Continuous and Delayed Photohemolysis Sensitized With Methylene Blue and Iron Oxide Nanoparticles (Fe₃O₄)

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Abstract. This research present the sensitization of methylene blue (MB), as a potential photodynamic therapy photosensitizer which showed phototoxicity for many tumor cells in vitro incorporated with iron oxide nanoparticles (Fe₃O₄, IO-NP), which offer magnificent interaction both inside and outside the surface of biomolecules together with red blood cells (RBC’s) with significant change in hemolysis process. The study investigated the sensitization of continuous photohemolysis (CPH) for MB and MB with IO-NP, delayed photohemolysis (DPH) at different irradiation temperature (Tirr). The photohemolysis rate for CPH at room temperature has a power dependence of 0.39 ± 0.05 with relative steepness of 1.25 ± 0.02 and for different concentration of MB and power dependent of 0.15 ± 0.03 with relative steepness of 1.34 ± 0.01 for different MB and IO-NP. Logistic and Gompertz functions were applied as appropriate mathematical models to fit the collected experimental data for CPH and DPH respectively, and to calculate fractional photohemolysis rate with minimum errors. The Logistic function parameter; İ, the hemolysis rate, increases with increasing concentrations of MB and decreases with increasing IO-NP concentrations in the presence of 6 µg/ml of MB. The parameter b; the time required to reduce the maximum number of RBCs to one half of its value, decreases with increasing MB concentration and increases with increasing IO-NP concentrations in the presence of 6 µg/ml of MB. In DPH at different Tirr, the Gompertz parameter; a, fractional hemolysis ratio, is independent of temperature in both case MB and MB plus IO-NP, while the parameter; b, rate of fractional hemolysis change, increases with increasing Tirr, in both case MB and MB plus IO-NP. The apparent activation energy of colloid-osmotic hemolysis is 9.47±0.01 Kcal/mol with relative steepness of 1.31 ± 0.05 for different MB and 6.06±0.03 Kcal/mol with relative steepness of 1.41 ± 0.09 for MB with iron oxide. Our results suggest that Logistic equation is the best fit for the CPH and Gompertz function for the DPH. Both models predict also that the relative steepness is independent of the light dose, sensitizer and IO-NP concentrations.

1. Introduction
Cancer is a complex deadly disease mainly caused by factors in the environment that mutate genes and encrypting decisive cell-regulatory proteins. The agents that cause cancer can be present in Food, chemicals, air and sunlight which people use in their daily activities [1, 2]. It has been predicted that, in
2020 it will be about 20 million new cases of cancer and almost twelve million deaths. Different methods of treatment and therapies were employed such as radiation therapy, cytostatic chemotherapy, surgical operation, and photodynamic therapy (PDT).

PDT appeared to be a promising mode of treatment for many diseases most especially localized cancer with minimal side effects [3, 4]. In PDT, light photosensitizing drug (photosensitizer), oxygen (O₂), and light are combined to have therapeutic effect. PDT is a kind of treatment that affects only the designated tissue. The activation of the photosensitizer by exposure to a carefully regulated dose of light of appropriate wavelength for a specified length of time elicits a cytotoxic action resulting in cell death [2]. PDT uses selective irradiation in therapeutic treatment which does not damage tissues that are healthy. It develops no resistance in case of repeated treatment. It can also be used to treat cancers that are resistance to other medication and heal very fast as the collagen contain in the tissue is not affected by photodynamic damage [5].

Nanoparticles provide new interaction with biomolecules on cutaneous and sub-cutaneous tissues which may raise cancer treatment and diagnostic. Nanoparticles are been recognized as a drug transporting system because of their size and extreme surface to volume ratio results to their usage in PDT [6], and their ability to concentrate the photosensitizer (PS) on the cells wall or diffuse inside the membrane. Researchers have shown that, magnetic carriers are widely applicable in drug targeting, isolation and sorting cell [7, 8]. Magnetic nanoparticles might be used as carriers for methylene blue (MB) photosensitizer because of their low toxicity, superparamagnetic properties and high saturation magnetization [9].

