JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 55 - 59

Jordan Journal of Biological Sciences

The Influence of Iron Oxide Nanoparticles on the Red Blood Cells Photohemolysis Sensitized with Photofrin: Temperature Effect

Mohammad-Ali H. Al-Akhras* and Duaa J. Al-Khalili

Bio-Medical physics laboratory, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan

Received: October 19, 2014 Revised: December 24, 2014 Accepted: January 4, 2015

Abstract

The present study aims to measure the temperature-dependence of *in vitro* continuous photohemolysis (CPH) photosensitized by Photofrin® in the presence or absence of Iron Oxide Nanoparticles (IONPs) and to evaluate the results using Gompertz function. Red Blood Cells (RBCs) were isolated from human healthy volunteers blood; they were then incubated with Photofrin® only or with IONPs for 45 minutes at 37 °C and then irradiated to a range of temperatures (4-41°C). The results show that Photosensitization of RBCs by Photofrin® with IONPs presence reduces the inhabitation effect of Photofrin® at the same irradiation temperature since the decreasing in activation energy and increment in t_{50} were obvious evidence for such result as well as the applicability of Gompertz function to the fractional photohemolysis ratio (a) and the rate of fractional photohemolysis (b), is found to be the most appropriate model to fit the experimental data with minimum parameters and minimum errors, Parameter (**a)** and the curves steepness were found to be temperature-independent. On the other hand, values of parameter (**b)** increased as irradiation temperature increased with or without IONPs presence. The apparent activation energy was found to be 18.85 ± 0.72 kcal/mol in the absence of IONPs and 17.29 ± 0.71 kcal/mol in the presence of IONPs. Our results indicated that Photofrin® incorporated with IONPs could be considered as a modality to improve cytotoxicity in photodynamic therapy and/or reduces the inhabitation effect.

Keywords: Continuous Photohemolysis (CPH), Gompartz parameters, Iron Oxide Nanoparticles (IONPs), Irradiation temperatures, Photodynamic Therapy (PDT)

1. Introduction

Photodynamic Therapy (PDT) is a promising treatment modality that has been successfully used in treating localized tumours, providing tumour selectivity and normal tissue sparing with almost no serious side effects. PDT employs the combination of light and photosensitizers to damage localized cancer cells (Sternberg *et al*., 1997; Dougherty *et al*., 1998). As light applied to an area to be treated; it chemically altered the photosensitizer which undergoes internal reactions with substrate that finally creates cytotoxic Reactive Oxygen Species (ROS) which then attack the main structural entities in the target cells. Therefore, molecular Oxygen existence is a key point in PDT (Henderson *et al*., 2000; Sil *et al*., 2004). Human erythrocytes were shown to be a primary target in PDT because they have a relatively simple structured model which enables the compounds to create photooxidation process, and the released haemoglobin, due to membrane damage, can be easily measured through spectrophotometrey (Ben-hur *et al*., 1986). The photohemolysis of erythrocytes (lysis of erythrocytes when they exposed to light) was tested by using many different natural and chemical photosensitive drugs, such as Chinese Corolla (Alzoubi *et al*., 2014), Cichorium pumilum (Al-Akhras *et al*., 2007) and Photofrin® (Al-Akhras *et al*., 1996). *In vitro* studies on photohemolysis rate, measurements were investigated based on two techniques: lysis of erythrocytes during irradiation (Continuous Photohemolysis; CPH) and lysis of erythrocytes after being given a controlled light dose (Delayed Photohemolysis; DPH) (Al-Akhras *et al*., 1996). Photofrin® (Porfimer Sodium) is considered to be among the most efficient and vastly used photosensitizers in clinical PDT, especially for localized tumour that accumulates in cancerous tissues in a much higher rate than in normal ones (Allison *et al*., 2004).. On the other hand, some studies reported that RBCs photohemolysis curves showed a good agreement with Gompertz function module (Al-Akhras *et al*., 1996; Choe *et al*., 2013; Al-Akhras *et al*., 2006). The Gompertz function is defined as:

$$H = H_o e^{-a e^{-bt}} \quad \text{.....} \quad \text{(Eq.1)}$$

Where: H is the percentage of hemolysis during the lysis time t (the time measured from start of hemolysis the RBCs

* Corresponding author. e-mail: alakmoh@just.edu.jo.

at dark incubation), H_o is the initial maximum number of cells, normalized to one, a is a fractional hemolysis ratio, and b is the rate of fractional hemolysis change.

