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Type I and Type II mechanisms of antimicrobial photodynamic therapy: An in vitro study on Gram-negative and Gram-positive bacteria

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Abstract

Background and Objectives—Antimicrobial photodynamic therapy (APDT) employs a nontoxic photosensitizer (PS) and visible light, which in the presence of oxygen produce reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$, produced via Type II mechanism) and hydroxyl radical ($HO^\bullet$, produced via Type I mechanism). This study examined the relative contributions of $^1O_2$ and $HO^\bullet$ to APDT killing of Gram-positive and Gram-negative bacteria.

Study Design/Materials and Methods—Fluorescence probes, 3'-(p-hydroxyphenyl)-fluorescein (HPF) and singlet oxygen sensor green reagent (SOSG) were used to determine $HO^\bullet$ and $^1O_2$ produced by illumination of two PS: tris-cationic-buckminsterfullerene (BB6) and a conjugate between polyethylenimine and chlorin(e6) (PEI–ce6). Dimethylthiourea is a $HO^\bullet$ scavenger, while sodium azide (NaN_3) is a quencher of $^1O_2$. Both APDT and killing by Fenton reaction (chemical generation of $HO^\bullet$) were carried out on Gram-positive bacteria (*Staphylococcus aureus* and *Enteroccoccus fecalis*) and Gram-negative bacteria (*Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.

Results—Conjugate PEI-ce6 mainly produced $^1O_2$ (quenched by NaN_3), while BB6 produced $HO^\bullet$ in addition to $^1O_2$ when NaN_3 potentiated probe activation. NaN_3 also potentiated HPF activation by Fenton reagent. All bacteria were killed by Fenton reagent but Gram-positive bacteria needed a higher concentration than Gram-negatives. NaN_3 potentiated Fenton-mediated killing of all bacteria. The ratio of APDT killing between Gram-positive and Gram-negative bacteria was 2 or 4:1 for BB6 and 25:1 for conjugate PEI-ce6. There was a NaN_3 dose dependent

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inhibition of APDT killing using both PEI-ce6 and BB6 against Gram-negative bacteria while NaN_3 almost failed to inhibit killing of Gram-positive bacteria.

Conclusion—Azidyl radicals may be formed from NaN_3 and $HO^{\bullet}$. It may be that Gram-negative bacteria are more susceptible to $HO^{\bullet}$ while Gram-positive bacteria are more susceptible to $^1O_2$. The differences in NaN_3 inhibition may reflect differences in the extent of PS binding to bacteria (microenvironment) or differences in penetration of NaN_3 into cell walls of bacteria.

Keywords

antimicrobial photodynamic inactivation; singlet oxygen; hydroxyl radical; sodium azide; Gram-positive and Gram-negative bacteria; antimicrobial photodynamic therapy; polyethylenimine chlorin(e6) conjugate; tris-cationic fullerene; singlet oxygen; hydroxyl radical; sodium azide

INTRODUCTION

Microbial infections globally remain the leading cause of mortality and the biggest single factor contributing to this pandemic of infectious diseases is the emergence of pathogenic bacteria with multiple drug resistance (1,2). This realization has led several authors to speak of the forthcoming “end of the antibiotic era” (3–7). Thus, the search for novel, more efficient antibacterial therapies has been a subject of intense and continuing research efforts (8). The use of photodynamic inactivation (PDI) as a non-antibiotic approach to inactivate pathogenic microorganisms seems to be very promising (9,10). Antimicrobial photodynamic therapy (APDT) has been proposed as an alternative treatment for localized infections (both experimentally (11) and clinically (12)) in response to the ever-growing problem of antibiotic resistance. APDT 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 reactive oxygen species (ROS) (13). These ROS can be classified into those produced by Type I photochemical mechanism: free radicals such as hydroxyl radicals ($HO^{\bullet}$) and those produced by Type II photochemical mechanism: singlet oxygen ($^1O_2$) that directly destroy those microorganisms. Both Type I and Type II reactions can occur simultaneously, and the ratio between these processes depends on the type of PS used and critically on the microenvironment of the PS molecule. Although vast progress has been made over the past few years, the mechanistic details of how the ROS generated during APDT affect microbial cells are not fully understood.

The use of $^1O_2$ quenchers such as sodium azide (NaN_3) and histidine, and of free radical scavengers such as thiourea and dimethylsulphoxide, represents a simple approach to determine which pathway(s) is (are) involved in photodynamic inactivation (14–16).

Regarding APDT it has been shown that Gram-positive bacteria are sensitive to photosensitization by many different dyes, while Gram-negative bacteria are more resistant. Since the early 1990s it was realized that Gram-negative bacteria were only efficiently killed by PS that had a pronounced cationic charge (17–19). The short diffusion distance of both $^1O_2$ and of most radicals produced during PDT explained the increased efficacy when the ROS were produced from dyes located attached to or inside the microbial cells.

There are reports of recently introduced fluorescent probes (20) (520-nm green fluorescence) available that are specific (or relatively specific) for the main reactive oxygen species (ROS) responsible for killing cells: hydroxyphenyl fluorescein (HPF) is supposed to be specific for $HO^{\bullet}$ (21), while singlet oxygen sensor green (SOSG) is supposed to be specific for $^1O_2$ (22). Azide has been frequently used as a physical quencher of $^1O_2$ (23,24)

and an inhibitor of APDT mediated killing of bacteria (25,26). Thiourea and dimethylthiourea are potent $HO^{\bullet}$ scavengers (27,28).

Over the last decade, APDT mechanisms of Gram-positive and Gram-negative bacteria using different PS have been extensively studied but few of these studies gave importance to the actual identity of the particular ROS involved (29–31). Many papers assume that $^1O_2$ is in fact the only ROS of importance in APDT (32,33), while other attribute the killing to radicals including $HO^{\bullet}$ (34). In this paper, we have attempted to determine which ROS is more effective for Gram-positive and Gram-negative bacteria by using PS that tend to undergo Type I mechanisms and PS that undergo Type II mechanisms. Then we should be able to determine whether Type I or Type II APDT reactions are better for killing various microbes.