Red blood cells (RBC), human erythrocytes, shown to be ideally targeted in PDT since they are somewhat simple structured design to study the ability of compounds to produce photo-oxidation process [10] in cells in vitro and amenable for hemotasis in the vascular bed of tumor. On the other hand, methylene blue (MB) as a photosensitizer has low toxicity and high quantum production of O₂ generation. It has fascinating photochemical and photo-physical properties that give magnificent results in in vivo and in vitro [9, 11].

This study investigated the RBC’s sensitization and compare the efficiency of continuous photohemolysis (CPH) for free MB and MB with IO-NP, delayed photohemolysis (DPH) at room temperature, at different irradiation temperature (Tirr) and at different irradiation time (tirr).

logistic function (Eq. 1) and Gompertz function (Eq. 2) were applied as appropriate mathematical models to calculate the fractional photohemolysis rate [12,13] and analyzes the resulting sigmoid curves to fit the collected experimental data for CPH and DPH respectively, with insignificant errors [14].

\[
N = \frac{A}{1 + \exp(-a(t - \beta))} \quad \text{(Eq. 1)}
\]

\[
H = H_0 e^{-ae^{-bt}} \quad \text{(Eq. 2)}
\]

Whereas: \( N \) and \( H \) is the percentage of hemolysis during the lysis time \( t \) (i.e. the time measured from the beginning of rupturing the RBCs), the number of RBCs, \( A \) and \( H_0 \) is the maximum number of RBCs (always normalized to one), \( a \) and \( a \) is the fractional hemolysis rate, and \( \beta \) is the time required to reduce the maximum number of RBCs to one half of its value, and \( b \) is the rate of fractional hemolysis change. The dependence of continuous photohemolysis on the photosensitizer concentration (MB) can be empirically determined by the power law [11] as:

\[
\frac{1}{t_{50}} = k C^p \quad \text{...............(Eq. 3)}
\]

Where: \( k \) is constant of time-intensity, \( C \) is the sensitizer concentration and \( p \) is the power dependent parameter.

The objective of this work was to investigate photosensitization of methylene blue as a drug for PDT and to present the IO-NP influence in the therapy. The MB has been selected in this work due to interested
optical properties and to its high sensitivity to temperature and its high ability to light absorption in UV-Vis regions.

2. Materials and Methods

Red blood cells were obtained from healthy donor and isolated by repeated centrifugation at 1000g for 8 min each time. The cells were then suspended in 7.4 pH Phosphate buffer saline (PBS). The light scattering absorption (optical density) of the isolated RBC at 680nm was approximately 2.0 which correspond to 7.86x10^6 cells/mm³ erythrocytes concentration as counted using haemocytometer. The light source used for irradiating the samples was a 200W Hg-Xe arc lamp (Power supply model: 68907) housed in an Oriel Research Arc Lamp focused to the sample spot size using a concave lens. Optical density of the samples was measured using Shimadzu 2450 UV-VIS spectrophotometer/Japan. Methylene blue (MB), an available commercial photosensitizing agent, was acquired in the form of powder from Aldrich (Milwaukee, WI). The iron (II, III) oxide nanopowder, 98% certified of 50nm size was purchased from Sigma-Aldrich (St Louis, MO, USA). Stock suspension was prepared by suspending 7.5 mg of iron oxide nanopowder in 10 ml of PBS to obtain a suspension of 0.75 mg/ml which is then diluted to 0.15 mg/ml and ultrasonic wave generator was used to completely suspend the particles. A concentration of 0.3 mg/ml of MB was prepared by dissolving 0.3 mg of MB soluble powder in 10 ml of PBS with all the measurement taking in darkness. The samples were a mixture of methylene blue (MB), iron oxide nanoparticles and Red blood cells (RBCs) suspended in PBS of 7.4 pH. Samples were incubated at 37°C for 30 min with gently shaking every 10 min to have a homogeneous solution and to allow the photosensitizer and IO-NPs to penetrate through RBCs membrane or to be bounded to the cell walls. The samples were centrifuged again at 1000g for 8 min to remove the aggregated and unbounded IO-NPs. Figure 1 represents these samples with two UV absorption peaks and four VIS peaks.

