

Full Length Research Paper

Influence of hydrogen peroxide or gold nanoparticles in protoporphyrin IX mediated antimicrobial photodynamic therapy on *Staphylococcus aureus*

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Antimicrobial photodynamic therapy (APDT) has been considered to treat skin diseases infected by drug resistant microorganisms. In this work, attempts were made to study the effectiveness of APDT with protoporphyrin IX (PPIX) in the presence of gold nanoparticles (GNPs) or hydrogen peroxide (H₂O₂) against the human bacterial pathogen, *Staphylococcus aureus*. To photoactivate the PPIX, xenon light source was used at 626 nm. The bactericidal effect was analyzed by standard plate counting method. Attempts were also made to study whether fluorescence spectroscopy can be used to characterize the damage at protein level. Results of the study revealed that PPIX with H₂O₂ has showed higher bactericidal effect than that of PPIX alone and PPIX with GNPs. From fluorescence spectroscopic characterization it was found that protein damage is one of the reasons of bactericidal effect as there is a considerable change in the intensity of emission and fluorescence lifetime of tryptophan present in the microorganism between pre and post APDT.

Key words: Photodynamic therapy, hydrogen peroxide, gold nanoparticles, *Staphylococcus aureus*, antimicrobial photodynamic therapy.

INTRODUCTION

Staphylococcus aureus is one of the serious human pathogens, which causes a wide range of diseases such as wound infections, septic arthritis, osteomyelitis and endocarditis (Chopra, 2003; Gemmell, 2004). Methicillin-resistant *S. aureus* (MRSA) strains are considered to be the most dangerous, as they cause and develop infections efficiently and they are also exhibit resistant to all types of β -lactam antibiotics and other antimicrobials (Nakonieczna et al., 2010). Further, MRSA infections may be life threatening to immunodeficiency patients in particular, HIV patients. Many studies are focused on the development of new treatment modalities to treat the drug

drug resistant pathogenic microorganisms such as MRSA.

Although, photodynamic therapy (PDT) is most commonly used for various oncological applications, it has been considered for many non-oncological applications (Wilson, 2004). Antimicrobial PDT (APDT) is one of the non-oncology applications. APDT is used against a wide range of bacteria, viruses and fungi, which are extremely harmful (Jori, 2004). PDT uses light activated chemical termed as photosensitizer (PS) and visible light of appropriate wavelength (Vera et al., 2012). The activation of the PS leads to the generation of reactive oxygen species (ROS) results in cytotoxic effect

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for bacterial cells mainly in the cytoplasmic membrane and in DNA (Hamblin and Hasan, 2004). Furthermore, microorganisms are found to be more susceptible to PDT when compared with mammalian cells (Zeina et al., 2002). Based on these, APDT has been used in dental applications for treating oral candidosis (Mima et al., 2010) gingivitis (Raghavendra et al., 2009) and root canal infections (Garcez et al., 2007).

Recently, many showed interest in the use of gold nanoparticles (GNPs) to improve the therapeutic efficacy (Zharov et al., 2005). It has been reported that GNPs enhance the effectiveness of the APDT by two possible mechanisms that is, enhancing the absorption of light /laser photons by the PS, by having absorbed the PS on its surface and by favoring the non-radiative decay process by forming excess ROS (Narband et al., 2008).

Hydrogen peroxide (H_2O_2) is an oxidizing agent used widely in removing dead tissue and cleaning wounds (Feuerstein et al., 2006). It is reported that, H_2O_2 can be combined successfully in APDT to enhance the effectiveness of the PS (McCullagh and Robertson, 2006). Garcez et al. (2011) studied the antimicrobial photodynamic effect of methylene blue (MB) in the presence of H_2O_2 to kill *S. aureus*, *Escherichia coli* and *Candida albicans*. They found that H_2O_2 is an interesting approach to improve the antimicrobial effect of MB.

Protoporphyrin IX (PPIX) has been used to produce bactericidal effect against several bacterial species including MRSA (Orenstein et al., 1997). Previous published studies (Grinholc et al., 2007) showed an effective antibacterial activity of PPIX under light irradiation even in low concentration of 10 μ M. However, to the best of our knowledge, there is no published data on the use of PPIX in combination with GNPs and/or H_2O_2 in APDT against *S. aureus*.