MATERIALS AND METHODS

2.1 Chemicals

NaN_3, ferrous sulfate ($FeSO_4$), 1,3-dimethyl-2-thiourea (DMT) and 30% hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO). The synthesis and molecular characterization of PEI-ce6 conjugate has been previously described in detail (35). It consists of a polythethylenimine with an average molecular weight of 10,000 with an average of one ce6 molecule attached (Figure 1A). The PEI-ce6 conjugate was stored as 2 mM solution in distilled water. The synthesis of BB6 was carried out by using methods previously described in the literature (36,37). It had three N-methyl-pyrrolidinium iodide groups attached to the fullerene cage and contained regioisomers (Figure 1B). BB6 was dissolved in N,N-dimethylacetamide to give stock solutions with concentration of 5 mM. All PS stock solutions were stored at 4°C refrigerator in the dark for no more than 4 weeks. The absorption spectra of the PS and the emission spectra of the two light sources are shown in Figure 1C. Other chemicals such as NaN_3, $FeSO_4$, H_2O_2 and DMT were prepared in distilled water immediately before experiments.

2.2 Fluorescence probe experiments

96-well black-sided plates were used for fluorescence probe experiments. SOSG or HPF (final concentration of 10uM) was added to 2uM BB6 or PEI-ce6 conjugate with and without addition of NaN_3 or DMT in 200uL PBS per well. Fluorescence spectrometry (SpectraMax M5 plate reader, Molecular Devices, Sunnyvale, CA) used excitation and emission at 504 and 525 nm for SOSG and 490 and 515 nm for HPF, respectively. Increasing fluences (J/cm^2) were delivered using white light (400–700 nm band pass filter, Lumacare, Newport Beach, CA) for BB6 or red light (660±15 nm band pass filter) for conjugate PEI-ce6 at an irradiance of 100 mW/cm^2 as measured with a power meter (model DMM 199 with 201 standard head; Coherent, Santa Clara, CA). Each time after an incremental fluence was delivered, the fluorescence was measured. For Fenton reagent aliquots of H_2O_2 and $FeSO_4$ were added to the wells and fluorescence measured immediately afterward.

2.3 Bacterial Strains and Culture Conditions

The bacteria used in this study were Gram-positive bacteria: *Staphylococcus aureus* 8325-4 and *Enterococcus faecalis* ATCC 29212 and Gram-negative bacteria: *Escherichia coli* K12, *Proteus mirabilis* ATCC 51393 and *Pseudomonas aeruginosa* ATCC 19660. The bacteria were routinely grown in brain-heart infusion (BHI) broth with aeration in an orbital shaker at 37 °C for 18 h. 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).

2.4 Fenton reagent ($FeSO_4/H_2O_2$) antimicrobial studies

Suspensions of bacteria (10(8) CFU/mL) were incubated at room temperature with various concentrations of Fenton reagent (equal concentrations of H_2O_2 and $FeSO_4$) with and without NaN_3 (0.5 mM) in pH 7.4 phosphate-buffered saline (PBS) for 60 min. At the completion of incubation aliquots (100 μl) were taken from each tube 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} to 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. (38). Plates were streaked in triplicate and incubated for 12–18 h at 37 °C in the dark to allow colony formation. Each experiment performed at least three independent times.

APDT studies

Suspensions of bacteria (10(8)/mL) were incubated in the dark at room temperature for 30 min with BB6 or conjugate PEI-ce6 and with or without various concentrations of NaN_3 in pH 7.4 PBS. In the case of BB6 the highest concentration used (4 uM) gave DMA concentrations of <0.1% that had no effect on the bacteria. 1 mL aliquots were transferred to a 24 well plate and illuminated from below in the dark at room temperature with a white light source (400–700 nm band pass filter) to deliver 10 J/cm^2 for BB6 or a red light source (660±15 nm band pass filter) to deliver 5 J/cm^2 for conjugate PEI-ce6 and at an irradiance of 100 mW/cm^2. Cells treated with BB6 or PEI-ce6 conjugate in the dark were incubated covered with aluminum foil for the same time as the PDT groups (30 min). At the completion of illumination (or dark incubation) aliquots (100 μl) were taken from each well to determine CFU as described above. A control group of cells treated with light alone (no BB6 or PEI-ce6 conjugate 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 PS to CFU of microbes treated with neither.

Statistics

Values are means of three separate experiments, and bars presented in the graphs are standard errors of the means (SEM). Differences between means were statistically analyzed by one way ANOVA and $p<0.05$ was considered significant.

RESULTS

Fluorescence probes to determine photochemical mechanisms

Although it is accepted that these probes (HPF and SOSG) are not entirely specific for either $HO^{\bullet}$ or $^1O_2$ (39), they can be employed to examine differences in photochemical mechanisms between two different PS. Figure 2 displays the results obtained from BB6 and PEI-ce6 using HPF and SOSG. By comparing Figs 2A and 2B it can be seen that while BB6 and white light activates both SOSG and HPF to give approximately equal levels of fluorescence, PEI-ce6 and red light showed markedly more fluorescence from SOSG than from HPF (Fig 2C and 2D). For BB6 and white light activation measured by both probes (Figs 2A and 2B), NaN_3 showed potentiation, while DMT showed quenching. When Figs 2C and 2D are compared it can be seen that for PEI-ce6 the results are very different. There was hardly any activation of HPF. Furthermore in the case of PEI-ce6 activation of SOSG there was a high degree of inhibition by NaN_3 and a much lesser degree of inhibition by DMT. From these probe results, we can infer that BB6 is a more likely to undergo Type I mechanism and PEI-ce6 is a more likely to undergo Type II mechanism.

Effect of NaN_3 on activation of HPF by Fenton reagent

It is well-known that Fenton reaction (H_2O_2/Fe^{2+}) can produce HO• (40). Because of the somewhat surprising observation that NaN_3 potentiated activation of HPF (and to a lesser extent activation of SOSG) by BB6 and light, we asked whether NaN_3 could also potentiate the activation of HPF by HO• generated by Fenton reagent. Figure 3 shows a high degree of potentiation of fluorescence generation by NaN_3 while DMT gave almost complete quenching (as might be expected from its ability to quench HO•). The explanation for the potentiation of HPF activation by NaN_3 may be that azide radicals (N3•) can be formed from HO• and azide anion (41). It remains to be proved whether azide radicals can react with HPF in the same manner as HO•.

