges but carboxyl (COOH) groups and phosphate groups are neutral. At pH 7.4 COOH groups, amino groups, and phosphate groups are neutral. But both bacterial COOH groups and phosphate groups have negative charges at pH 10.0.

There are two types of quenching that can occur to adversely affect the photoactivity of the ce6 molecules. The first is intermolecular or dynamic quenching that is concentration dependent. In this form of quenching the ce6 molecules transfer energy between each other without being constrained by covalent attachment to the PEI backbone. When the concentration of the conjugate is raised the chance of ce6 molecules being in close proximity is higher. The second type of quenching is intramolecular or static quenching. This process is independent of concentration but is increased with higher substitution ratio. The ce6 molecules in PEI-ce6 11 are forced closer together than they are in PEI-ce6 9 and 10 and more likely to quench each other. It should be noted that only PEI-ce6 11 can undergo static quenching because both PEI-ce6 9 and PEI-ce6 10 have only one ce6 per PEI chain. Both types of quenching can be reduced by increasing the mutual repulsion of the ce6 molecules by ionizing their carboxyl groups and creating negative charges.

The shape of the killing curves for PEI-ce6 9 and 10 against both Gram-positive bacteria at pH 5.0 showed relatively more killing for the same PDT dose administered by illuminating the cells at 100 nM concentration and less at a PDT dose ten times higher at 1000 nM concentration. However at pH 10.0 (and to a lesser extent at pH 7.4) there was relatively more killing at 1000 nM. We interpret these data to mean that dynamic quenching is more important at pH 5.0 where the ce6 molecules do not repel each other. PEI-ce6 11 does not show this enhanced activity specifically at low concentration at pH 5.0, consistent with the idea that static quenching is more important than dynamic quenching in this molecule.

We compared the PDT efficacy of the conjugates as reported above with the efficacy after the conjugate in solution had been washed out (centrifugation of bacteria after incubation and resuspension in same volume of PBS before illumination) (data not shown). In the case of Gram-negative *E. coli* the PDT effect of the washed bacteria at 10 uM ce6 concentration in conjugate was slightly (0–1 log) higher than that found with the no-wash protocol. The explanation for this was that at the higher concentration there is some optical shielding from the ce6 in solution that absorbs light without generating effective ROS because the conjugate is not near enough to the bacteria. In the case of Gram-positive *S. aureus* there was no difference between wash and no-wash at the lower maximum concentration employed (1 uM ce6 equivalent). The relative difference between wash and no-wash was independent of pH and conjugate structure showing that quenching of the conjugate in solution did not play a role in the PDT activity of the conjugates.

It is considered that the PDT efficacy (especially toward Gram-negative bacteria) of these conjugates between ce6 and polycationic polymers such as pL and PEI increases with increasing molecular weight of the amino-containing polymer. The presence of aliphatic amino groups in both these polymers suggests that their pKa values should be comparable. This was previously shown [45] by a comparison of pL-ce6 conjugates where the average number of lysine residues in the pL chain was either 8 or 37. Conjugates with 37 lysine residues were more effective than those with eight lysines against Gram-negative *E. coli*. Another paper [36] compared three PEI-ce6 conjugates: a linear PEI with an average of eight amino groups, and two cross-linked PEI polymers, one with 16 amino groups and the other equivalent to the present PEI-ce6 9 (280 amino groups).

The molecular weight of PEI in PEI-ce6 10 and 11 is the same (1400 amino groups) but is higher than PEI-ce6 9 (280 amino groups). However we did not notice any difference

between the effectiveness of PEI-ce6 9 and 10 against either Gram-positives or Gram-negatives. This finding implies that the size of PEI-ce6 9 had reached a maximum with regard to the ability to increase penetration into Gram-negatives. The proposed explanation for the previous results [36,45], therefore, only applies up to a certain limit of polymer size.

We found significant differences between Gram-positive and Gram-negative bacteria (in addition to the well-known need to use higher concentrations of PEI-ce6 for Gram-negatives). Several studies have shown that Gram-negative bacteria are more resistant to PDT due to their special wall structure [9,15,46]. For Gram-positives (especially at low concentrations) pH 5.0 was best, while for Gram-negatives, pH 10.0 was better. It is possible that the pH affects the bacterial cells differentially, with Gram-positives becoming better able to bind the neutral ce6 molecules at low pH (more hydrophobic cell surface), while Gram-negatives remain hydrophilic regardless of pH. The original idea that at low pH, the amino groups on the PEI would become more cationic and better able to allow the conjugates to penetrate Gram-negative bacteria appears to be incorrect.

However, in the present study, the CFU assay revealed that PEI-ce6-mediated PDT had a strong antimicrobial effect against Gram-negative *E. coli*. The effectiveness of PEI-ce6 conjugates 9, 10, and 11 all increased with pH value. PDI effect was higher at high pH 10.0 and lower at low pH 5.0. The explanation is at pH 10.0 ce6 exists predominantly as negatively charged entity by deprotonation of ionizable COOH groups. So PEI-ce6 compounds disaggregate and produce more killing on *E. coli* due to increased quantum yield of ROS generation. At pH 5.0 the efficiency of these PEI-ce6 conjugates has less effect due to aggregation and reduction of ROS quantum yield. This phenomenon (difference of effectiveness between pH 5.0 and 10.0) is especially noticeable with low concentrations of PEI-ce6 conjugates 9 and 10. This is because low concentrations of PEI-ce6 conjugates 9 and 10 tend to minimize ce6 aggregation.