![Fig. 1: Absorption spectrum of suspension containing RBCs, MB and IO-NP.](image)

3. Results and Discussions

In the past decades, metallic inorganic nanoparticles have taken the attention of many nanobiotechnologist due to the effect they shown on biological imaging and bioconjugation in delivering drug
and diagnostic work. This metallic particle assists in the interaction within the range of drug molecules due to their wide surface area relative to the volumes [15]. One of the ideal reasons set to improve the novel photosensitizers was the expansion and their improvement in cancer treatment using photodynamic therapy (PDT). It involves selective transfer of photosensitizer such as methylene blue to tumors, and when irradiated with a suitable wavelength of light, the MB produces a singlet- oxygen ($^1O_2$) and reactive oxygen species (ROS) that cause damage to tumor cell with minimal effect to normal tissue. Sensitization of RBC for continuous and delayed photohemolysis in vitro has been used as a medium of $^1O_2$ and ROS generation. Therefore, this work demonstrates the photohemolysis behavior of RBCs and methylene blue incorporated with iron oxide nanoparticles.

There are many researches that show MB mediated phototherapy against fungal, tumor and bacterial infections [9, 11, 16, 21, 22, 25]. It was discovered that, small amount of MB concentration inhibits high percentage of bacteria on irradiation, which is consistence with the results of this study; whereas MB incorporated with iron oxide nanoparticles regulate the inhibition of normal cell rupture [16].

3.1. Continuous Irradiation of MB and MB with IO-NP:

Five sets of samples were prepared with different MB concentration of 2 - 10 $\mu$g/ml. Another six sets of samples were prepared with fixed concentration of 6 $\mu$g/ml MB and different concentration of IO-NP (0 - 40 $\mu$g/ml). The prepared samples are covered with aluminum foil and kept in refrigerator to prevent it from exposure to light and temperature then irradiated continuously sample by sample at room temperature to the total rupture of the RBCs. The typical sigmoidal CPH curves are shown in Fig. 2. The curves represent the effect of different concentrations of methylene blue (2 – 10 $\mu$g/ml) on RBCs under irradiation at room temperature.

For all curves, the points in the figures represent the experimental data while the lines show their best fit with logistic function (Eq. 1), which was applied as best fit equation among several equations [17-20] for CPH curves. The power law, (Eq. 3) gives the relationship between the drug concentration and the time to rupture 50% of RBCs ($t_{50}$). Therefore; the dependence of $1/t_{50}$ versus methylene blue (MB) concentration ($C$) that corresponds to Fig. 2 is presented in Fig. 4. Table 1A, shows the experimental and the theoretical times required for 50% lysis of red blood cells sensitized with different concentrations of MB. Logistic function fitted parameters corresponding to CPH measurements, average values of power dependent parameter ($P$), and relative steepness for lysis curves ($S$)which is defined as; $S = t_{80}/t_{30}$, where $t_{50}$ and $t_{30}$ are the time to lysis 80% and 30% of the RBC’s respectively.

In Fig. 2, the increasing concentration of MB leads to decrease in time of generating ROS which in turn increases the cell death time. The relationship shown in Fig. 4 indicates that, $t_{50}$ increases with increasing MB concentration. The high concentration of MB is relatively associated with high time percentage of cell rupture under irradiation and vice versa. Similar results were found by Fahmida et al [21]. The power dependence found to be 0.39±0.05 and a typical hemolysis curves with steepness above one was observed at different MB concentrations. The hemolysis rate, $a$ increases with increasing concentration of MB while the time required to reduce the maximum number of RBCs to one half of its value, $b$, decreases with increasing MB concentration. Resemblance of such result was obtained by Ankita at el. [22] that
concludes, $\beta$ depend on MB concentration, were $\beta$ decreases with increasing MB concentration (See Table 1A).

