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On the low photo-inactivation rate of bacteria in human plasma
II. Inhibition of methylene blue bleaching in plasma and effective bacteria destruction by the addition of dilute acetic acid to human plasma

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Abstract

Methylene blue (MB) and other photo-sensitizer molecules have been recognized as effective means for the inactivation of bacteria and other pathogens owing to their ability to photo-generate reactive oxygen species (ROS) including singlet oxygen. These reactive species react with the membrane of the bacteria causing their destruction. However, the efficiency of MB to destroy bacteria in plasma is very low because the MB 660 nm absorption band, that is responsible for the ROS generation, is bleached. The bleaching of MB, in plasma, is caused by the attachment of a hydrogen atom to the central ring nitrogen of MB, which destroys ring conjugation and forms Leuco-MB which does not absorb in the 600 nm region. In this paper we show that addition of dilute acetic acid, ~ 10^{-4} M, to human plasma, prevent H-atom attachment to MB, allowing MB to absorb at 660 nm, generate singlet oxygen and thus inactivate bacteria. The mechanism proposed, for preventing MB bleaching in plasma, is based on the oxidation of cysteine to cystine, by reaction with added dilute acetic acid, thus eliminating the availability of the thiol hydrogen atom which attaches to the MB nitrogen. It is expected that the addition of acetic acid to plasma to be effective in the sterilization of plasma and killing of bacteria in wounds and burns.

1. Introduction.

The excessive use of antibiotics, in recent years, has brought a threat to public health [1] and the prediction, by some experts in this field, that the end of the antibiotics era is approaching [2-4]. Therefore, alternative means for the eradication of harmful bacteria, which do not have the disadvantages of drugs that induce immune strains, are been seriously considered. Among them, photodynamic therapy (PDT), a widely known method for bacteria inactivation achieved by the illumination of photosensitive molecules with visible light, is now frequently used [5]. The treatment of bacterial infection by PDT has been rather successfully employed in dermatology and other areas of medicine [6-8]. In the experiments described in this paper, MB molecules are excited by 6-8 mW/cm^2, 661 nm LED light, to the strongly absorbing first excited singlet state ($\varepsilon_{664\ nm}$ =7.7x10^4 cm^{-1} M^{-1}), which decays with a 10^9 s^{-1} rate to the long lived MB triplet state [9, 10]. The triplet state in turn transfers electrons (reaction type I) or energy (reaction type II) to ground state atmospheric molecular triplet oxygen, $^3O_2$, to form OH radicals and singlet oxygen, $^1O_2$ ($^1\Delta_g$), respectively[11]. Both of these species are very reactive, so called reactive oxygen species(ROS), as made evident by their reactive mean free paths of ~10 A for OH radicals and ~100 A for singlet oxygen [12, 13]. This short reaction range places a limit to the distance that must be maintained between bacteria and MB in order for the ROS to react efficiently with and destroy the bacteria membranes.

Bacteria membranes vary in composition: Gram negative bacteria are enveloped by negatively charged membranes walls, therefore photosensitive molecules with cationic substituents are expected be more effectively attached to bacteria than to mammalian cells. In contrast, Gram-positive bacteria may be inactivated by attachment to anionic or neutral photosensitive molecules. Most Gram-positive bacteria dispersed in 10^{-5} M MB/ phosphate-buffered saline(PBS) solution are inactivated within a few, 10-15, minutes, when irradiated with the 8 mW/cm^2, 661 nm LED light and even faster in slightly alkaline (pH 8 or 9) solutions [14]. The inactivation rate of Gram-

negative bacteria is much slower than that of Gram-positive bacteria and both become highly resistant to photo-inactivation when dispersed in plasma as shown in Table 1.Sterilization of pathogens in pooled human plasma is achieved, to a large extent, by solvent detergent methods [15, 16], while single donor human plasma, to be used for transfusion, is often sterilized by photo-generated ROS, mostly singlet oxygen [17]. Similarly, when the bacterial infection is caused by burns or other means where the bacteria reside in plasma, MB has been shown to be an ineffective bacterial killing agent [18] because its 660 nm absorption band is bleached in the presence of plasma. The bleaching of MB in plasma is thought to be caused by the attachment of a hydrogen atom onto the MB central ring nitrogen [19] which destroys ring conjugation and forms leuco-methylene blue (LMB) that does not absorb in the red region of the spectrum and therefore does not generate singlet oxygen when illuminated with 660 nm light, Scheme 1. The drastic decrease of the 660 nm absorption band optical density (OD), of MB in plasma, as a function of illumination is shown in Figure 1. This figure also shows that in other media, including water and PBS, the optical density of the 660 nm band remains constant with illumination. In fact, we find that using MB/plasma solutions that contain 10^7 colony forming units (CFU) per cm^3 Gram-negative bacteria, only about 1-3 log of bacteria were killed after two hours of LED illumination. The bacteria that were used in this research are listed in Tables 1 and 2. Table 1 shows the MB photo-inactivation of Gram positive and Gram negative bacteria in PBS and plasma as a function of illumination time. Inactivation time is defined in this paper, as the period of time necessary to reduce the 10^7 live bacteria to 10^2. These data clearly show that under the same experimental conditions, practically all bacteria tested in this study are destroyed at a much higher rate in PBS than in plasma solutions.