In this context, the aim of the present study was to compare the antimicrobial photodynamic effect of PPIX in the presence of 16 nm GNPs or H_2O_2 against *S. aureus*. Standard plate counting method was used to determine the number of surviving bacteria after different treatments. Further, attempts were also made to study the steady state and time resolved fluorescence spectroscopic characterization of *S. aureus* dully pre and post APDT to understand the molecular changes in particular at protein level due to APDT.

MATERIALS AND METHODS

Bacterial culture

S. aureus (clinical isolates) was obtained from Chettinad Hospital and Research Institute, Chennai. The bacterium was maintained in tryptic casein agar (Himedia, Mumbai, India) in stock culture plates till it is used for the experiment. Before the experiments, the microorganism was grown in Muller–Hinton broth (MHB) (Himedia, Mumbai, India) at 37°C with shaking at 200 rpm in LS 500 incubator/shaker (Neolab, Mumbai, India) for 16 h. The culture was then harvested by centrifugation at 10,000 rpm for 10 min and washed

three times using normal saline (NS) (PH 7.0). Subsequently the bacterium was diluted in 0.9% NS at an optical density (OD) of 0.05 at 600 nm, which corresponded to approximately 2×10^6 colony-forming units (cfu)/mL.

Synthesis of gold nanoparticles

GNPs were synthesized using the reduction of chloroauric acid ($HAuCl_4$) with sodium citrate as reported by Turkevich et al. (1951) and Kimling et al. (2006). In brief, 2 mL of 10 mM $HAuCl_4$ was made into 100 mL in double distilled water and the solution was boiled under stirred condition. Subsequently, 3 mL of 1% sodium citrate in aqueous medium was added and the boiling continued for further 30 min till the color changed from light purple to wine red. The solution was cooled to room temperature. UV/VIS absorption spectrophotometer (Lambda 35, Perkin Elmer, USA) was used to characterize the nanoparticles. The absorption spectrum of the GNPs is shown in Figure 1. The peak at 522 nm indicates that the diameter of the GNPs is 16 nm (Wang et al., 2011; Kimling et al., 2006).

Photosensitizer and light source

PPIX (Sigma-Aldrich, St. Louis, USA) and hydrogen peroxide (Merck, Mumbai, India) were used for the sensitization of *S. aureus*. PPIX and H_2O_2 were used at concentration of 30 μ M and 100 mM, respectively. The light source used in this study was the 300 W Xenon lamp (Mini-Crimescope-MCS400, ISA Jobin Yvon-Spex, Edison, NJ) with a filter wheel and a liquid light guide arrangement. The broad band of the Xenon lamp was filtered using a band pass filter centered at a wavelength of 630 ± 20 nm. The light intensity of the lamp was measured by the Field Maser GS power meter (Coherent INC, USA) and it was found to be 20 mW. The emission spectrum of the lamp was measured using the spectrometer (USB 4000-VIS-NIR, Ocean Optics, USA) and the emission peak was found at 626 nm. The emission spectrum of the light source is shown in Figure 2.

Lethal photosensitization

To investigate the photodynamic effect of PPIX (30 μ M) in the presence or absence of GNPs (5.28×10^{11} particle/mL) or H_2O_2 (100 mM), 0.1 mL of bacterium was mixed with 0.1 mL of PPIX-GNPs or PPIX- H_2O_2 mixture and incubated for 15 min in 96 well plate (Tarsons, Kolkata, India). Bacterium samples were irradiated separately with xenon light source for 15 min (light dose 18 J/cm²). Samples were irradiated in the dark under aseptic conditions in a laminar flow. A number of samples were kept as controls which include bacterium alone, bacterium illuminated with light and bacterium mixed with PPIX or H_2O_2 or GNPs alone without light illumination and bacterium mixed with PPIX irradiated with light.

Bacterial survival assay

Standard plate counting method was used to determine the number of CFU/mL of bacterium before and after the different treatments. 0.1 mL was taken from each sample and 1:10 serially diluted six times in NS, spread on nutrient agar plates and incubated at 37°C for 48h.