**Table 1.** (A); RBCs with different concentrations of MB, (2, 4, 6, 8, and 10 $\mu$g/ml). (B) RBCs with different concentration of IO-NPs (0, 10, 20, 30, and 40 $\mu$g/ml) and fixed concentration of 6 $\mu$g/ml MB. The results are presented as mean ± S.D.

| Group | $(t_{50})_{exp}$ C | Logistic function parameters |
|-------|-------------------|-----------------------------|
|       | (min) | (μg/ml) | $A$ | $a$ (min)$^{-1}$ | $\beta$ (min) |
| A     | 98.167 | 2 | 0.089±0.006 | 98.07±0.82 |
|       | 82.833 | 4 | 1.014 | 0.104±0.007 | 82.56±0.67 |
|       | 79.500 | 6 | ± | 0.104±0.006 | 79.19±0.64 |
|       | 68.000 | 8 | 0.029 | 0.121±0.007 | 67.83±0.49 |
|       | 62.833 | 10 | 0.113±0.008 | 62.58±0.66 |

Power dependent $P = 0.39 ± 0.05$, and Steepness $S = 1.25 ± 0.02$

| B     | 51.50 | 0 | 0.145±0.009 | 51.20±0.34 |
|       | 68.17 | 10 | 1.032 | 0.104±0.005 | 69.33±0.62 |
|       | 81.17 | 20 | ± | 0.075±0.003 | 82.44±0.76 |
|       | 85.17 | 30 | 0.008 | 0.067±0.003 | 87.44±1.06 |
|       | 92.17 | 40 | 0.085±0.003 | 93.18±0.47 |

Power dependent $P = 0.15 ± 0.03$, and Steepness $S = 1.34 ± 0.01$
Fig. 2: Photosensitization of CPH for RBCs with different concentrations of MB irradiated at room temperature. The solid lines are theoretical fitted with Logistic function and the points are the experimental data.

The effect of different concentration of IO-NP (0 - 40 µg/ml) and fixed concentration of 6 µg/ml MB incubated at 37º C and irradiated at room temperature are presented in Fig. 3. The dependence of $I/t_{50}$ versus IO-NP concentrations are shown in Fig. 4. Table 1B shows the experimental and theoretical times required for 50% lysis of Red blood cells sensitized with different concentrations of IO-NP, Logistic function parameters corresponding to CPH measurements, average values of power dependant parameter ($P$), and relative steepness for lysis curves ($S$).
Fig. 3: Photosensitization of CPH for RBCs with different concentration of IO-NPs and fixed concentration of 6 µg/ml MB irradiated at room temperature. The solid lines are theoretical fitted with Logistic function and the points are the experimental data.

Fig. 4 Relation between \( I/t_{50} \) of continuous irradiation of MB and the concentrations results to power dependence of 0.393±0.002 and relation between \( I/t_{50} \) of continuous irradiation of IO-NP with fixed 6µg/ml MB and the concentration led to power dependence of 0.159±0.003.

The size of the spherical iron oxide nanoparticles has an average diameter of 50nm. In agreement with Aravind et al. [23], a mixture of MB with iron oxide indicated the succeeding of \(^1\text{O}_2\) production and cell death efficiency in vitro, whereas the MB loaded nanoparticles are targeted directly to the cell membrane [11, 24]. Fig. 3 gives the typical photohemolysis curves of RBCs sensitized with MB and IO-NP [17, 18, 20]. Fig. 5 demonstrates high lysis time of RBCs with increasing IO-NP concentration and low percentage of cell rupturing time at low iron oxide concentration. This indicates that, the interaction that occur between MB and iron oxide influence the lysis of cell rupture as also represented by Sonal et al. [25]. This
interaction enhances the activities of protein peroxides as well as reducing the intensity of light entering into the cells that reduces normal cell membrane damage.