In recent years, there has been much research on the utilization of nanomaterials in biomedical applications. Because of their high surface ratio, small size (near to bio-macromolecules), fast diffusion that improved their chemical reactivity and multi-functioning for biomaterials, their ability to fit into dimensions of micron (Amirnasr *et al*., 2011), IONPs have become widely applied to biomedicine and biotechnology cell sorting and isolation, delivery vehicles and diagnostic agents (Yang *et al*., 2013), drug targeting, magnetic resonance imaging (Patrick *et al*., 2009; Morales *et al*., 2003), as some results showed that the interaction of IONPs with RBCs, which serves as main target in biomedical applications, do not affect some important physiological parameters such as the pH and Ca^{2+} content (Moersdorf *et al*., 2010). In the present study, the temperature dependence of *in vitro* CPH photosensitized by Photofrin® was measured in the absence and presence of IONPs and the results were evaluated using Gompertz function.

2. Materials and Methods

2.1. Materials

RBCs were isolated from human healthy male mature volunteers by repeated centrifugation at 4000 rpm and resuspension in pH7.4, 10 mM Phosphate Buffer Saline (PBS). IONPs stock with 0.75 mg/ml solution diluted to 0.15 mg/ml and ultrasonic wave's generator was used for 25 minutes to ensure dissolving. Photofrin® stock was prepared by dissolving 1mg of Photofrin® powder in 10 ml PBS, Iron oxide (Fe_3O_4) nanopowder, 98% with a radius of 50 nm purchased from Sigma-Aldrich (St Louis, MO, USA). Photofrin® powder (Porfimer Sodium) was purchased from Cyanamid (Pearl River, New York).

2.2. Methods

The washed RBCs was diluted in PBS to get Optical Density (OD) about 2 cm^{-2} at 680 nm, which corresponds to 7.86 x 10^6 cells/mm^3 erythrocytes concentration, measured using haemocytometer. CPH measurements were conducted onto two types of samples; RBCs incubated with 2 µg/ml Photofrin® and 50 µg/ml IONPs and RBCs incubated with 2 µg/ml Photofrin® only, both types were incubated at 37˚C for 45 minutes with shaking each 15 minutes then they continually irradiated to a range of irradiation temperatures (T_{irr}) for 4-41˚C until reaching absorbance stability. The irradiated sample was contained in a 2 cm X 2 cm cylindrical quartz cuvette and mounted in a stand which has a water circulation for controlling temperature set (AL-Akhras *et al*., 1996). The samples were located at 29 cm from light source and the output intensity the samples reached was about 60 W/cm^2 as measured by Filed Max II Laser Power/Energy Meter/Coherent/USA**.** The photohemolysis light source was 200 W high-pressure Hg/Xe arc lamp, housed in an Oriel Research Arc Lamp Housing model: 66903, with Oriel Digital Arc Lamp Power Supply model: 68907.

The absorbance rate was measured by calibrating the SHIMADZA, UV-Vis -2450 spectrophotometer to read 100% transmittance at 680 nm for PBS solution and comparing the intensity of transmitted beam through reference sample (PBS) with a transmitted beam through test sample (our sample), which is detected by photomultiplier for each sample, and, finally, the data were read by a designed computer connection through UV Probe-[Spectrum] computer program version 2.33.

3. Results and Discussion

Continuous Photohemolysis (CPH) curves for samples with and without IONPs irradiated at different temperatures were obtained by studying the relationship between fractional photohemolysis and time of exposure as shown in Figures 1 and 2, respectively.