Steady-state fluorescence measurements

Steady state fluorescence spectral characteristics of tryptophan

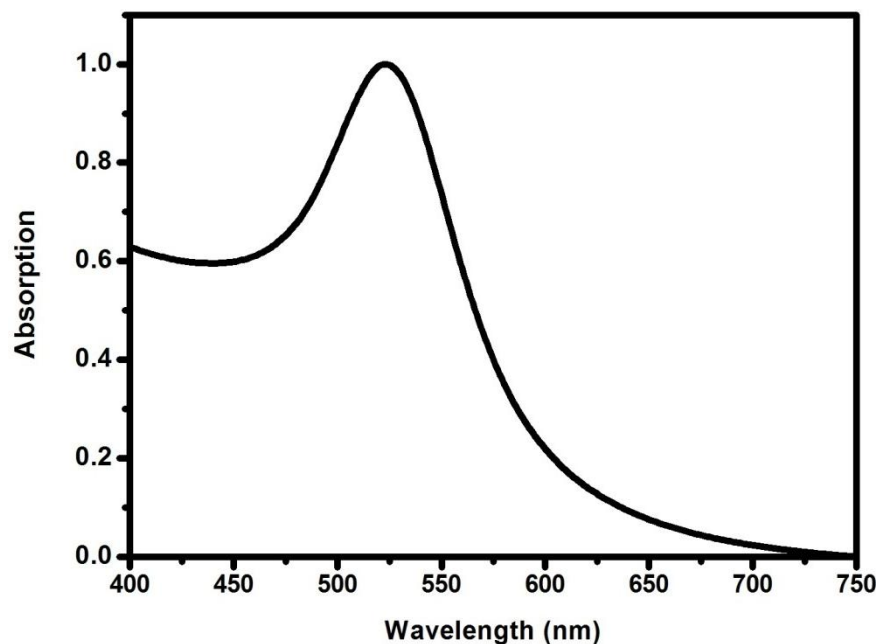


Figure 1. Absorption spectrum of 16 nm GNPs.

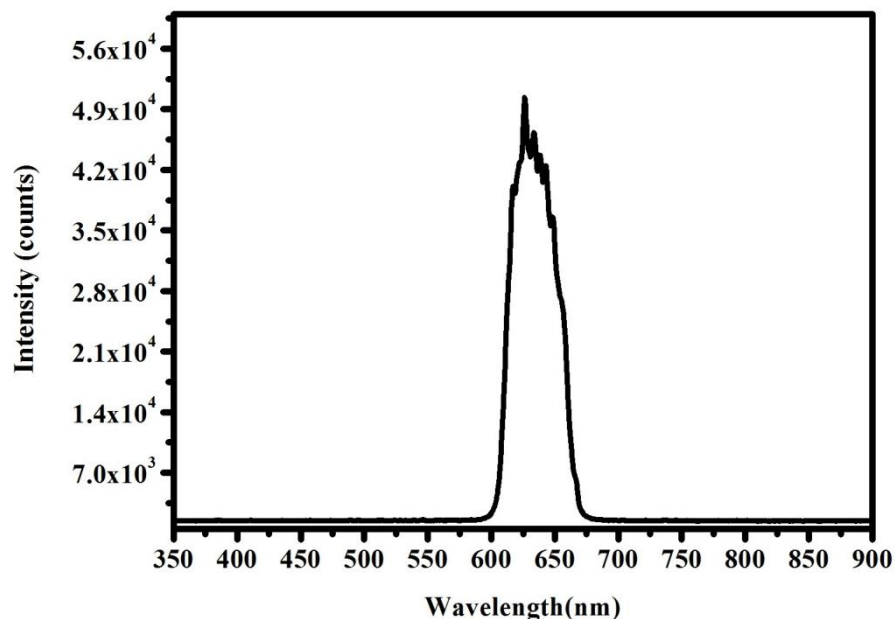


Figure 2. Emission spectrum of xenon lamp.

from the treated and un treated *S. aureus* was recorded using spectrofluorometer (FluoroMax-2, ISA Jobin Yvon-Spex, Edison, NJ) as reported by Ebenezer et al. (2010). The excitation source (150 W ozone-free xenon arc lamp) coupled to the excitation monochromator to obtain the light of a desired wavelength and the fluorescence emission was collected using the emission monochromator connected to a photomultiplier tube (R928P, Hamamatsu, Shizuoka-Ken, Japan). The gratings in the excitation and emission monochromators have a groove density of 1200 grooves/mm.