Effect of added NaN_3 on Fenton reagent-mediated killing of bacteria

Because of the observation that NaN_3 could potentiate activation of HPF probe by Fenton reagent, we asked what was the effect of NaN_3 on killing of bacteria by HO• generated from Fenton reagent? We initially established a protocol for dose dependent bacterial killing consisting of a 1-hour incubation time and adding equal concentrations of H_2O_2 and Fe_2SO_4 up to 11 mM (the concentration needed to kill Gram-positives). We also showed that NaN_3 alone incubated for 1 hour with bacteria did not begin to show any toxicity until the NaN_3 concentration reached 50 mM (data not shown). We used 0.5 mM NaN_3 concentration in combination with Fenton reagent as much higher concentrations such as 10 mM gave some precipitation (perhaps by azide anion reacting with ferrous iron). Figures 4A and 4B shows the data for both the Gram-positive species, *S. aureus* and *E. faecalis*. Killing began to be apparent at 8 mM Fenton and was almost complete at 11 mM. For both species, NaN_3 potentiated killing by between 1–4 logs. Figures 4C–4E show the data for the three Gram-negative species (*E. coli, P. aeruginosa* and *P. mirabilis).* It can be seen that the Gram-negative species were more susceptible than the Gram-positive species, with killing becoming apparent at 4 mM and becoming almost complete at 9 mM. Interestingly *P. aeruginosa* (considered to a resistant species to most antimicrobials) was the most susceptible. For all three Gram-negative species NaN_3 potentiated killing by between 1–4 logs.

Effect of NaN_3 on APDT-killing of bacteria mediated by PEI-ce6 conjugate

We selected parameters that produced approximately 4–5 logs of killing of all bacterial species. These parameters were 400 nM PEI-ce6 and 5 J/cm^2 red light for Gram-positives and 10 μM PEI-ce6 and 5 J/cm^2 for Gram-negative species. The ratio of PS concentration necessary for equal light killing of Gram-negative:Gram-positive bacteria was 25:1. A dose response of inhibition of killing by addition of NaN_3 was then constructed. The data for Gram-positves are displayed in Figure 5A and 5B. There was virtually no inhibition of killIng apparent until the concentration of 9–10 mM NaN_3 was reached, and even then it was only 1 log or less. The data for Gram-negatives are shown in Figure 5C–5E. It is apparent that inhibition of killing appears as low as 500–1000 μM NaN_3 and at 10 mM the inhibition reached 3 logs.

Effect of NaN_3 on APDT-killing of bacteria mediated by BB6

We selected parameters to obtain 3–4 logs of killing of the Gram-positive and Gram-negative species. These parameters were 1 μM BB6 and 10 J/cm^2 white light for *S. aureus* and *E. faecalis,* and 2 μM BB6 for *E. coli* or 4 μM BB6 for *P. aeruginosa* and *P. mirabilis* combined with 10 J/cm^2 white light. The ratio of PS concentration necessary for equal light killing Gram-negative:Gram-positive bacteria was 2 or 4:1. The dose-response of inhibition of killing by addition of NaN_3 for Gram-positives is shown in Figures 6A and 6B. Slight inhibition was apparent at 5-mM NaN_3 but even at 10-mM the inhibition did not exceed 1

log. The data for Gram-negatives is shown in Figures 6C–6E. Inhibition (circa 1 log) was seen as low as 500 μM NaN_3, this had risen to 2 logs by 3 mM and at 10-mM the degree of inhibition of killing had risen to 3 logs.

DISCUSSION

We report interesting findings concerning the difference between the bacterial killing effects of photodynamically generated $HO^{\bullet}$ or $^1O_2$ against Gram-positive and Gram-negative bacteria. Some researchers (42,43) have reported that $^1O_2$ (Type II mechanism) plays a very important role in APDT. Other studies have shown that Type I mechanisms (particularly $HO^{\bullet}$) can also be equally important as Type II pathways (15,16). However these studies did not clearly discuss whether different ROS are more effective for killing Gram-positive or Gram-negative bacteria. Our data suggest for first time that Gram-positive bacteria are more sensitive to $^1O_2$ and Gram-negative bacteria are more susceptible to $HO^{\bullet}$.

The introduction of fluorescence probes to characterize the identity of different ROS produced during PDT has aroused some controversy in the literature (39,44). This is because some authors assume that these probes have a higher degree of specificity than is actually the case. We have found that HPF is actually more specific for $HO^{\bullet}$ than is the alternative fluorescein derivative probe 3'(4-aminophenyl) fluorescein (APF) (21) (data not shown) and this is in agreement with results from Price et al (44). However SOSG does not appear to be very specific for $^1O_2$, as it is significantly activated by Fenton reagent (data not shown). The use of quenchers of different ROS to identify the precise ROS involved in PDT is also fraught with difficulty. Most quenchers can react with any oxidizing agent and the two highly active oxidizing agents involved in PDT ($^1O_2$ and $HO^{\bullet}$) are able to indiscriminately oxidize many of these quenchers. DMT was the most specific quencher for $HO^{\bullet}$ that we found, but even so the specificity was not complete.

Side-by-side comparison of the probe studies however, showed that BB6 was a PS more likely to undergo Type I mechanism, while PEI-ce6 was a PS that was more likely to undergo Type II. Several previous studies have examined the photochemical mechanisms of photoactivated fullerenes. Arbogast et al showed that fullerenes dissolved in organic solvents in the presence of oxygen seem to preferentially produce $^1O_2$ (45). By contrast Yamakoshi et al found that in polar solvents, especially those containing reducing agents (such as NADH at concentrations found in cells), illumination of various fullerenes will generate different ROS produced by Type 1 mechanism such as superoxide anion and hydroxyl radical (46). Mroz et al (47,48) showed that photoactivation of a mono-cationic fullerene BB4 (a monosubstituted version of the tris-cationic BB6) produced ROS from Type I mechanism as well as Type II. Despite much theoretical discussion it is still not clear what physical or chemical factors influence the Type I/Type II balance amongst different PS (49). It is considered likely that superoxide anion is first formed by electron transfer to oxygen, and superoxide then dismutates to hydrogen peroxide. Thereafter the mechanism is less certain but has been suggested by Vakrat-Haglili et al (50) and by Silva et al (51) to be basically a photoinduced electron transfer to H_2O_2 to form $HO^{\bullet} + HO^{-}$. Moreover the situation is complicated by the fact that photochemically generated $^1O_2$ can react with biological substrates (such as unsaturated fatty acids) to form secondary radicals such as lipid peroxide radicals (52). The fact that the microenvironment influences this Type I/Type II balance (at least in cell free solutions) is clear from the papers on fullerenes referred to above (46). It is sometimes claimed that Type I mechanism is oxygen independent, but it is more accurate to say that its dependence on oxygen is less than the Type II mechanism, but oxygen is still required to form $HO^{\bullet}$. Experiments that measure oxygen consumption can be used to gain information about photochemical mechanisms in solution.