In vitro study of RBCs photohemolysis is based on CPH, in which the suspension of RBCs along with Photofrin® and IONPs is exposed to a direct dose of light source with photohemolysis measurements in dark that may be assayed by hemoglobin release. Photohemolysis of RBCs is related to the generation of Reactive Oxygen Species (ROS) that accumulate with biomolecules of cell membrane, such as lipids and DNA proteins that lead to cells swelling and, finally, rupturing (Al-Akhras *et al*., 1996). Photosensitization of RBCs with Photofrin® conjugated with IONPs inhibits erythrocytes membrane lysis, the decreasing in activation energy and increment in t_{50} were obvious evidences for such a result. A similar result was reported by Reddy *et al*. (2006)**.** They noted that the rates sensitized with Photofrin® under irradiation causes death within 13 days but rates sensitized with Photofrin® in the presence of IONPs stayed 33 days. Experimental data were mathematically modelled using Gompertz function (see Table 1). For all curves, the points represent the experimental data while the solid lines show their best fitting with Gompertz function. Relative steepness for lysis curves (S) is defined as t_{80}/t_{30} where t_{80} is the time required to 80% lysis of cells and t_{30} is the time required to 30% lysis of cells and it does not seem to be affected by IONPs presence.

The irradiation temperature (T_{irr}) and the time required to lysis 50% of the cells (t_{50}) are clear to be inversely proportional. The value of t_{50} is larger at lower values of T_{irr} and much larger with the presence of IONPs at same T_{irr}. The applicability of Gompertz function to the fractional photohemolysis ratio, (**a)** and the rate of fractional photohemolysis, (**b)** is found to be the most appropriate model to fit the experimental data with minimum parameters and minimum errors. The parameter (**a)** was showed to be independent of temperature and the values of parameter (**b)** increases with increasing irradiation temperature for 2 µg/ml Photofrin® and 50 µg/ml IONPs. Similarly, the parameter (**a)** was showed to be independent of temperature, while the parameter (**b)** increases with increasing irradiation temperature for 2 µg/ml Photofrin® in the absence of IONPs.

The Arrhenius equation can thoroughly analyze the dependence of temperature and cells killing (Alzoubi *et al*., 2014). Arrhenius equation is defined as $1/t_{50} = A\, e^{-E/kT}$, where $1/t_{50}$ reciprocal to the time required to 50% lysis of cells that acts as the activation rate which is described by the steepness of the killing curve; A is

constant, E is the activation energy, k is the Boltzmann constant, and T is the absolute temperature. Hyperthermia measurements with human RBC led to no hemolysis after 30 h at a temperature below 37°C (Gershfeld and Murayaman, 1988). The typical curves of $1/t_{50}$ Vs 1/T are shown in Figure 3. The curves were fitted with Arrhenius equation to calculate the activation energy. The apparent activation energy destabilization of the RBC membrane by photosensitization for 4 – 41°C were quantitatively calculated and found to be 18.85 ± 0.72 kcal/mol in the absence of IONPs and 17.29 ± 0.71 kcal/mol in the presence of IONPs. It was also noticed that the activation energy for the samples without IONPs are greater than the activation energy with IONPs. On the other hand, the presence of IONPs decreases the photohemolysis process and increases the t_{50}.

The generally accepted colloid-osmotic mechanism postulates that the photochemical damage to the RBC membrane leads to cation efflux, followed by cell swelling and rupture (Pooler, 1985). A speculative connection to colloid-osmotic lysis is that ***a*** and ***b*** are related to the rate of the damaged band 3 protein sites that act in concert to form a K^+ leak. An additional consideration is that singlet oxygen, generated by strong bound Photofrin® with IONPs, may react with membrane targets *via* the external medium. Figure 4 showed microscopic images of RBCs with the presence and absence of IONPs: Figure 4 (a) the RBCs incubated with 2µg/ml Photofrin® only and Figure 4 (b) incubated with 2 µg/ml Photofrin® and 50µg /ml IONPs. The images were taken using NIKON inverted microscope. Strong bounded IONPs, on the cell surface with possible diffusions inside the membrane, are clearly shown in Figure 4 (b). In conclusion, our results indicate that Photofrin® incorporated with IONPs could be considered as a modality to improve cytotoxicity in photodynamic therapy or reduces the inhabitation effect.