During the fluorescence data acquisition, the excitation and the emission slit widths are set at 5 nm with an integration time of 0.1 s. The collected signal is transferred to the PC through an RS232 interface. The data were processed by the Windows-based data acquisition program Data Max powered by GRAMS/386®. Two hundred microlitres of bacterium was added to 200 μ L of PPIX-GNPs or PPIX- H_2O_2 mixture. Two samples were used as controls, bacterium alone and bacterium mixed with PPIX and illuminated with light. Samples were irradiated separately with light for 15 min

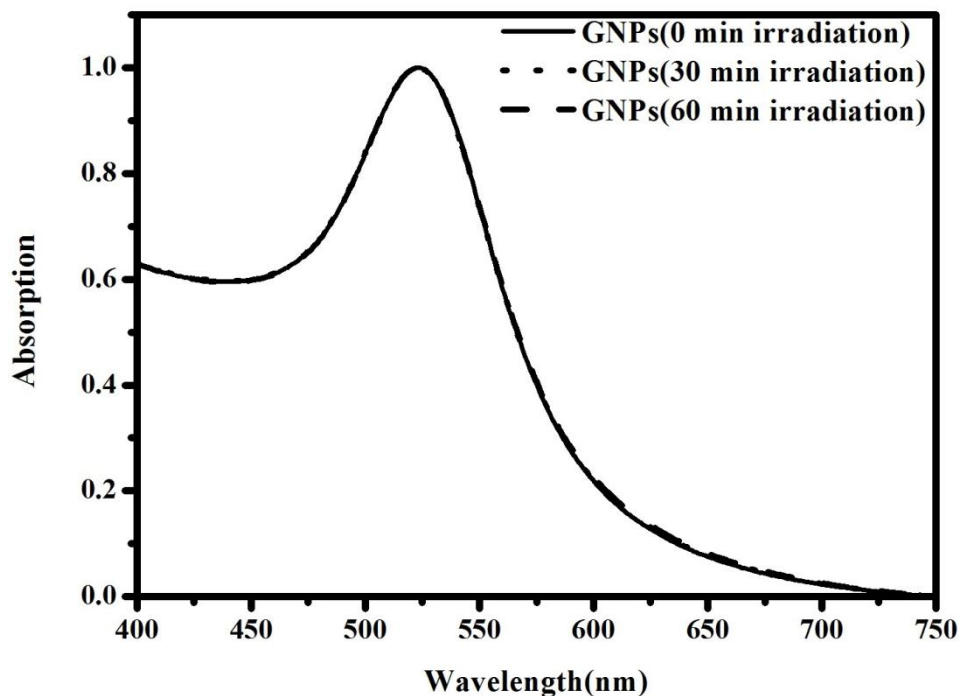


Figure 3. Normalized absorption spectral changes of GNPs after 0, 30 and 60 min of irradiation.

in 12 well plate. Appropriate volume of NS was added to bring the total volume of each sample in the cuvette to 1.50 mL. Samples were excited at 280 nm and emission spectrum was collected in the wavelength range of 300 to 540 nm.

Fluorescence lifetime measurements

Fluorescence lifetime measurements were made using Time Correlated Single Photon Counting System (TCSPC, HORIBA JOBIN YUVON IBH, UK) by exciting the samples using 280 nm Nano LED (Pulse width: <1ns), with a fast response red sensitive PMT (Hamamatsu Photonics, Japan) detector. The fluorescence emission was collected at 90° from the path of the light source. The electrical signal was amplified by a TB-02 pulse amplifier (Horiba), fed to the constant fraction discriminator (CFD, Phillips, The Netherlands). The first detected photon was used as a start signal by a time-to-amplitude converter (TAC), and the excitation pulse triggered the stop signal. The multichannel analyzer (MCA) recorded repetitive start-stop signals from the TAC and generated a histogram of photons as a function of time-calibrated channels (55.48 ps/channel) until the peak signal reached 10,000 counts for 340 nm emission. The instrument response function was obtained using a Rayleigh scatter of Ludox-40 (40wt. % suspension in water; Sigma Aldrich) in a quartz cuvette at 280 nm excitation. Decay analysis software (DAS6 V6.0, Horiba) was used to extract the lifetime components. The goodness of fit was judged by chi-square values, Durbin-Watson parameters, as well as visual observations of fitted line, residuals and autocorrelation functions.

Statistics

Each experiment was performed twice and at least in triplicate. Values are expressed as means \pm standard deviation.