In the present study, the APDT experiments revealed the PS concentration ratio necessary for killing between Gram-positive and Gram-negative bacterial species is 1:2 or 1:4 for BB6 and 1:25 for PEI-ce6 conjugate. In other studies we have shown that approximately ten times higher concentration of typical antimicrobial PS with cationic charges is needed to kill Gram-negatives than to kill Gram-positives (19,53). Taken together with the higher susceptibility of Gram-negatives to Fenton reagent, the higher ratio killing obtained against Gram-negatives compared to Gram-positives obtained with the Type I mechanism PS (BB6) compared to that found with the Type II mechanism PS, (PEI-ce6), we may infer that Gram-positive bacteria are more susceptible to $^1O_2$, while Gram-negative bacteria are more sensitive to $HO^\bullet$. There have been a few reports that discuss the role of Type I and Type II mechanisms in the PDT killing of microbial cells. Dahl et al (54) showed that gas-phase $^1O_2$ (generated from solid-support bound Rose Bengal and allowed to diffuse across an air-gap) was ten times more toxic to Gram-positive *S. aureus* than it was to Gram-negative *E. coli.* Similar results were reported by Valduga et al (55). Papers from Martin et al (34,56) attributed the PDT killing (mediated by various photosensitizing dyes) of *E. coli* to radical-mediated processes. It is difficult to distinguish between the effect of the Gram-negative permeability barrier excluding the PS and increasing the resistance because the $^1O_2$ is generated outside, and the effect of the barrier effect in excluding extracellularly generated $^1O_2$, that would also increase resistance (55). While it might be thought that $^1O_2$ would easily diffuse through the type of biomolecules that make up bacterial cell walls (lipopolysaccharide (LPS), peptidoglycans, lipoteichoic acids), it is possible that Gram-negative cell walls (but not Gram-positive cell walls) have a component that preferentially reacts with $^1O_2$. This component that reacts with $^1O_2$ could well be LPS that contains mono-unsaturated fatty acids (57)

NaN_3 is well known as a physical quencher of $^1O_2$ (24) and as an inhibitor of PDT induced cell killing caused by $^1O_2$ produced from PS that undergo Type II mechanism refs. We found an increase of HPF probe activation when NaN_3 was added to both Fenton reagent and BB6-mediated PDT. The report from Price et al (44) also mentioned a strong increase in HPF fluorescence upon addition of NaN_3 to Fenton reagent. We believe that a possible explanation of this finding is the production of azide radical ($N_3^\bullet$) from N_3^- and $HO^\bullet$. The lower reactivity of $N_3^\bullet$ (redox potential 1.33V) vs $HO^\bullet$ (redox potential 1.985V) may mean that $N_3^\bullet$ radicals survive long enough to react better with the fluorescence probe that the extremely short-lived $HO^\bullet$. Interestingly NaN_3 significantly potentiated the Fenton-mediated killing of both bacterial classes (and by approximately the same amount). If we are correct in presuming that $N_3^\bullet$ are formed, then these less reactive radicals may also survive long enough to penetrate deeper into microbial cells compared with the more reactive $HO^\bullet$ that may all expend themselves at the outer surface of the bacterial cells. There have been a few papers that discuss the possibility of azide radical being formed from $HO^\bullet$ and NaN_3 and potentiating various kinds of biological damage. Roberts (58) found that addition of 10 mM azide potentiated hemolysis by ^{60}Co ionizing radiation, and since this potentiation was prevented by $HO^\bullet$ scavengers, attributed the observation to production of $N_3^\bullet$ from $HO^\bullet$. Land and Prutz (59) found that addition of 10 mM azide potentiated the inactivation of enzymes by pulse radiolysis and attributed the finding to production of $N_3^\bullet$ from $HO^\bullet$. Ali et al (60) reported that the hemolysis of human red blood cells by a photoactivated (cool fluorescent light) riboflavin–Cu(II) system was enhanced by addition of 150 μM azide. They attributed their observation to inhibition of the photodegradation of riboflavin, but commented that “as the riboflavin–Cu(II) system has been shown to produce $HO^\bullet$ the intermediacy of N3• could not be ruled out”.

We found that NaN_3 inhibited APDT of Gram-negative species much more efficiently than it inhibited the killing of Gram-positive species. How can we explain this surprising finding? If we believe (as discussed above) that Gram-positive species are more susceptible to $^1O_2$

than Gram-negative species, we would have expected that the inhibitory effect of NaN_3 in quenching $^1O_2$ would have been more pronounced against Gram-positive bacteria than against Gram-negative bacteria. We propose two possible explanations. The first explanation is that the cell wall of Gram-positive bacteria is more porous than that of Gram-negative bacteria, which is why PS are able to kill Gram-positives much more effectively than Gram-negatives. This difference in permeability might mean that if N_3^- ions are excluded from penetrating into the cell wall of Gram-positives, they may not be able to reach the sites where $^1O_2$ is produced lessening the efficiency of quenching, whereas in Gram-negatives both the PS and the N_3^- may be confined to the same location in the outer layers of the cell wall where the quenching of the $^1O_2$ would therefore be more efficient. The second explanation concerns the microenvironment of the PS. Some Gram-negative bacteria (but not all) have a more pronounced negative charge than Gram-positive bacteria do (61). Therefore the cationic charged PS may be supposed to bind more strongly to Gram-negatives than they do to Gram-positives. It is likely that tightly bound PS undergo a more Type II photochemical mechanism i.e. produce more $^1O_2$ than $HO^\bullet$, while PS in aqueous solution (as a greater proportion of PS molecules will be if incubated in the presence of Gram-positive bacteria) undergo more Type I photochemical mechanism i.e. produce more $HO^\bullet$ than $^1O_2$. If PS in the presence of Gram-positive bacteria were acting by a more Type I mechanism then this consideration may explain why NaN_3 was less efficient in inhibiting the killing than it was in the case of Gram-negatives where the bound PS were acting in a more Type II mechanism. It should be noted that the effect of different photochemical mechanisms and ROS in APDT may be very different when it is carried out in a biological environment such as blood, serum or infected tissue. In particular the availability of targets for ROS in the host tissue outside of the bacterium may affect the ratio of $HO^\bullet$ and $^1O_2$ available for effective APDT.