Table 1. Effect of irradiation temperature on RBCs photosensitized by Photofrin® with presence and absence of IONPs. **Group I**: RBCs incubated with 2 µg/ml Photofrin® and 50 µg/ml IONPs**, Group II:** RBCs incubated with 2 µg/ml Photofrin® only

	$(t_{50})_{exp}$	$(t_{50})_{th}$	T_{irr}	*S*	Gompertz function parameters			R
Group	(min)	(min)	(˚C)		H_o	*a*	*b* $(min)^{-1}$	%
I	47.23	45.93	4.0		1.10±0.03	52.46±12.65	0.09±0.01	0.997
	42.68	41.90	18.0	1.37	1.17±0.04	33.09±6.73	0.08±0.01	0.997
	37.86	37.08	25.0	±	1.18±0.05	50.89±15.29	0.12±0.01	0.996
	32.26	31.70	35.0	0.02	1.12±0.04	57.26±17.27	0.14±0.02	0.997
	25.75	25.23	41.0		1.14±0.04	38.85±10.21	0.15±0.01	0.998
II	39.04	39.04	4.0		1.25±0.07	43.05±13.05	0.10±0.01	0.996
	33.22	32.74	18.0	1.38	1.27±0.07	44.66±13.57	0.12±0.01	0.997
	28.53	28.21	25.0	±	1.15±0.04	59.88±17.41	0.15±0.01	0.998
	21.73	21.43	35.0	0.09	1.44±0.14	20.00±5.25	0.16±0.02	0.997
	15.54	14.98	41.0		1.21±0.09	45.90±23.40	0.26±0.04	0.996

Results are expressed as mean ±S.D. ***S:*** Curves Steepness defined as t_{80}/t_{30} Gompertz Function (Eq.1) Parameters: H_0 Initial maximum number of cells (normalized to one). ***a*** Fractional hemolysis ratio. ***b*** The rate of fractional hemolysis change. $(t_{50})exp$: the experimental value of time required to lysis 50% of cells $(t_{50})th$: the theoretical value of time required to lysis 50% of cells T_{irr}: Irradiation temperature **R:** correlation coefficient

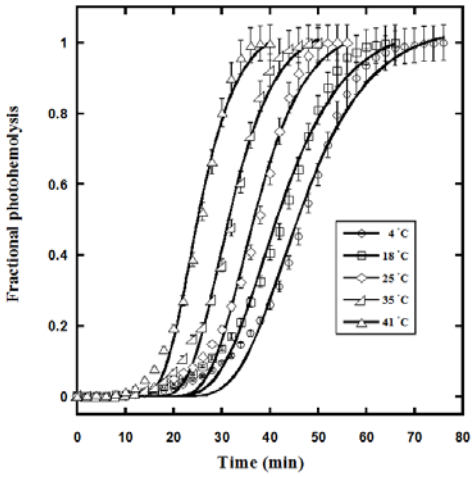
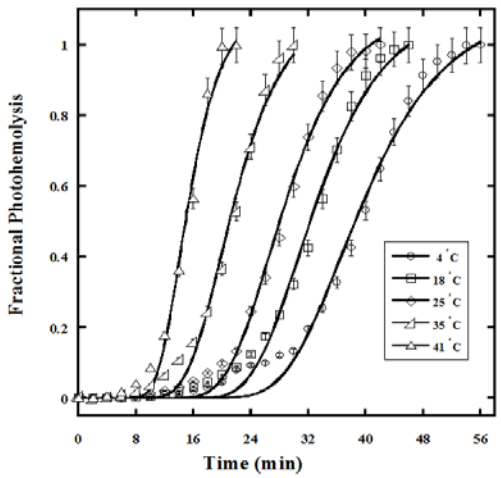


Figure 1. Photosenstization of CPH by 2 µg/ml Photofrin® with 50 µg/ml IONPs, irradiated to range of temperature at fixed incubation temperature. The solid lines are the Gompertz function fitting.



Figuer 2. Photosensitization of CPH by 2 µg/ml Photofrin®, irradiated to range of temperature at fixed incubation temperature. The solid lines are the Gompertz function fitting.