In this report we propose a mechanism and specific reactions, supported by experimental data, for the bleaching of MB in plasma. Our data also show that the addition of dilute, ~10^{-4} M, acetic acid,, into the bacteria/plasma solution is sufficient to stop the bleaching of the MB 660 nm absorption band and allow for the formation of sufficient MB triplet states and singlet oxygen concentration to destroy bacteria in plasma at a rate equivalent to that in PBS solution. Dilute acetic acid, 10^{-4} M, did

not have any deleterious effect on the biofluids used in this study or others that we are cognizant of. This is important because human plasma transfusion is a most frequent event, throughout the world, therefore bacterial and viral decontamination at any level cannot be overstated. Even though the patient sepsis caused by the transfusion of bacteria and virus contaminated blood is thought to be small [20], it cannot be dismissed because new and more drug-resistant strains of bacteria are continuously encountered[21, 22].

2. **Experiments**.

The human plasma used in our experiments was commercially available, pooled plasma that was not useful, any longer, for human transfusion. It was kept refrigerated until used, following the procedures approved by the Institutional Review Board. Pure grade albumin, gamma-globulin, fibrinogen cysteine and methylene blue were purchased from Aldrich-Sigma. 9, 10-anthracene diproprionic acid (ADPA) salt, used to determine the amount of singlet oxygen generated in various solutions, was also purchased from Aldrich-Sigma as ADPA and made to a salt by addition of 10^{-4} M NaOH. The samples were placed in 0.5 cm optical path length quartz cells, unless otherwise stated, and their absorption spectra were recorded by a Shimatsu 1600 UV spectrophotometer. The solutions were poured into open quartz cuvettes placed, on ice to slow down bacteria growth, and illuminate with 8 mW/cm^2, 661 nm LED light for predetermined periods of time. The absorption spectra and change in OD of each solution were recorded before and after each irradiation periods. The acetic acid was added first to the plasma, followed by the addition of MB. The concentration of acetic acid was adjusted to be sufficient enough to oxidize all of the cysteine thiol, S-H, groups, in the solutions studied. The concentration of bacteria in the samples tested was 10^{7} CFU unless otherwise stated. Solution of plasma that does not contain albumin were also illuminated and found to behave as the PBS solutions, namely did not bleach the 660 nm of MB as was the case with human plasma solutions. For plasma experiments without particular proteins, the plasma was ultrafiltered (exclusion 5000 daltons) and then added back independently of albumin, gamma globulin and fibrinogen. Thus we had utrafiltrates with either gamma globulin or fibrinogen

but no albumin.

3. **Results and discussion**.

3.1 Relationship between bleaching MB and singlet oxygen generation

The relative amount of singlet oxygen generated by each MB solution, after 661 nm LED illumination was determined by the slope of the decay of the 400 nm ADPA band OD as a function of irradiation time [23]. ADPA is known to react with singlet oxygen, to form endoperoxide, ADPA-O_2, which does not absorb in the 400 nm region where ADPA has its first excited singlet state absorption band [23, 24], Scheme 2. Therefore the decrease of the 400 nm ADPA absorption band is a measure of the amount of singlet oxygen formed. We may note that ADPA does not react with atmospheric ground state triplet molecular oxygen. Solutions of human plasma**,** 2.0 x 10^{-5} M MB and 10^{-4}M ADPA were placed in 0.5 cm optical path length quartz cells and their absorption spectra recorded between 800 nm and 200 nm. These solutions were subsequently exposed to 661 nm LED light for intervals of time ranging from 10 sec. to 2 minutes and their spectra recorded after each light exposure. Figure 2, shows the decay of the 660 nm absorption band of MB and the 400 nm absorption band, of ADPA, as a function of irradiation time with the 661 nm LED light.

These time resolved spectra changes indicate that while the 660 nm MB band OD decreases with light exposure at a rather high rate, the intensity of the 400 nm ADPA absorption band remains constant. These data are in agreement with previously published data [24] that attribute the bleaching of the MB excited state to the loss of the MB ring conjugation, possibly, by the addition of an H-atom to the MB central ring nitrogen atom, forming the leuco-MB type molecule which does not absorb at 660 nm, Scheme 1. The expected very low photo-generation of singlet oxygen, if any, in the plasma/MB solutions is substantiated by the unaltered OD of the 400 nm absorption band of ADPA

with illumination, Figure 2. As pointed out previously, the 400 nm band of ADPA decreases as ADPA reacts with singlet oxygen, Scheme 2 and remains constant if singlet oxygen is not present in the solution.

3.2 Preventing the bleaching of MB in plasma

It has been proposed [24] that in plasma/MB solutions the H-atom that attaches onto the MB nitrogen is the hydrogen-atom of the cysteine amino acid thiol, S-H group. Cysteine is an amino acid of albumin, which the major protein component of human plasma. The most common reaction of the cysteine thiol group is reversible oxidation. In this oxidation reaction two cysteine molecules combine after oxidation to form the well-known S-S disulfide bridge of the cystine dimer, Scheme 3. Such redox reactions occur frequently in proteins and constitute the mechanism that stabilizes proteins and in plasma the cysteine/cystine redox reaction cycle is in fact considered to be a major regulating for cell survival [25, 26]. Considering this cysteine/cystine redox reaction, and assuming that the hydrogen that reacts with MB and attaches to its central nitrogen is the cysteine H-atom of the plasma protein albumin, we have investigated means by which this reaction may be inhibited, thus preventing the bleaching of the 660 nm MB band. We find that the other plasma proteins, fibrinogen and gamma-globulin, that do not contain cysteine amino acids, do not bleach the 660 nm MB band. To that effect we oxidized the cysteine thiol, S-H, group to cystine, S-S dimer bridge using dilute, $\sim 10^{-4}$ M, acetic acid in order to eliminate the availability of the S-H hydrogen atom for attachment to MB and thus prevent bleaching of MB and formation of LMB and allows the MB molecule in plasma solutions to be excited to its strongly absorbing 660 nm first excited singlet state and photo-generate sufficient amounts of singlet oxygen, by type II reaction and kill, efficiently, bacteria and other pathogens. The drastic difference in the bleaching of the 660 nm MB absorption band in plasma with and without the addition acidic acid is evidenced in Figure 2. It is rather important to note that the acetic acid is added to the plasma before the addition of MB to the plasma bacteria solution. This figure, also, shows that in plasma/MB/acetic acid suspension the 660 MB absorption band OD remains constant throughout the 160 sec. irradiation interval, while the