Further work will be necessary to confirm some of these hypothetical explanations for our experimental observations. Nevertheless we believe that a relatively simple experimental system (APDT of suspensions of different types of microbial cells) has the potential to reveal mechanistic insights that could be of importance to the PDT field as a whole.

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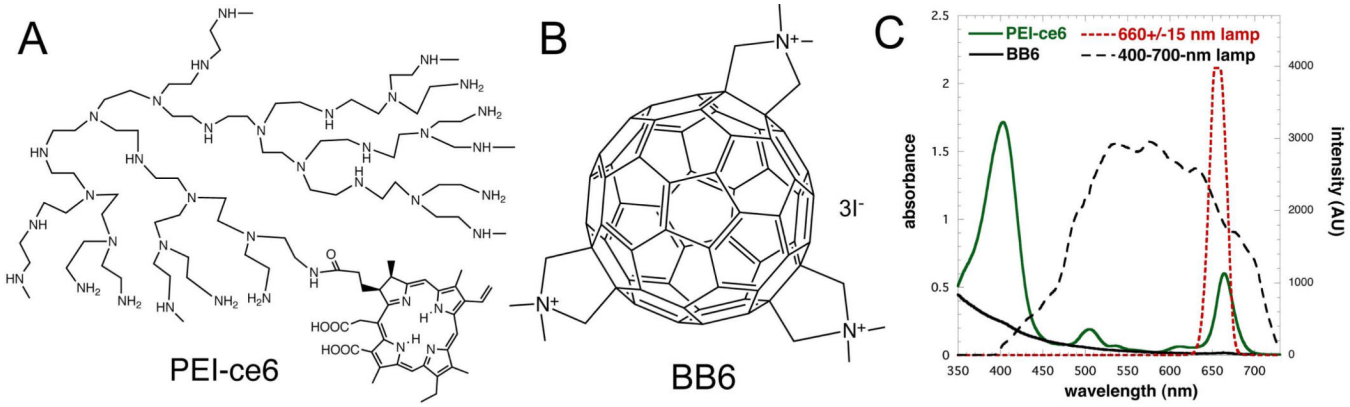

Figure 1. Chemical structures and absorption spectra

(A) PEI-ce6; (B) BB6; (C) Absorption spectra recorded at 10 μM in H_2O or DMA together with emission spectra of the 400—700-nm white light and the 660+/−15-nm red light source.