Soukos et al. [32] proposed that the pronounced photodynamic effect of the cationic conjugate on bacterium *Actinomyces viscosus* may be due to the electrostatic attraction between the conjugate and the negatively charged membrane of the bacterium. Many researchers reported that the positive charge on the PS molecule promotes a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cells, increasing the efficiency of the photoinactivation process [13,47–49].

There is, to the best of our knowledge, no report that the PDI effect of polycationic conjugates depends on the pH values. A number of investigations have been done on pH dependent photophysics of chlorin derivatives [50,51]. From our present study we can see that the reaction of the cells to different pH values is different for Gram-positive and Gram-negative bacteria. It seems *S. aureus* and *E. fecalis* were relatively more susceptible than *E. coli* at pH 5.0.

From the fluorescence spectra study we found that higher pH values produced higher fluorescence in these three different PEI-ce6 conjugates. The order of fluorescence intensity was pH 10 > pH 7.4 > pH 5.0. PEI-ce6 10 and 11 both have the same molecular weight of PEI, but the substitution ratio of ce6 is different. From these results we can see that PEI-ce6 10 had higher fluorescence than PEI-ce6 11. The difference in fluorescence was especially large at neutral pH. This is because PEI-ce6 11 has the ce6 molecules to be in close proximity and therefore quench each other. From these fluorescence results we can explain why PEI-ce6 11 had less antimicrobial PDT effect than PEI-ce6 9 and 10 conjugates on Gram-positive and Gram-negative bacteria.

There were no differences in cells' uptake between different pH values in three PEI-ce6 conjugates. These results suggest that binding of the PEI-ce6 conjugates to Gram-positive and Gram-negative bacteria were similar in these different pH values.

In conclusion, based on the findings in the present study, pH values affected the PDT efficacy of PEI-ce6 conjugates. The reactions of the cells to different pH values were different for Gram-positive and Gram-negative bacteria. There were no differences in PDI effect on different polymer molecular weight in this study, but substitution ratio of ce6 affects PDI effect against these tested bacteria *S. aureus*, *E. fecalis*, and *E. coli*.

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Fig. 1. Structure and characterization of conjugates. (A) Partial chemical structure of PEI-ce6 conjugates. (B) TLC used for routine monitoring of purity. (C) Polyacrylamide gel electrophoresis of PEI-ce6 conjugates. (D) Agar gel electrophoresis of PEI-ce6 conjugates.

Fig. 2.

Photoinactivation of Gram-positive and Gram-negative bacteria. Bacterial cells were incubated with stated concentrations of conjugate for 30 minutes in PBS adjusted to three different pH values. Cells were then either exposed (light) or not (dark) to 10 J/cm² of light (660 ± 15 nm) at an irradiance of 100 mW/cm². Cells were then serially diluted and plated to count CFU. Survival fractions are expressed relative to CFU of pH alone (no conjugate, no light). (A) *S. aureus* PEI-ce6 9; (B) *S. aureus* PEI-ce6 10; (C) *S. aureus* PEI-ce6 11; (D) *E. faecalis* PEI-ce6 9; (E) *E. faecalis* PEI-ce6 10; (F) *E. faecalis* PEI-ce6 11; (G) *E. coli* PEI-ce6 9; (H) *E. coli* PEI-ce6 10; (I) *E. coli* PEI-ce6 11.

Fig. 3.

Uptake of PEI-ce6 into cells. Bacterial cells were incubated with concentrations of conjugate for 30 minutes in PBS adjusted to three different pH values. Cells were dissolved in SOLVABLE. Fluorescence was quantified and compared with 27 separate calibration curves and protein in cell extract measured. Results are expressed in nmol of ce6 per mg of cell protein. (A) *S. aureus* PEI-ce6 9; (B) *S. aureus* PEI-ce6 10; (C) *S. aureus* PEI-ce6 11; (D) *E. faecalis* PEI-ce6 9; (E) *E. faecalis* PEI-ce6 10; (F) *E. faecalis* PEI-ce6 11; (G) *E. coli* PEI-ce6 9; (H) *E. coli* PEI-ce6 10; (I) *E. coli* PEI-ce6 11.

Fig. 4.

Fluorescence of PEI-ce6 conjugates. Fluorescence emission spectra after excitation at 405 nm were recorded between 600 and 750 nm in buffers of specified pH after six twofold dilutions starting at 10 μM . **A–C**, PEI-ce6 9; **D–F**, PEI-ce6 10; **G–I**, PEI-ce6 11.

TABLE 1

Conjugates Employed in This Study Showing Molecular Weight of PEI and Substitution Ratio

Conjugate	PEI mol. weight	Substitution ratio	Mol wt. ratio ce6:PEI
PEI-ce6 9	10,000	1	0.06
PEI-ce6 10	60,000	1	0.01
PEI-ce6 11	60,000	5	0.05