RESULTS

Test GNPs stability under light irradiation

As the synthesized GNPs were subjected for photo irradiation in the presence of PS, it is required to know whether the GNPs undergo any change in the size and shape due to illumination or not. In this context, the stability and aggregation of GNPs (if any) during the illumination was tested at various energy densities of the light source (0 J/cm², 36 J/cm² and 72 J/cm²) at 626 nm. The maximum absorption of GNPs at 522 nm did not change even after 60 min of exposure (at 72J/cm²). Figure 3 shows the absorption spectra of GNPs exposed at different energy densities. The results are correlated with that reported by Wang et al. (2011).

Photodynamic therapy

Figure 4 shows the colony forming units (CFU) of *S. aureus* under dark environment but with either PPIX, GNPs or H₂O₂ and after APDT with PPIX in the presence and absence of GNPs or H₂O₂. From Figure 4, it is observed that there are no considerable changes in the CFU due to individual interaction of light, PPIX, GNPs and H₂O₂. However, due to APDT with PPIX it is observed that there is 1.5 log reduction of *S. aureus* with respect to the control and dark. When the bacterium was irradiated in the presence of PPIX-GNPs mixture, the

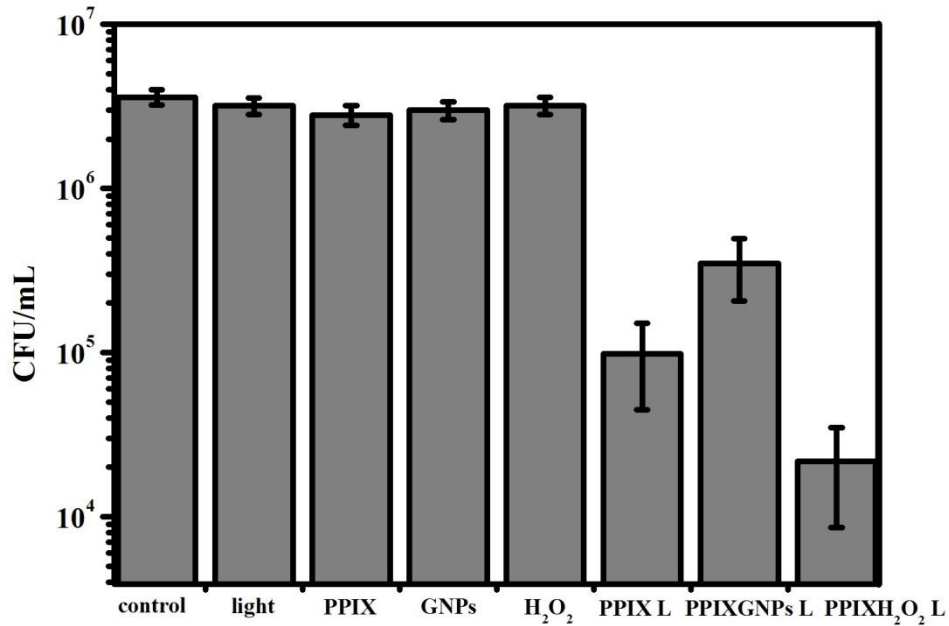


Figure 4. Colony forming unit (CFU) of *S. aureus* before and after APDT.