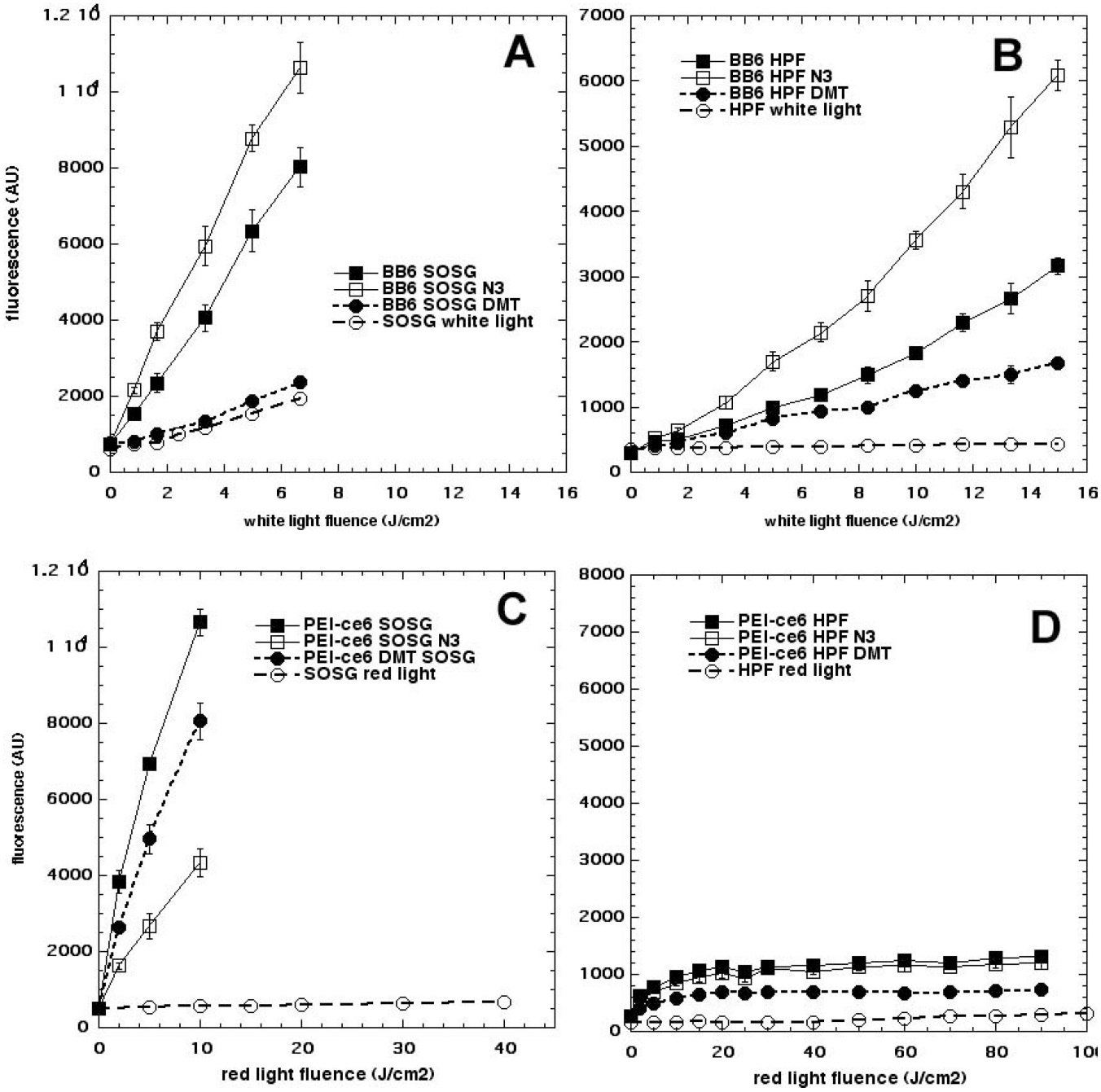

Figure 2. Fluorescence ROS probe studies with photoactivated PEI-ce6 and BB6

Fluorescence generated from probes (10 μM) and PS (2 μM) in water without addition, and with addition of 10 mM NaN3 or 100 mM DMT. (A) BB6 and SOSG, white light; (B) BB6 and HPF, white light; (C) PEI-ce6 and SOSG, 660-nm light; (D) PEI-ce6 and HPF, 660-nm light. The fluences of white light have been corrected to show photons absorbed by the BB6.

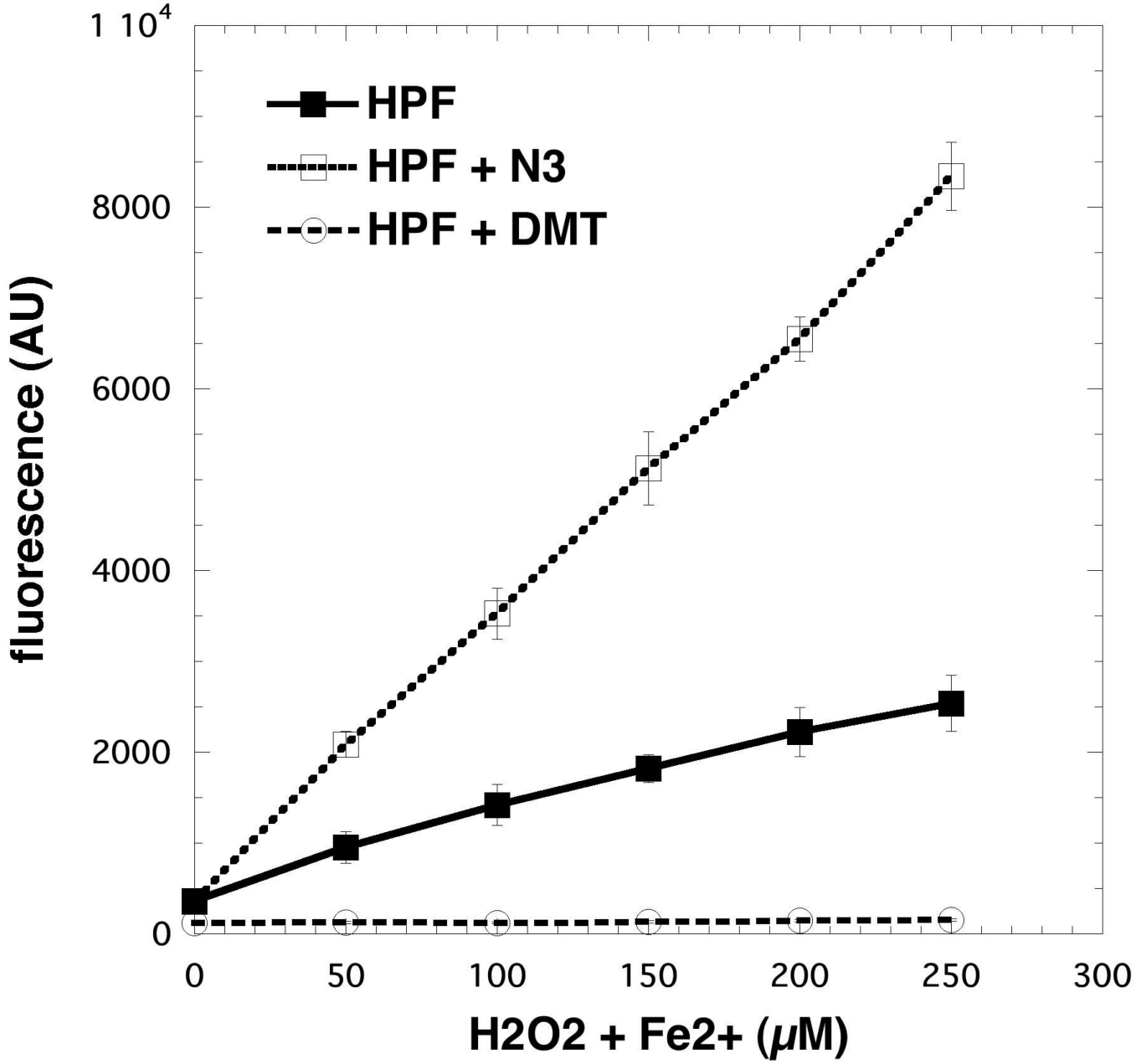

Figure 3. HPF fluorescence probe study with Fenton reagent

Stated concentrations of equimolar $FeSO_4$ and H_2O_2 were added to 10 μM HPF without addition or with addition of 10 mM NaN3 or 200 mM DMT and fluorescence read immediately.