400 nm ADPA absorption band OD decreases at a high rate. The slope of the 660 nm band is practically zero, while the ADPA 400 nm band decreases with a slope of 3.4 x 10^{-3} s^{-1} during the first 40 seconds of irradiation, indicating that a high yield of singlet oxygen is formed during the irradiation time of in the plasma/MB solution. The decay rate of the 400 nm ADPA OD suggests that the addition of acetic acid induces the MB/plasma solution to generate singlet oxygen at a rate comparable to that measured in MB/PBS solution where cysteine is absent. This data shows that without the addition of acetic acid, the 400 nm ADPA absorption band OD remains practically constant, after 661 nm illumination for the same period of time, indicating that no singlet oxygen is generated.

3.3 Mechanism of the inhibition of MB bleaching and recovery of singlet oxygen generation

The mechanism that we propose for the photo-generation of singlet oxygen in plasma is based on the oxidation the cysteine S-H thiol group, to cystine S-S disulfide bridge dimer by the addition of dilute acetic acid to plasma, Scheme 3. This oxidation reaction, in effect, removes the availability of the thiol hydrogen which attacks the MB central nitrogen, thus preventing the bleaching of the 660 nm MB band and allowing the generation of MB triplet radicals and the subsequent formation of singlet oxygen and other reactive species. To verify that the cysteine hydrogen atom of the thiol, S-H, group, is responsible for the singlet oxygen inhibition in plasma, we performed similar experiments to those conducted with plasma/ADPA/MB solutions, using either albumin or cysteine, instead of plasma solutions, with all other experimental conditions remain exactly the same. Albumin/ADPA/MB solutions, without acetic acid, were irradiated with the same LED 661 nm light intensity and the absorption spectra were recorded after each illumination period. The data plotted in Figure 3 make it clear that the ADPA 400 nm absorption band does not decrease unless acetic acid is added. These measurements are in good agreement with previously published experimental results [18], which showed that the MB 660 nm band is bleached in albumin/MB solution, and the ADPA 400 nm absorption band intensity did not change with 661 nm irradiation.

Therefore we conclude that irradiation of albumin/MB solution with red light does not generate singlet oxygen and is the reason that bacteria in plasma are not destroyed by MB PDT. Further support of the S-H hydrogen atom attachment to central ring nitrogen mechanism, is provided by the data displayed in Figure 4, where solutions of cysteine/ ADPA/MB with and without addition of acetic acid addition were illuminated with the same LED light and the changes in the 660 nm MB absorption OD and 400 nm ADPA absorption OD were recorded as a function of irradiation time. The 660 nm absorption band of the solutions that did not contain acetic acid is found to be bleached within 30 seconds, Figure 4, in agreement with literature data [24], while the ADPA 400 nm band remains unaltered in shape and position with a decay slope slower than 6.0×10^{-4} s^{-1}. However, as was the case for the plasma, albumin and cysteine solutions, the MB and ADPA absorption band OD changes were reversed after the addition of dilute acetic acid. Namely, the optical density of the 660 nm MB band remained constant with irradiation, while the 400 nm ADPA OD decreased with a slope of 1.6×10^{-3} s^{-1}, Figure 3, and 1.1×10^{-2} s^{-1}, Figure 4, in albumin and cysteine solutions respectively. These data suggest that singlet oxygen was formed in the acid containing cysteine/MB solutions. We attribute this effect to the oxidation of cysteine to cystine and the removal of the S-H hydrogen atom from the cystine molecule to form the S-S bridge. Figure 5 shows the decrease of the 664 nm MB absorption band OD and the growth of the 255 nm cystine absorption band, in a cysteine/MB/acetic acid solution so as a function of 661 nm LED irradiation time. These time resolved spectra further substantiates the cysteine oxidation and formation of cystine made evident by the growth of the cystine S-S 255 nm absorption band and decay of the 660 nm MB singlet as a function of 661 nm illumination time. Note that the time interval of this experiment is 1.5 hr. in contrast to the previous published data, which show that without acetic acid the 660 nm MB band is bleached completely within 2 minutes [24]. The absorbance of cystine at this wavelength, 255 nm, is very low, ~120 cm^{-1} M^{-1}. Similar experiments using gamma-globulin and fibrinogen, the two other plasma proteins, which do not contain cysteine, behaved the same as those in PBS and water solutions, namely, the MB 660 nm band was not bleached and singlet oxygen was generated at a rate similar to MB/water solutions after irradiation with the 661 nm LED light for

the same period of time as the plasma and albumin samples.