![Fig. 5 Arrhenius plot for CPH and DPH dependence rate on 20 min irradiation temperatures of 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C.](image)

Similar results using Chinese chlorella were reported by Al-Akhras et al. [26] and Fahmida et al. [21] using MB. The curves have power dependence of 0.15 ± 0.03 and relative steepness of 1.34± 0.01 with the presence IO-NP concentration. Values of the Logistic Function parameter; $\alpha$ decreases with increasing iron oxide concentration and the fitting parameter $\beta$; increases with increasing iron concentration. That is, $\beta$ is dependent on iron oxide concentration. (Table 1B) Al-Akhras et al. [27] obtained similar using Cichorium pumilum.

3.2. Delayed Irradiation of MB and MB with IO-NP:

Eight samples were prepared with fixed concentration of 6 µg/ml MB and another set of eight samples prepared with fixed 6 µg/ml MB and fixed 20 µg/ml IO-NP. Samples were irradiated at different temperatures (5°C - 40°C) for 20 min at each temperature.
The Gompertz curves for delayed photohemolysis (DPH) of fixed 6 µg/ml methylene blue are shown in Fig. 6. The behavior of the RBC lysis for fixed 6 µg/ml methylene blue with 20 µg/ml IO-NP incubated at 37°C and irradiated at different temperatures varying from 5 - 40°C (Fig. 7). The dependence of \(1/t_{50}\) for the concentrations versus different temperature of irradiation obtained from the curves (Fig.6 and 7) is represented in Fig. 5. Gompertz function fitted parameters corresponding to DPH measurements (Fig. 6 & 7), average values of power dependant parameter (P) and relative steepness for lysis curves (S) are shown in Table 2. The DPH rate was known to rely on concentration of the photosensitizer, incident fluence, \(T_{\text{inc}}\) and \(T_{\text{irr}}\). In the measurements, the accelerating outcome at high \(T_{\text{inc}}\) is as a result of thermal activation of colloid-osmotic hemolysis. Higher \(T_{\text{irr}}\) is attributed to achieving practical \(t_{50}\) but photobleaching may occur at low \(T_{\text{irr}}\). This result came abreast with that of Al-Akhras [13] using protoporphyrin IX. In DPH curves, (Fig. 6 and 7) the curves represent the photohemolysis of MB and MB with IO-NP both in which at low \(T_{\text{irr}}\), higher hemolysis rate is experienced. It also reveals that, by increasing the \(T_{\text{irr}}\), the ROS generation also decrease which decrease the cell damage. The values of the Gompertz function parameter, \(a\) decreases with increasing MB and IO-NP concentration. The parameter, \(a\) and \(b\) are almost independent of \(T_{\text{irr}}\). For further analysis of the temperature dependence of intoxicating cells and to calculate the activation energy, the Arrhenius equation has been applied and described as:

\[
(1/t_{50}) = Ae^{-E/RT}
\]

(Eq. 4)

Where \(1/t_{50}\) is the activation rate which is described by steepness of the killing curve and \(A\) is constant; \(E\) is the activation energy; \(R\) is the gas constant, and \(T\) is the absolute irradiation temperature.

The linear plot of \((1/t_{50})\) vs the reciprocal of irradiation temperature \((1/T_{\text{irr}})\) leads to an apparent activation energy equal to 9.47±0.01 Kcal/mol for the MB with \((R=0.98)\) and 6.06±0.03 kcal/mol for the MB+IO-NP with \((R=0.97)\) (Fig.5). In this Figure, the \(t_{50}\) decreases with increasing irradiation temperature, \(T_{\text{irr}}\). The typical sigmoidal curves of DPH are shown in Fig. 6 and Fig. 7 fitted with Gompertz function. the fitting parameters from the curves shows that, the parameter \(a\) increase with increasing MB and iron concentration while the parameter, \(b\) is dependent on MB and MB with IO-NP concentrations (Table 2).