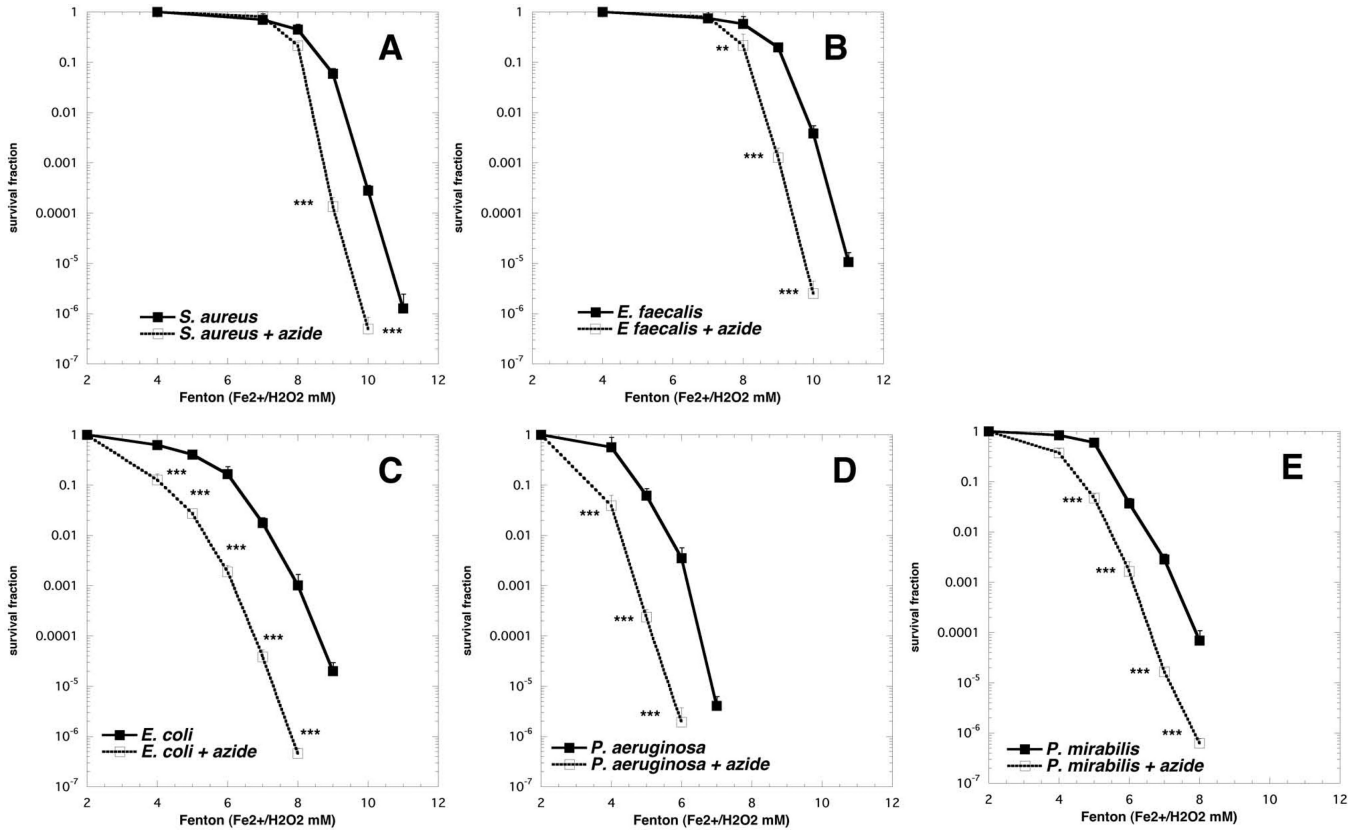

Figure 4. Killing of bacteria by Fenton reagent and effect of NaN_3

Suspensions of bacteria (10(8)/mL) had stated concentrations of equimolar $FeSO_4$ and H_2O_2 added, with or without the addition of 0.5 mM NaN_3 and after 60 min aliquots were removed for CFU determination. *** P<0.001; ** P<0.01 one way ANOVA. (A) *S. aureus;* (B) *E. faecalis;* (C) *E. coli;* (D) *P. aeruginosa;* (E) *P. mirabilis*.