3.4 Bacteria photo-inactivation in plasma assisted by acetic acid

Previous experimental data have shown, that MB radical and singlet oxygen formation are diminished in plasma solutions resulting in a very low rate of bacteria inactivation, especially the highly resistant Gram-negative bacteria such as, *Serratia marcescens* (SM). Data presented in this report show that the addition of a very small amount of acetic acid prevented the bleaching of MB and restored, to a large extend, its generation of singlet oxygen in plasma . Preliminary data, Table 1, suggest that the inactivation of SM bacteria is 10 times faster when 10^{-3} M acetic acid is added to the SM/plasma/MB solution compared to the same solution without the acid' Such high bacteria inactivation rate in plasma with acetic acid added is equivalent to the inactivation rate in PBS solution. To understand the effect of acetic acid in inhibiting the bleaching of MB quantitatively, we oxidized the cysteine amino acid of albumin using various concentrations of dilute acetic acid as shown in Figure 6. In order to bleach the MB at a rate fast enough to record this effect at a short period, we increased the concentration of cysteine to 0.1 M, which is 3 to 4 orders of magnitude higher than the found in human plasma [27, 28]. Figure 6 shows that increasing the concentration of acetic acid from 10^{-4} M to 0.1 M, the bleaching of MB by cysteine is slowed down remarkably. When the concentration of acetic acid is made to be similar to or larger than that of cysteine, the bleaching process is significantly retarded, although not completely is topped. This suggest that in order to reduce the bleaching effect of cysteine, the concentration of acetic acid added should be equivalent to that of the cysteine, which is ~ 10^{-5} M-10^{-4} M in the plasma [27, 28]. This is consistent with the amount of acetic acid, we added in order to restore the photo-generation of singlet oxygen. Table 2 lists the inactivation of *Serratia marcescens* bacteria in various plasma solutions to which MB and acetic acid have been added. It is evident from this table that the inactivation of bacteria in plasma is greatly increased by the addition of dilute acetic acid.

Photodynamic therapy has received regulatory approval for cancer and other diseases in several

countries [29]. Our proposed mechanism and reactions are intended for external use and bacteria decontamination of human plasma and the subsequent removal of the MB before its use for transfuse.

In summary, in this report we have presented data which show that in plasma/MB solutions, the methylene blue 660 nm absorption band was bleached and no detectable singlet oxygen was photo- generated. However, the addition of dilute acetic acid to plasma prevented the bleaching of the 660 nm singlet MB and allowed the formation of singlet oxygen at practically the same level as MB in PBS solutions. This process makes possible the efficient destruction of bacteria and other pathogens in plasma by illumination of MB with 661 nm light. In addition, the slope of the 400 nm ADPA absorption band decrease indicated that singlet oxygen formation is similar in plasma, albumin and cysteine solutions. This observation supports the mechanism proposed, that the hydrogen of the cysteine thiol group, S-H, is responsible for the bleaching of the MB 660 nm band by attaching to the central ring nitrogen thus destroying conjugation. Further support for the mechanism proposed is provided by data which show that gamma-globulin and fibrinogen, the other two plasma proteins, that do not contain cysteine amino acids, do not bleach MB and behave the same as PBS solutions. It is expected that this procedure, namely, the addition of dilute acetic acid, will also be effective in killing bacteria and other pathogens in open wounds and burns [30, 31].

4. Conclusion:

Data presented in this report show that the ineffective MB photo-inactivation of bacteria in plasma is due to the bleaching of the 660 nm absorption band of MB in plasma. This process can be inhibited by the addition of dilute acetic acid to plasma. Addition of acetic acid to plasma/MB solutions makes possible the photo-generation of both radicals and singlet oxygen, by MB. ROS react with the walls of bacteria causing their destruction. The mechanism for the prevention of MB bleaching in plasma is shown to be the oxidation of cysteine thiol, S-H group to form the disulfide bond, S-S, bridge of cystine. This process removes the availability of the thiol group H-atom which attaches to

the central ring nitrogen atom of the MB molecule, and destroys the MB ring conjugation. Experiments show that the addition of dilute acetic acid to the SM/plasma solutions causes the destruction of bacteria at about the same rate as in PBS solution. Data presented, on the absence and presence of photo-generated singlet oxygen in plasma, albumin and cysteine solutions with and without acetic acid, support the proposed mechanism for the inactivation of bacteria in plasma and the effect of dilute acetic acid in restoring efficient photo- inactivation of bacteria by MB.

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Competing interests: None declared

Ethical approval: Not required

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Tables：

Table 1. Inactivation of Gram-positive and Gram-negative bacteria in MB/PBS and MB/plasma solutions as a function of irradiation time with 8 mW/cm^2, 661 nm LED light. Inactivation time is defined as the period of time necessary to reduce the 10^7 live bacteria to 10^2.

Bacteria type-solution	Gram +/−	Inactivation time(min)
ATCC 12228 *coagulase-negative Staphilcocci epidermidis* - PBS	+	10
ATCC 12228 *coagulase-negative Staphilcocci epidermidis* - plasma	+	30
ATCC 13477 *Serratia marcescens* - PBS	-	35
ATCC 13477 *Serratia marcescens* - plasma	-	>250
ATCC 13477 *Serratia marcescens* - plasma/acetic acid	-	30

Table 2. The effect of acetic acid on the inactivation of *Serratia marcescens* in plasma/PBS (1:1) by methylene blue under 6.7 mW/cm^2, 661 nm LED irradiation for 150 minutes.

