OPTIMIZATION OF CELL DEATH USING RESPONSE SURFACE METHODOLOGY OF THE EFFECT OF TIME AND CONCENTRATION ON Sn(IV) CHLORIN E6 DICHLORIDE TRISODIUM PHOTOSENSITIZER

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The absorption bands dark toxicity and the effect of Sn(IV) chlorine e6 dichloride trisodium salt photosensitizer on the viability of MCF7 and T47D breast cancer cell lines were investigated. Response surface methodology (RSM) was carried out to evaluate the effect of time and concentration of Sn (IV) chlorine e6 on the viability of MCF7 and T47D breast adenocarcinoma cell lines. The results of RSM showed that the effect of time and photosensitizer concentration was significant and a second-order model was a suitable model to describe the behavior of viability. The results also showed that 97% to 99% of the total variation in viability was explained by the second-order mode. Minimum viability for MCF7 was achieved at incubation time of 6 hours and concentration of photosensitizer between 33 - 34 μg/ml using light dose of 70 J/cm^2 at 635 nm wavelength, while for T47D the best conditions for minimum viability was achieved at 35 μg/ml. Maximum absorption was achieved at 410 nm (soret band) and 635 nm (Q band). The results also showed that the photosensitizer exhibited a lack of dark toxicity which could be used in photodynamic therapy.

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1. Introduction

Photodynamic Therapy (PDT) is a modality of cancer treatment based on the selective accumulation of light sensitive drug named photosensitizer in the tumor tissue, as compared with the intact surrounding tissue [1-7]. In PDT combination of the photosensitizer and light at particular wavelength in the presence of oxygen produce chemical reactions of type I (generating radical oxygen species ROS) [8] and type II (generating toxic singlet oxygen) [9] which causes cell damage. The effect of photosensitizer depends on its concentration, incubation time, the presence of oxygen, the lifetime of the molecule in the triplet state and other factors [10]. In this study optimization *in vitro* for incubation time and photosensitizer concentration was investigated using response surface methodology (RSM) with red light dose of 70 J/cm^2 at 635 nm wavelength. This wavelength allows good penetration through tissues and maximum absorption for the photosensitizer, as shown in Fig. 1.

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2. Methodology

2.1 Materials

Photosensitizer (Sn (IV) chlorine e6 dichloride trisodium salt) was purchased from (Frontier Scientific, Inc. Logan, USA). MCF-7 and T47D breast cancer cell lines were purchased from (ATCC, USA). EMEM culture medium, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and fetal bovine serum (FBS), were used. All these chemicals were purchased from (GIBCO, Malaysia). Xenon lamp was used as the source of light equipped with 635 nm red spectrum filter.

2.2 PDT Treatment

The Photosensitizer was directly dissolved in PBS (1mg/ml) before adding to the cells. MCF7 breast cancer cell lines were grown in EMEM medium supplemented with 10% FBS and incubated at 37^{o}C and 5% CO_2. T47D cell lines were grown in RPMI 1460 medium supplemented with 10% FBS at the same conditions as well. The cells were incubated in a 96-well plate with 2×10^4cell/well for one night incubation to be attached. Photosensitizer was added on the next day to the medium with different concentrations and different incubation times as showed in the experimental design. Then the medium was replaced with fresh medium to remove the unbounded photosensitizer. Each well was irradiated at 635 nm light and dosage of 70 J/cm^2.

2.3 MTT Assay

After irradiation, the cells were incubated at 37^{o}C with 5% CO_2. Next, after 24 hours of incubation, MTT solution was prepared for an amount of 5 mg in 1ml of PBS. The 20 µg of the prepared solution was added to each well of the 96-well plate and the plates were incubated at 37^{o}C with 5% CO_2. After four hours of incubation, the medium was discarded and replaced by 100 µl DMSO/well and the results were recorded by using ELISA plate reader.