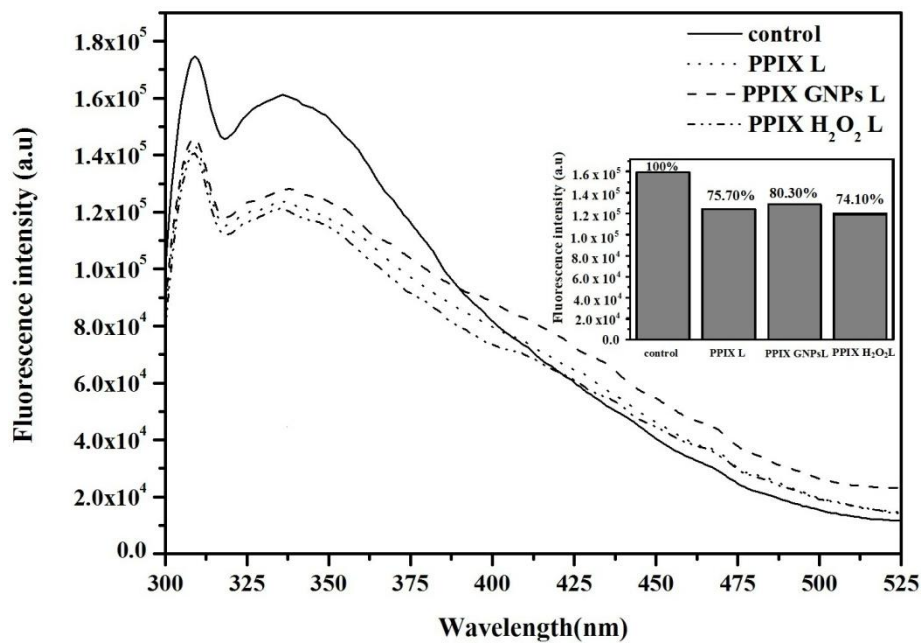


Figure 5. Autofluorescence spectra of *S. aureus* at 280 nm excitation from the control and different APDT groups. The inset shows the reduction of fluorescence intensity after APDT.

reduction was only 1.0 log. APDT in the presence of PPIX-H₂O₂ induced more bacterium reduction (2.0 log).

Steady- state fluorescence

In order to know whether autofluorescence spectroscopy

may be considered for monitoring the APDT, steady-state fluorescence emission spectra under different experimental conditions were measured at 280 nm excitation and they are shown in Figure 5. From the figure, it is found that the overall fluorescence from post treated bacteria showed significant reduction in the emission intensity in wavelength region from 300 to 525 nm. The sharp