Acetic Acid Concentration (M)	Bacterial Colony Counts (CFU)			
	Methylene Blue Plus Acetic Acid	Methylene Blue Alone	Acetic Acid Alone	Control
10^{-1}	$<10^2$	10^7	10^7	10^7
10^{-2} Plus Regular Additions*[1]	10^2	10^7	10^7	10^7
10^{-3} Plus Regular Additions*[1]	$<10^2$	10^7	10^7	10^7
10^{-4} Plus Regular Additions*[2]	10^6	10^6	10^7	10^7
10^{-4}	10^6	10^6	10^7	10^7

*[1] 1.5% increments of Acetic acid at 10^{-1} M concentration added every 15 minutes

*[2] 1.5% increments of Acetic acid at 10^{-2} M concentration added every 15 minutes

Figures:

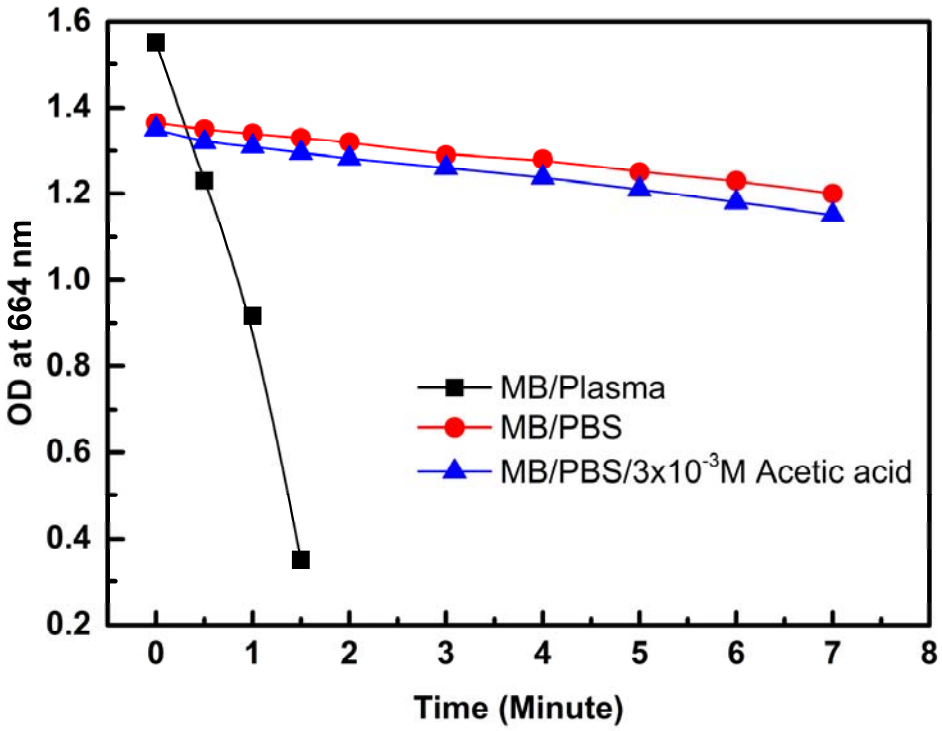


Figure1. Optical density change of MB 664 nm absorption band as a function of irradiation time:

■ MB in plasma; ● MB in PBS; ▲ MB in PBS/3x10^{-3} M acetic acid.

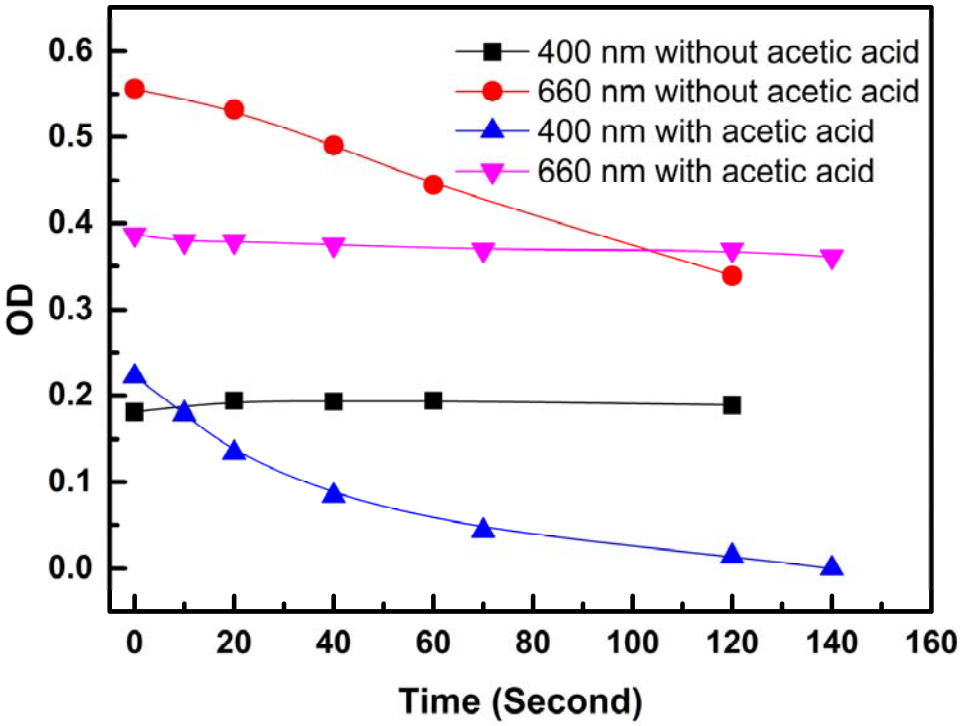


Figure 2. Optical density of MB/ADPA/plasma solution at 400 nm and 660 nm as a function of irradiation time: ■ 400 nm without acetic acid; ● 600 nm without acetic acid; ▲ 400 nm with acetic acid; ▼ 600 nm with acetic acid.