3. Experimental design

RSM is a collection of mathematical and statistical techniques that are useful for modeling and analysis of problems in which a response (y) of interest is influenced by several independent variables (x_i). The objective of RSM is to find the levels of independent variables that optimize the response.

RSM was employed to study the effect of two factors (independent variables) time (x_1), and the concentration of a new photosensitizer Sn(IV) Chlorine dichloride e6 (x_2) on viability of MCF7 or T47D cancer cell lines (y).

The response is a function of the levels of independent variables (time and photosensitizer concentration), say

$$y = f(x_1, x_2) + \varepsilon \tag{1}$$

where ε represents the error observed in the response y. The expected response is $E(y) = f(x_1, x_2) = \eta$, and the surface represented by

$$\eta = f(x_1, x_2) \tag{2}$$

η is called a response surface.
Experimental data was analyzed to fit the following second-order model

$$\eta = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j \quad (3)$$

where, β_0, β_i, β_{ii}, and β_{ij} are regression coefficients, and x_i are the coded variables. The relationship between the natural variable ξ_i and the coded variables x_i is

$$x_i = \frac{\xi_i - (High\ \ level + Low\ \ level)/2}{(High\ \ level - Low\ \ level)/2}$$

A central composite design (CCD) with two indepentent variables was used (10). Thirteen runs were required for each cell line to cover all possible combination of factors levels. The data were collected from 2^2 factorial augmented by five center points and four extra points (axial points or star points). The experiments were run in random order to minimize the effects of unexpected variability in the observed responses. The experimental range for each indepentent variable was based on the preliminary trials, incubation time levels are (4-8) hr and photosensitizer concentration levels are (25-35) µg/ml.

4. Result and discussion

The light dose was fixed at 70 J/cm^2 based on preliminary study, in which the photosensitizer absorbed sufficient energy to pump its molecules to be at excited state as shown in Figure 1. The effect of time and photosensitizer concentration on viability of MCF7 and T47D cancer cell lines was studied using response surface methodology (RSM). The results of central composite design showed that a second-order model is suitable to describe the relationship between viability as a response and time and photosensitizer concentration as an influential factors on viability. The second-order models that describe the behavior of viability to optimize the process by finding the best setting of time and photosensitizer concentration that minimize the viability are given in Equations 4 and 5 in terms of coded variables for MCF7 and T47D cancer cell lines respectively.

$$\eta_1 = 48.2 - 3.44x_1 - 3.77x_2 + 4.21x_1^2 + 3.46x_2^2 - x_1x_2 \quad (4)$$

$$\eta_2 = 46.8 - 6.01x_1 - 3.31x_2 + 4.79x_1^2 + 1.04x_2^2 + 2x_1x_2 \quad (5)$$

The second-order regression models obtained for viability are satisfied since the value of the coefficient of determination (R^2) is high and close to 1. The values of R^2 for viability models are 0.99 and 0.97 for MCF7 and T47D respectively. This shows that 0.97-0.99 of the total variation is explained by the models and only 0.01-0.03 of the total variation is unexplained.

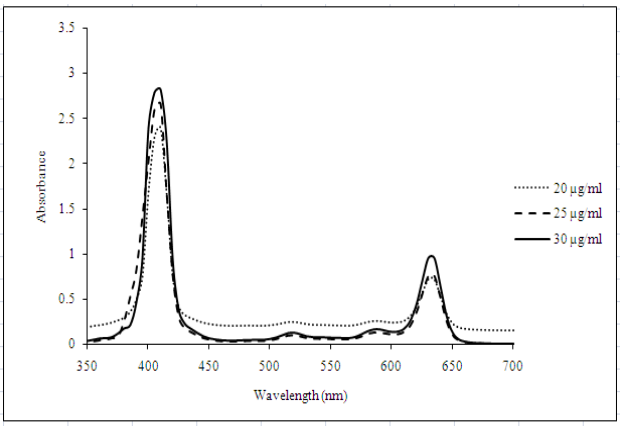

Fig. 1. Absrption spectra for Sn(IV) chlorine e6 for the indicated concentrations.