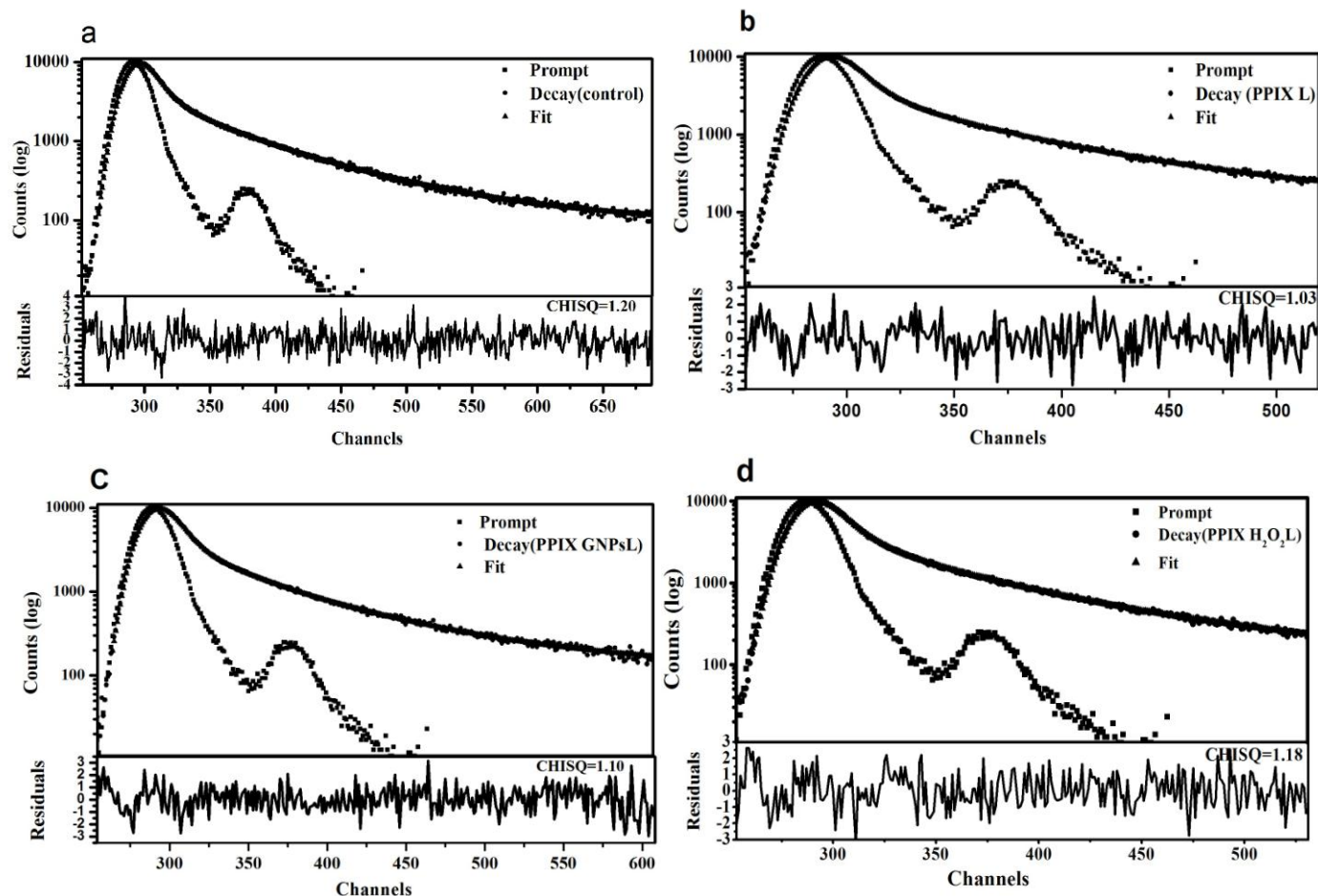


Figure 6. Fluorescence lifetime spectra of tryptophan from *S. aureus*, control (a) *S. aureus* treated with PPIX L (b) PPIX GNPs L (c) PPIX H₂O₂ L (d).

peak at 310 nm for all the treatments is due to the water Raman. The beak at 340 nm is due to the key amino acid, tryptophan which is present in the bacteria. From the inset of Figure 5, it is found that there is a reduction in the peak emission at 340 nm due to APDT with respect to control group. The reduction is more due to APDT with PPIX and H₂O₂ and the reduction of intensity of the different conditions of APDT is in the order of APDT with PPIX and H₂O₂ > APDT with PPIX > APDT with GNPs. The results of autofluorescence characteristics of tryptophan for control and APDT group correlates with that of CFU results.

Fluorescence lifetime

As there is a significant difference in the tryptophan fluorescence between APDT and control *S. aureus*, the excited state kinetics of tryptophan at 280 nm was carried out. Figure 6 shows fluorescence lifetime spectra of tryptophan from *S. aureus* before and after APDT under

different conditions. Tryptophan fluorescence lifetime values for 340 nm decay from *S. aureus* are shown in Table 1 and τ_1 , τ_2 and τ_3 represent the fast, the fastest and the slow decay component respectively. Their relative amplitudes are also given in Table 1. From Table 1 it is found that there is considerable variation in the lifetime values between control and APDT groups. However it is further observed that there is more reduction in the lifetime values for APDT with H₂O₂ than that of APDT with GNPs indicating that APDT with PPIX and H₂O₂ produces more alteration in the key protein than that of other conditions of APDT and with respect to control.

DISCUSSION

Although it is well known that PPIX produce strong bactericidal effect when illuminated with light, the results obtained in present study indicates that the bactericidal effect of PPIX can still be enhanced in the presence of H₂O₂. The bactericidal effect was considerably reduced

Table 1. Fluorescence lifetime values and corresponding amplitudes of tryptophan from *S. aureus* for 340 nm emission.

Treatment	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	A ₁	A ₂	A ₃
control	1.79 ± 0.01	0.36 ± 0.04	4.91 ± 0.05	0.20 ± 0.05	0.41 ± 0.08	0.39 ± 0.03
PPIX L	1.42 ± 0.21	0.32 ± 0.03	4.31 ± 0.12	0.17 ± 0.01	0.42 ± 0.03	0.41 ± 0.01
PPIX GNPs L	1.69 ± 0.03	0.38 ± 0.05	4.64 ± 0.45	0.20 ± 0.05	0.41 ± 0.04	0.38 ± 0.01
PPIX H ₂ O ₂ L	1.14 ± 0.33	0.30 ± 0.02	4.03 ± 0.45	0.16 ± 0.04	0.37 ± 0.0	0.47 ± 0.03

Values are means ± standard deviations for three independent experiments. τ_1 , τ_2 and τ_3 are the lifetime values. A₁, A₂ and A₃ are the lifetime amplitudes; (ns) nanoseconds.