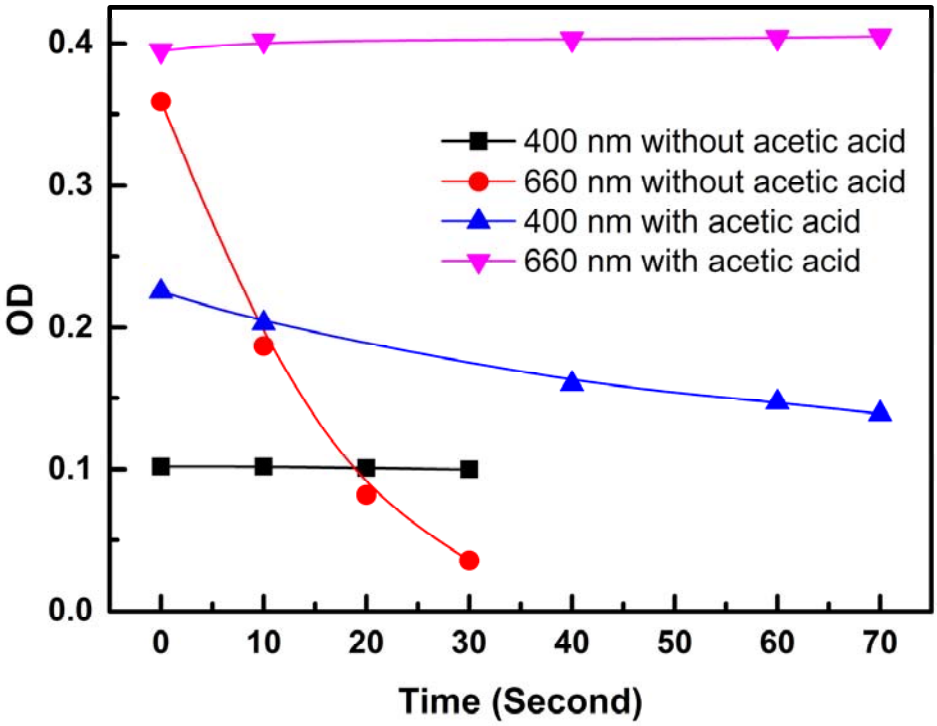


Figure 3. Optical density of MB/ADPA/albumin solution at 400 nm and 660 nm as a function of irradiation time: ■ 400nm without acetic acid; ● 600 nm without acetic acid; ▲ 400nm with acetic acid; ▼ 600 nm with acetic acid.

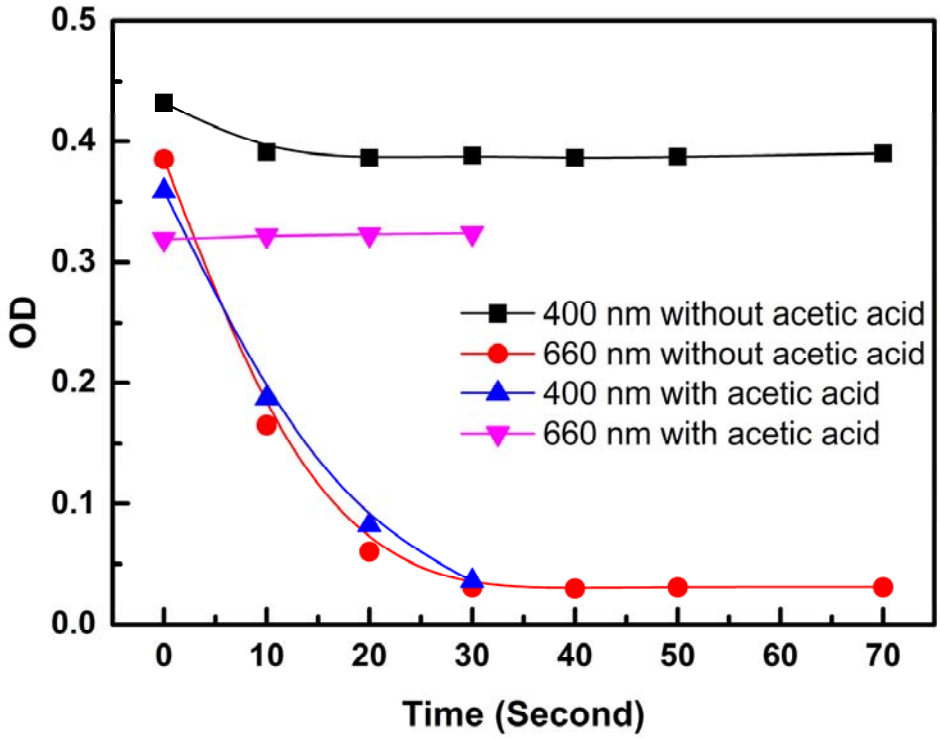


Figure 4. Optical density of MB/ADPA/cysteine solution at 400 nm and 660 nm as a function of irradiation time: ■ 400nm without acetic acid; ● 600 nm without acetic acid; ▲ 400nm with acetic acid; ▼ 600 nm with acetic acid.