The result of analysis of variance (ANOVA) for viability of MCF7 and T47D cancer cell lines is given in Table 1. The analysis of variance revealed that a second-order model adequately fitted the experimental data for MCF7 and T47D. The linear effect of time (x_1) and concentration of photosensitizer (x_2) were significant. The contribution of quadratic effect over the linear effect for time (x_1^2) and concentration of photosensitizer (x_2^2) was significant. Besides the significant effect of the main effect and quadratic terms the interaction between incubation time and photosensitizer concentration exhibited a significant effect on viability (Table 1). Significant interaction shows that the factors do not work independently. For T47D cancer cells the main effect of time and photosensitizer concentration, quadratic term for time and the interaction between incubation time and photosensitizer concentration were significant. However, the quadratic term for photosensitizer concentration did not show a significant effect on viability. The used light dose provided more absorption energy to photosensitizer molecules, which concentrated in cells with suitable incubation time, to excite to higher levels. This mechanism leads to increase the chemical reactions directly and indirectly. Subsequently, the generation of singlet oxygen and reactive oxygen species can be enhanced to increase the biological damages in the cancer cells. The light dose and photosensitizer concentration with sufficient incubation time increased the number of excited molecules, which lead to more cellular damage for the cancer cells. The relative contribution of each factor to viability of MCF7 and T47D cancer cell lines was directly measured by the regression coefficient in the fitted model of Equations 4 and 5. A positive sign for the regression coefficient in the fitted model shows the ability of the factor to increase the viability, while the negative sign shows the ability of a factor to decrease the viability.

Table 1. ANOVA for Response Surface Quadratic Model (Analysis of variance table [Partial sum of squares])

MCF7					
Source	Sum of squares	DF	Mean squares	F value	P-value
Model	395.99	5	79.20	112.28	< 0.0001
Time	94.92	1	94.92	134.57	< 0.0001
concentration	113.57	1	113.57	161.01	< 0.0001
Time ×Time	123.44	1	123.44	175.01	< 0.0001
Con.× con.	83.40	1	83.40	118.24	< 0.0001
Time × con.	4.00	1	4.00	5.67	0.0488
Residual	4.94	7	0.71		
Error	2.80	4	0.070		
Total	400.92	12			

T47D					
Source	Sum of squares	DF	Mean squares	F value	P-value
Model	552.84	5	110.57	38.24	< 0.0001
Time	288.50	1	288.50	99.77	< 0.0001
concentration	87.68	1	87.68	30.32	0.0009
Time ×Time	159.44	1	159.44	55.14	0.0001
Con.× con.	7.49	1	7.49	2.59	0.1516
Time × con.	16.00	1	16.00	5.53	0.0509
Residual	20.24	7	2.89		
Error	2.80	4	0.70		
Total	573.08	12			

The three-dimensional response surface plots for viability of MCF7 and T47D are given in Figures 2 and 3 respectively, which show the effect of incubation time and concentration of photosensitizer on viability. In conclusion, the viability exhibited a clear surface, suggesting that the best condition for minimum viability of MCF7 and T47D cancer cell lines is well defined inside the design boundary. The minimum percentage of viability of MCF7 was achieved at incubation time of more than 6 hours and photosensitizer concentration between 33 - 34 µg/ml, as shown in Fig. 2. For T47D, the minimum percentage of viability was achieved at incubation time of more than 6 hours and photosensitizer concentration of 35 µg/ml, as shown in Fig. 3.