(1.0 log) due to the addition of 16 nm GNPs with PPIX than that with PPIX alone, which is 1.5 log. However, higher bacterial killing (2.0 log) was observed when PPIX-H₂O₂ mixture was added to the bacterium followed by light irradiation. The enhanced bacterial killing of PPIX-H₂O₂ mixture may be explained as suggested by Funk and Krise (2007) which reported that H₂O₂ may alter the membrane permeability and hence the probability of cellular accumulation of the PS, or it may be due to the fact that the photoreaction would cause membrane disruption which in turn facilitate the penetration of H₂O₂ into the cell (Caetano et al., 2007). Seaver and Imlay (2011) suggested that higher effect of PDT in the presence of H₂O₂ could be because PS has an easier transit across the bacterial cell membrane, or the presence of H₂O₂ may increase the amount of molecular oxygen which improves the ROS formation as suggested by Garcez et al. (2011).

Narband et al. (2008) reported the ability of GNPs to enhance the antimicrobial effectiveness of the PS, toluidine blue (TBO) against *S. aureus* when illuminated with light. They observed higher bacterial killing at a concentration of 20 μ M of TBO in the presence of 2 or 15 nm GNPs. They suggested that in the presence of GNPs, TBO relaxes to the ground state by a non-radiative decay and that this process is primarily through formation of other reactive oxygen species—possibly hydroxyl radicals which increase the bacterial kill. Furthermore, Khan et al. (2012) reported the GNPs-enhanced photodynamic therapy of methylene blue (MB) against *Candida albicans* biofilm and they found that GNPs of 21 nm diameter conjugated to MB showed significant reduction of biofilm. In our study, the presence of 16 nm GNPs did not produce any enhancement; on the other hand, the antibacterial activity of PPIX in the presence of GNPs was reduced. This result is in good agreement with the result obtained by Perni et al. (2010). They investigated the antimicrobial activity of light-activated silicon containing methylene blue (MB) and GNPs of different sizes. In their study, they found that only GNPs of 2 nm diameter enhanced the antibacterial activity of MB while 20 nm GNPs reduced the antibacterial activity of MB. The authors referred the reduction of the antimicrobial activity to the light scattering or shielding effect in the presence of large size GNPs. Only at specific ratio between GNPs and PS

the antibacterial activity can be enhanced as explained by Narband et al. (2008). Hence, it is suggested that GNPs of 16 nm size may either scatter or shield the incident photons and hence suppress the photon excitation of PPIX.

It is well known that numerous intrinsic fluorophores such as tryptophan is retained in bacteria and give intense fluorescence when excited in the UV region (Cantor and Schimmel, 1980). Fluorescence from tryptophan has been used extensively to study and characterize proteins (Lakowicz, 1999). In this study, fluorescence spectroscopy has also utilized to know whether it provides additional information in particular the antibacterial activity due to APDT. To the best of our knowledge, there is only one study which reported the changes in steady-state fluorescence of tryptophan from *S. aureus* under different bactericidal agents. Alimova et al. (2005) measured the steady-state fluorescence of tryptophan for five species of bacteria (including *S. aureus*), subjected to three bactericidal agents. They found that the addition of hydrogen peroxide (H₂O₂) to *S. aureus* produced 33% decrease in tryptophan fluorescence intensity. In this study, the largest reduction of tryptophan steady-state fluorescence intensity was 25.90% from *S. aureus* when added with PPIX-H₂O₂ mixture and irradiated with light. Based on these observations, the decreases in tryptophan fluorescence intensity may correspond to protein damage (Manpreet et al., 1998).

In order to study further, excited state kinetics of tryptophan were also carried out due to pre- and post-APDT. Tryptophan residues in protein have a multi-exponential decay which indicates the existence of multiple ground states conformations, dynamic motion of quenchers or acceptors or time-dependent spectral relaxation (Lakowicz, 2000). The effect of reducing the slow fluorescence lifetime component arises from the existence of hydrogen bonding in proteins which limit the ability of tryptophan residues to rotate. The change in fluorescence lifetime for five species of bacteria (including *S. aureus*) subjected to different bactericidal agents was reported by Alimova et al. (2005). They found that bactericidal agents decreased both the fast and the slow decay components accompanied by increase and decrease of the fast and the slow relative amplitude components, respectively. Our results are in good agreement with this study as bacterium treated

with PPIX alone or PPIX-H₂O₂ followed by light irradiation showed a decrease in both, the fast and slow components

Conclusion

In summary, PPIX with H₂O₂ has showed higher bactericidal effect than APDT with PPIX alone. However, APDT with GNPs exhibit lesser antimicrobial effect. Steady and excited state fluorescence spectroscopy may be considered to characterize the molecular changes at proteins level due to pre and post APDT as well as monitor APDT efficiency.

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