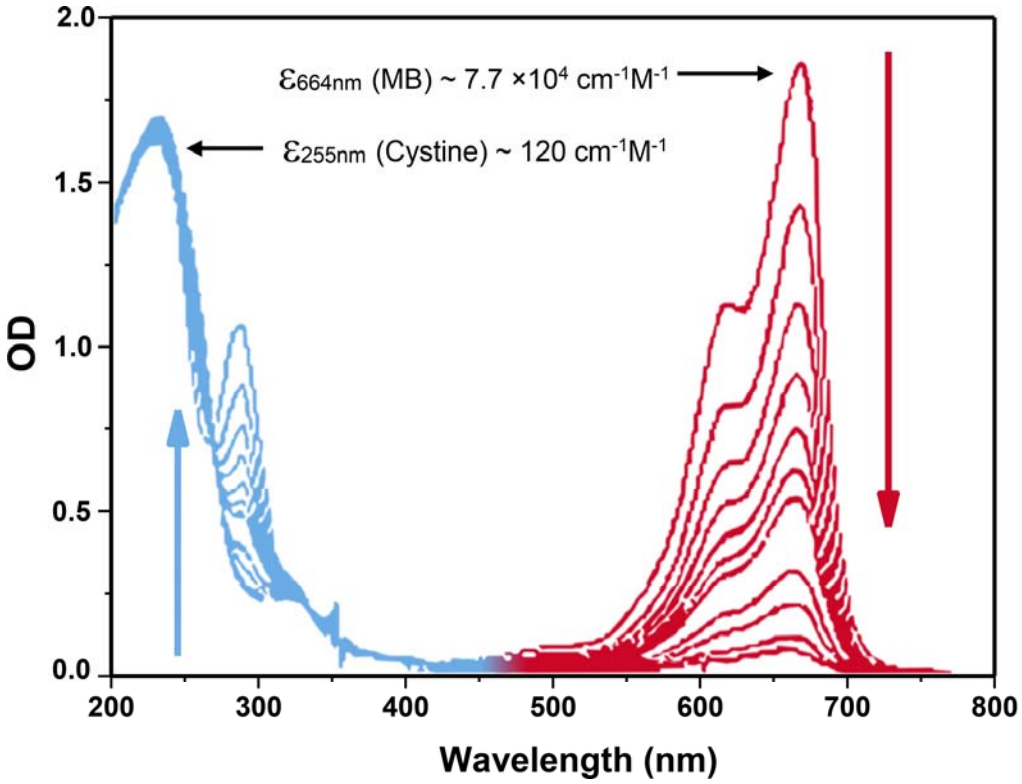


Figure 5. Time-resolved spectra of the 255 nm cystine band OD increase and 664 nm MB absorbance decrease after a cysteine/MB aqueous solution in a 1.0 cm optical cell was irradiated with 661 nm LED light.