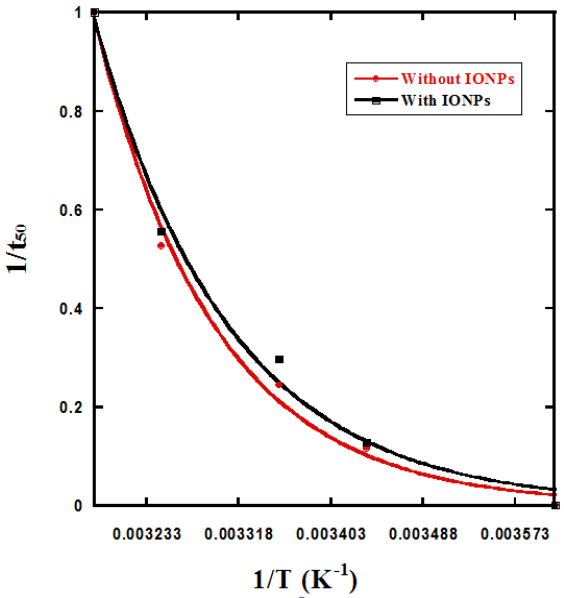
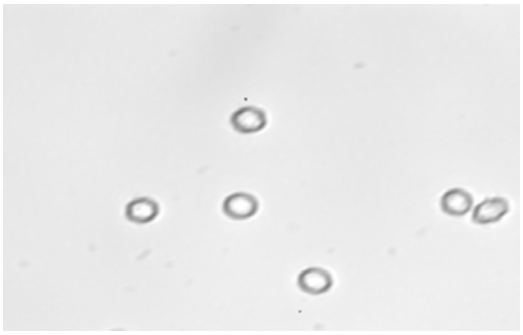
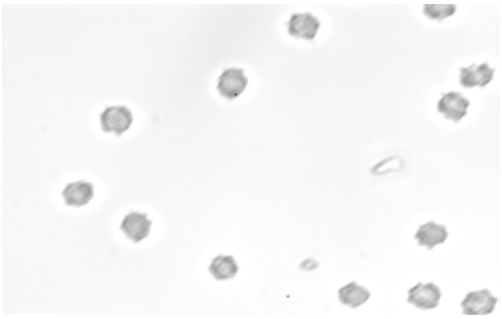


Figure 3. Arrhenius plot for the dependence of $1/t_{50}$ and temperatures corresponds to CPH curves with and without IONPs. The solid lines are the Arrhenius equation fitting. The apparent activation energy was found to be 18.85 ± 0.72 kcal/mol in the absence of IONPs and 17.29 ± 0.71 kcal/mol in the presence of IONPs



(a)



(b)

Figure 4. Microscopic images of RBCs with presence and absence of IONPs. (a) RBCs incubated with 2µg/ml Photofrin® only; (b) incubated with 2 µg/ml Photofrin® and 50µg /ml IONPs. The images were taken using NIKON inverted microscope.

4. Conclusion

Photosensitizations of RBCs with Photofrin® conjugated with IONPs are investigated. Gompertz function serves as a typical mathematical model for CPH that leads to suitable modelling parameters and is found to be in a very good agreement with the experimental parameter. The notable increase in the rate (***b***) suggests that additional membrane targets are accessible to singlet oxygen generated in the external medium or might be attributed to membrane lysis which combines the effects of cell swelling induced by a damage to the anion transport protein and a thermally activated photochemical damage to structural membrane proteins.

Acknowledgements

This work was supported by a grant from the Deanship of Scientific Research at Jordan University of Science and Technology (JUST): Grants number 113/2013.

References

Al-Akhras MA and Grossweiner LI 1996. Sensitization of photohemolysis by hypericin and photofrin, *J Photochem Photobiol B*. **34(2-3):**169-75.

Al-Akhras M A, Amin A, Mohammad K, Al-Haddad Fand Allaaeldin H. 2007. *In vitro* studies on the effect of phototoxicity of a new photosensitizer extracted from flowers and aerial parts of *Cichorium pumilum*. *Am J Pharmacol Toxicol* **2 (2)**: 39-45

Al-Akhras M A. 2006. A new application of Gompertz function in photohemolysis: the effect of temperature on red blood cell hemolysis photosensitized by protoporphyrin IX. *Med Bio Eng Comput*. **44**:703–710

Allison, R R, Downie G H, Cuenca R, Hu, XH, Child, C JH and Sibata C H. 2004. Photosensitizers in clinical PDT, *Photodiagnosis and Photodyn Ther.*, **1***:* 27-42.

Alzoubi, F Y, AL-Akhras M A, Al-Omari H A and Aljarrh K. 2014. Evaluating the photosensitivity of the Chinese Chlorella on Red Blood Cells. *J Mol Pharm Org Process Res*. **2**:2.