The slight decrease in this energy for MB with and without IO-NP is due to temperature and the light effect can be seen clearly in which higher irradiation time accelerates hemolysis much faster compared to incubation. This came alongside with the results obtained by AL-Akhras [13, 28]. From both curves, the relative average steepness are \(S= 1.31±0.05\) and \(1.41±0.09\).
**Fig. 6** Photosensitization of delayed photohemolysis (DPH) of fixed 6 μg/ml MB irradiated with arc lamp for 20 min. The solid lines are theoretical fitted with Gompertz function and the points are the experimental data.

**Fig. 7** Photosensitization of delayed photohemolysis (DPH) of fixed 6 μg/ml MB and fixed 20 μg/ml IO-NPs irradiated at different temperatures for 20 min each. The solid lines are theoretical fitted with Gompertz function and the points are the experimental data.
4. Conclusion:

In this work we reported the photosensitivity of the methylene blue associated with Iron Oxide Nanoparticles (Fe₃O₄) on red blood cells. Both delayed and photohymolysis were assessed under different concentration and different irradiation temperature. Logistic and Gompertz functions were valid to fit the experimental results and to predict the cell lysis parameters. For both CPH fitted with Logistic equation and DPH fitted with Gompertz function, the combination of different concentration of MB and Iron-Oxide nanoparticles inversely affect the RBC’s \( t_{50} \) for different \( T_{irr} \). Moreover the Photoactivation of MB associated with iron oxide nanoparticles causes a delay in cells membrane rupturing. Iron-Oxide nanoparticles in combination with MB have no signif icant effect on the RBC’s rupturing rate. The power dependent factor for CPH has less effect in MB with and without iron oxide concentrations but has significant effect at \( T_{irr} \) for DPH. Furthermore, the \( T_{irr} \) has more impact on the photohemolysis rate in the presence of MB as compared to that of MB plus IO-NP.

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| Group | \( (t_{50})_{exp} \) (min) | \( T_{irr} \) (°C) | Gompertz function parameters | \( Ho \) | \( a \) | \( b \) (min)\(^4\) |
|-------|-----------------|---------------|-----------------|--------|--------|----------------|
| A     | 80              | 5             | 12.33±0.25      | 0.0352±0.0003 |
|       | 65              | 10            | 9.49±0.21       | 0.0383±0.0004 |
|       | 49              | 15            | 4.58±0.16       | 0.0345±0.0012 |
|       | 33              | 20            | 5.18±0.11       | 0.0558±0.0009 |
|       | 23              | 25            | 4.27±0.08       | 0.0784±0.0011 |
|       | 18              | 30            | 3.41±0.13       | 0.0825±0.0032 |
|       | 15              | 35            | 3.85±0.31       | 0.1156±0.0074 |
|       | 14              | 40            | 4.96±0.25       | 0.1399±0.0043 |

Activation energy \( E = 9.47±0.01 \) Kcal/mol, and \( S = 1.31 ± 0.05 \)

| Group | \( (t_{50})_{exp} \) (min) | \( T_{irr} \) (°C) | Gompertz function parameters | \( Ho \) | \( a \) | \( b \) (min)\(^4\) |
|-------|-----------------|---------------|-----------------|--------|--------|----------------|
| B     | 62              | 5             | 4.95±0.12       | 0.0279±0.0007 |
|       | 48              | 10            | 4.28±0.06       | 0.0343±0.0006 |
|       | 41              | 15            | 4.89±0.19       | 0.0438±0.0015 |
|       | 32              | 20            | 4.79±0.15       | 0.0557±0.0016 |
|       | 23              | 25            | 3.32±0.10       | 0.0644±0.0022 |
|       | 22              | 30            | 3.50±0.12       | 0.6882±0.0023 |
|       | 21              | 35            | 4.35±0.14       | 0.0849±0.0021 |
|       | 19              | 40            | 3.96±0.26       | 0.0888±0.0480 |

Activation energy \( E = 6.06±0.03 \) Kcal/mol, and \( S = 1.41 ± 0.09 \)
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