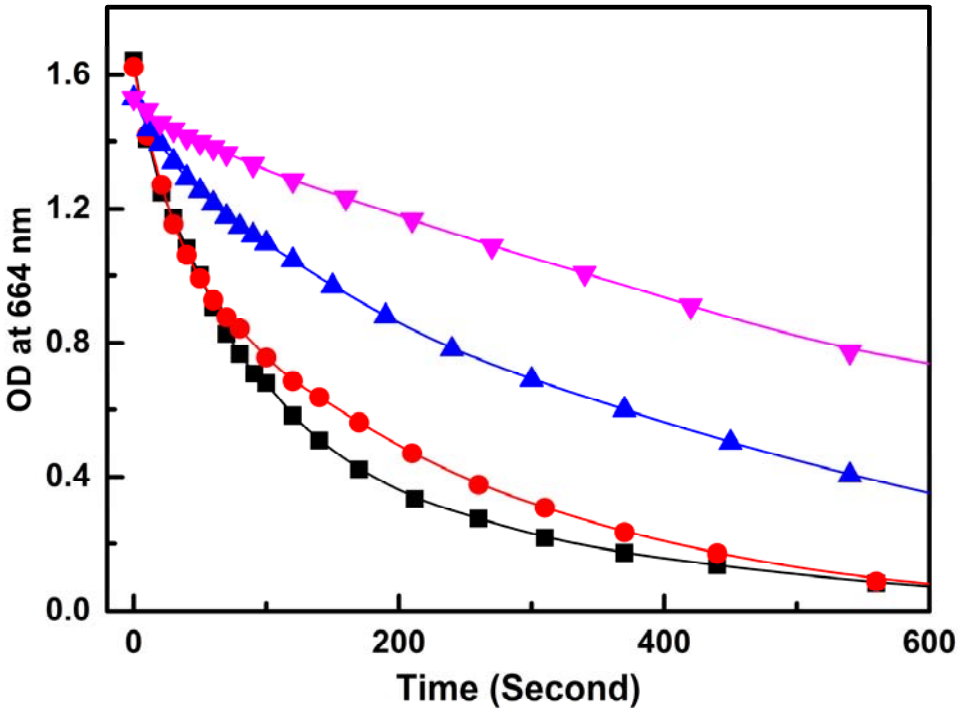


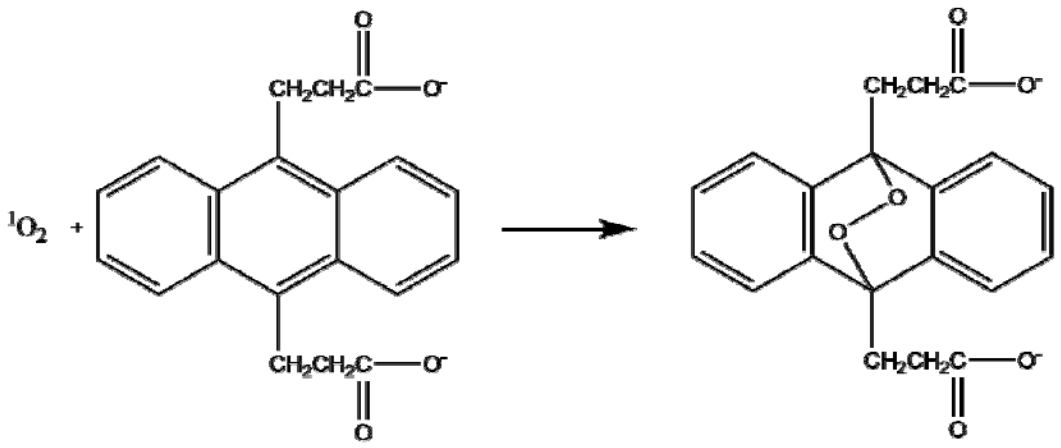
Figure 6. Optical density at 664 nm of 2.0×10^{-5} M MB/ 0.1 M cysteine solutions in a 1.0 cm optical cell as a function of irradiation time with different concentrations of acetic acid: ■0 M; ● 10^{-4} M; ▲ 10^{-2} M; ▼ 0.1 M.

Schemes:

Scheme1

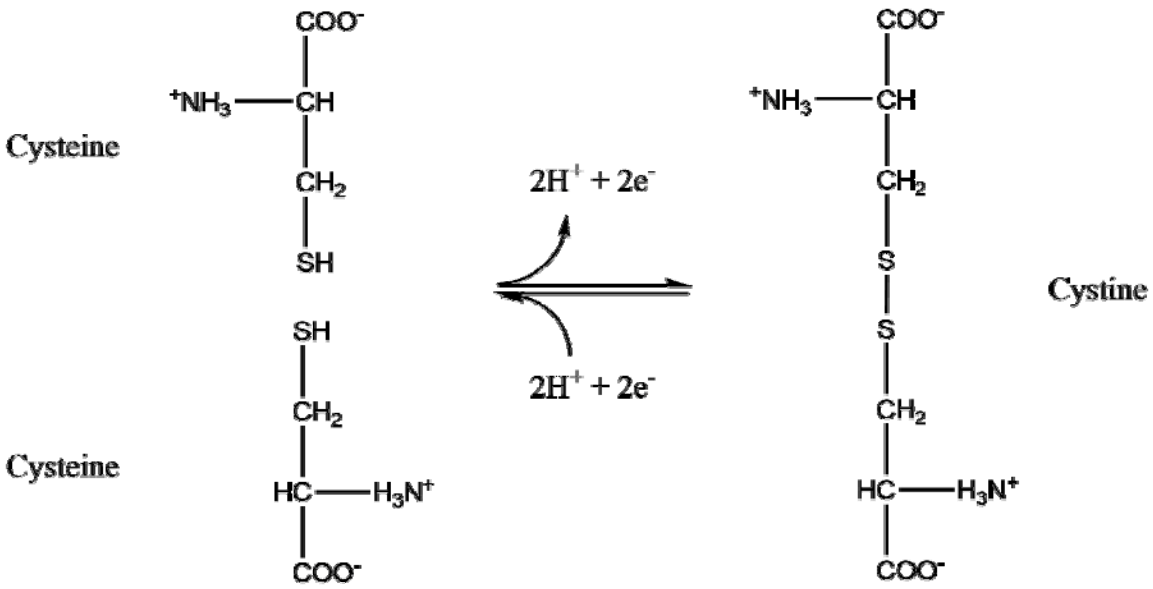
Methylene Blue Leucomethylene Blue

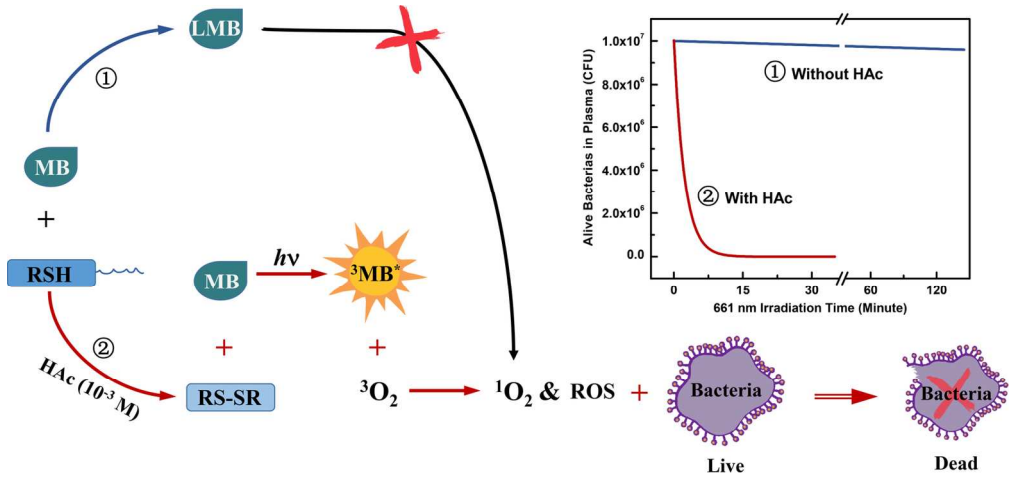
Scheme 2



ADPA $ADPAO_2$

Scheme 3. Cysteine-Cystine redox cycle





149x70mm (300 x 300 DPI)