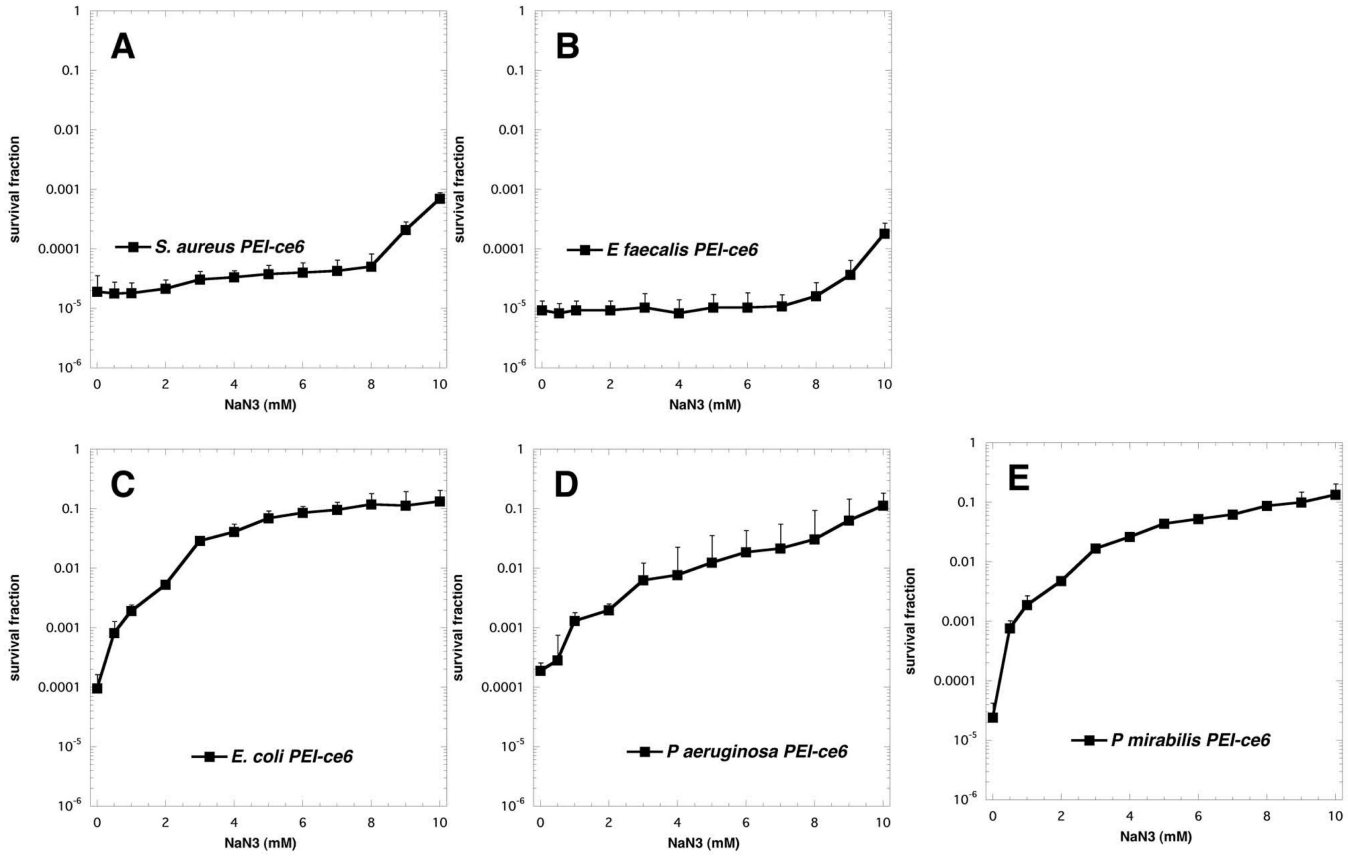

Figure 5. APDT of bacteria mediated by PEI-ce6 and effect of NaN_3

Suspensions of bacteria (10(8)/mL) were incubated with PEI-ce6 for 30 min followed by addition of NaN_3 (0–10 mM) and illumination with 660-nm light and CFU determination. (A) 400-nM PEI-ce6 and *S. aureus;* (B) 400-nM PEI-ce6 and *E. faecalis;* (C) 10 μM PEI-ce6 and *E. coli;* (D) 10 μM PEI-ce6 and *P. aeruginosa;* (E) 10 μM PEI-ce6 and *P. mirabilis*.

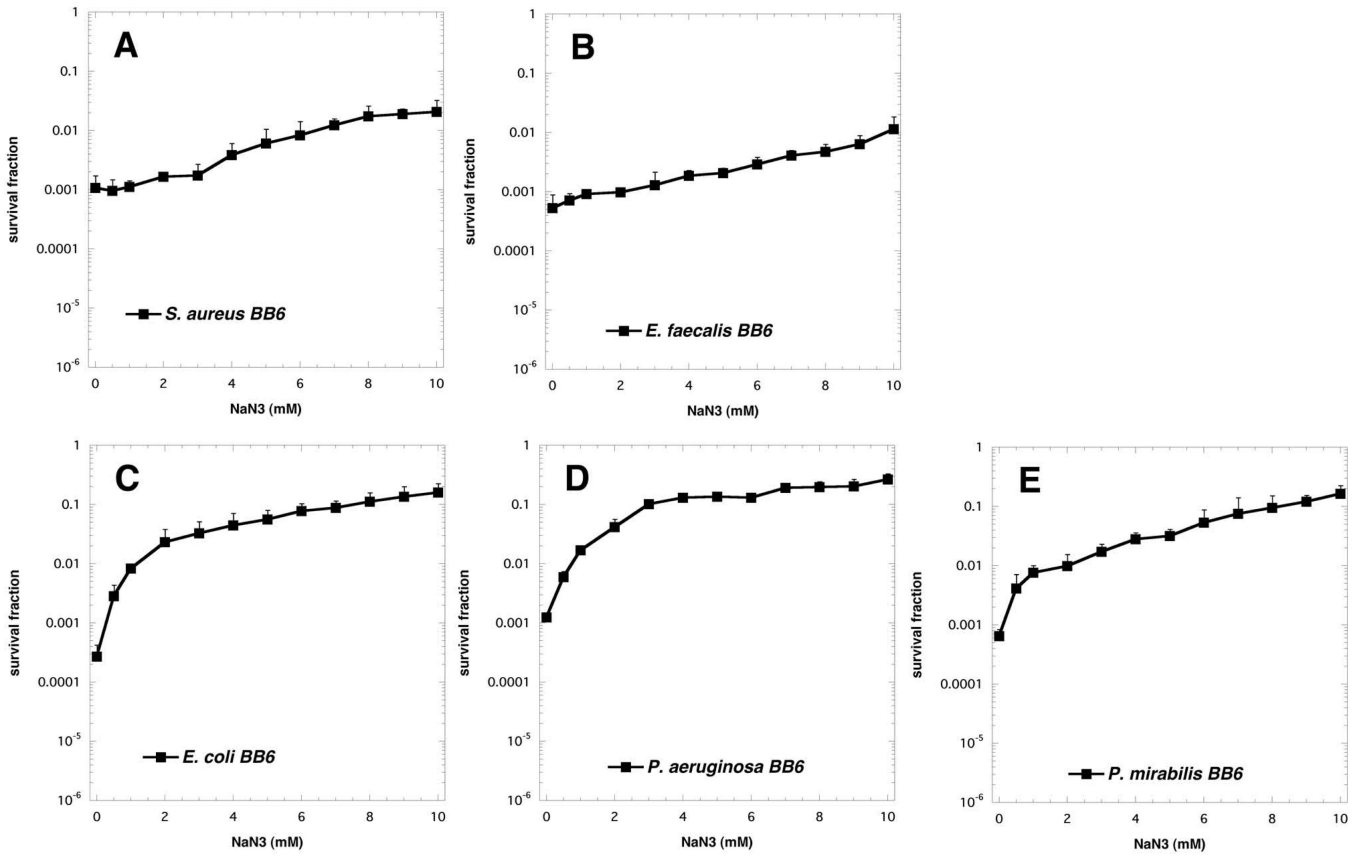

Figure 6. APDT of bacteria mediated by BB6 and effect of NaN_3

Suspensions of bacteria (10(8)/mL) were incubated with BB6 for 30 min followed by addition of NaN_3 (0–10 μM) and illumination with white light (400–700-nm) and CFU determination. (A) 1 μM BB6 and *S. aureus;* (B) 1 μM BB6 and *E. faecalis;* (C) 2 μM BB6 and *E. coli;* (D) 4 μM BB6 and *P. aeruginosa;* (E) 4 μM BB6 and *P. mirabilis*.