Amirnasr A, Emtiazi G, Abasi S and Yaaghoobi M. 2011. Adsorption of hemoglobin, fatty acid and glucose to iron nanoparticles as a mean for drug delivery, *J Biochem Tech*, **3(2)***:*280-283.

Ben-hur E and Rosenthala I. 1986. Photohemolysis of Human Erythrocytes Induced by Aluminum Ph'i'halocyanine Tetrasulfonate, *Cancer Letters*, **30**: 321-327.

Choe S-W , Terman D S, Rivers A E, Rivera J, Lottenberg R and Sorg B. S. 2013. Drug-loaded sickle cells programmedex vivofor delayed hemolysis target hypoxic tumor microvessels and augment tumor drug delivery. *J Control Release*. **171**: 184–192.

Dougherty T J. Gomer C J, Henderson B W, Jori G, Kessel D, Korbelik M, Moan J and Peng Q. 1998. Photodynamic Therapy, *J Natl Cancer Inst*, **90**: 889–905.

Gershfeld, N. L. and Murayama M.1988. Thermal instability of red blood cell membrane bilayer: temperature dependence of hemolysis. *J Membr Biol.* **101**: 67-72

Henderson B W, Busch T M, Vaughan L A, Frawley N P, Babich D, Sosa T A, Zollo J D, Cooper M T, Bellnier D A, Greco W R and Oseroff A R, 2000. Photofrin photodynamic therapy can significantly deplete or preserve oxygenation in human basal cell carcinomas during treatment, depending on fluence rate, *Cancer Res.*, **60**: 525-529.

Morales, P. Verdaguer, S. Teresita, G. and Serna, C. (2003). The preparation of magnetic nanoparticles for applications in biomedicine, *J. Phys. D: Appl. Phys.* 36: 182–197.

Moersdorf D, Hugounenq P, Truonc Phuoc L, Mamlouk-Chaouachi H, Felder-Flesch D, Begin-Colin S, Pourroy G and Bernhardt I. 2010. Influence of magnetic iron oxide nanoparticles on red blood cells and Caco-2 cells. *Adv Biosci Biotechnol*. **1**:439-443.

Patrick A L, Qian Y, Shao R, Guo N L, Schwegler-Berry D, Pacurari M and Flynn D C. 2009. Iron oxide nanoparticles induce human microvascular endothelial cell permeability through reactive oxygen species production and microtubule remodeling. *Particle Fibre Toxicol.,* **6(1)**: 1.

Pooler J. 1985. The kinetics of colloid osmotic hemolysis. II. Photohemolysis, *Biochim Biophys Acta*, **812:** 199-205.

Tartaj P, Morales M P, Veintemillas-Verdaguer S, Alez-Carre˜ T G and Serna C J. 2003. The preparation of magnetic nanoparticles for applications in biomedicine. *Phys D: Appl Phys*.,**36**: 182–197.

Reddy G. R, Bhojani M S. McConville P, Moody, J, Moffat B A, Hall, D E, Kim G, Koo, Y-E, Woolliscroft M J, Sugai, J V, Johnson T D, Philbert M A, Kopelman R, Rehemtulla A and Ross B D. 2006. Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res.*, **12 (22)**: 6677-6686

Renno R Zand Miller J W. 2001. Photosensitizer delivery for photodynamic therapy of choroidal neovascularization, *Advanced Drug Delivery Rev.*, **52**: 63–78.

Sil S, Bose T, Roy D and Chakraborti A S. 2004. Protoporphyrin IX-induced structural and functional changes in human red blood cells haemoglobin and myoglobin. *J. Biosci.* **29**: 281–291.

Sternberg, Dolphin, David .1997. Porphyrin-based photosensitizers for use in photodynamic therapy, *Tetrahedron.* **54**: 4151-4202.

Yang C, Wang J, Chen D, Chen J, Xiong F, Zhang H, Zhang Y, Gu N, Dou J. 2013. Paclitaxel-Fe_3O_4 nanoparticles inhibit growth of $CD138^-$ $CD34^-$ tumor stem-like cells in multiple myeloma-bearing mice, *Int J Nanomedicine*, **8**: 1439–1449.