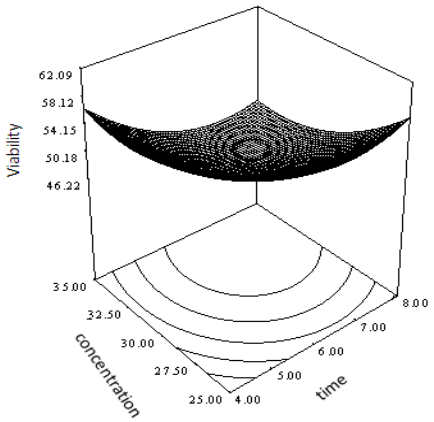

Fig. 2. Three dimensional response surface plot for viability of MCF7 cell lines

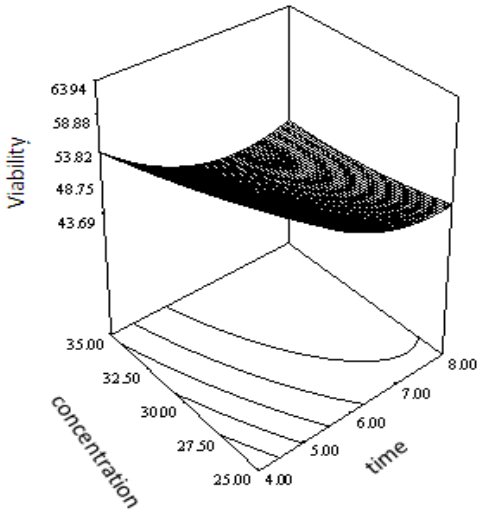

Fig. 3. Three dimensional response surface plot for viability of T47D cell lines

4.1. Optimization of the experiment

Based on the above results and discussion, it is better to run an optimization study to find out the best conditions for viability. The model has been developed and checked for adequacy, the optimization criteria can be set to find the best viability conditions. The criterion is to keep viability of MCF7 and T47D cancer cell lines as minimum as possible. Minimum viability for MCF7 and T47D cells were achieved at incubation time of 6 hours and photosensitizer concentration between 33- 34 µg/ml for MCF7 and for T47D is 35 µg/ml. The viability increases at levels beyond the best condition. Within the best conditions of incubation time and concentration the cellular uptake of the photosensitizer by the cells expected to be maximum, which result in maximum damage of cancer cells when it is irradiated with light. The lack of cellular uptake will cause less chemical reactions and incomplete damage of the cells. The increasing of Sn(IV) chlorine e6 concentration or incubation time more than 30 µg/ml and more than 6 hours, respectively, did not make a significant difference in the viability, which means that the cells were saturated with the photosensitizer. A comparison was made to study the effect of the photosensitizer in the dark and in the presence of light is shown in Figures 4 and 5. The viability of the photosensitizer is very high, which means that there is a poverty of dark toxicity to photosensitizer. The dark toxicity for T47D cells is little higher than the dark toxicity for MCF7 cells, but it is less than 13% at concentration of 30 mg/ml. The small dark toxicity at high concentration can be explained on the basis that the photosensitizer causes a small damage for the plasma membrane at high concentrations without light, thus gives an advantage to this photosensitizer to be suitable for PDT. The light has no effect on MCF7 and T47D cells without photsensitizer. There is no significant different between the effect of light on MCF7 and T47D cancer cells. There is a lack of phototoxicity in both cancer cells without photosensitizer, which can be explained that the light does not make toxic interactions in the cell.

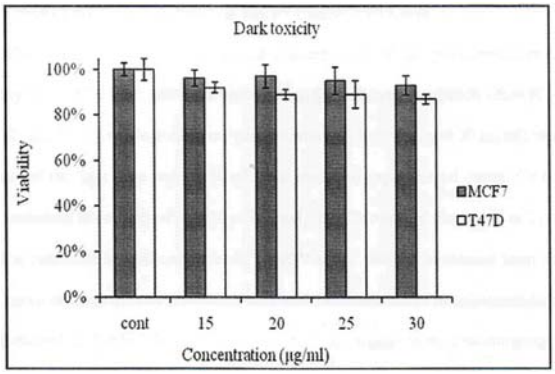

Fig. 4: viability of MCF7 and T47D cells at different concentration of Sn(IV) chlorine e6 without light irradiated

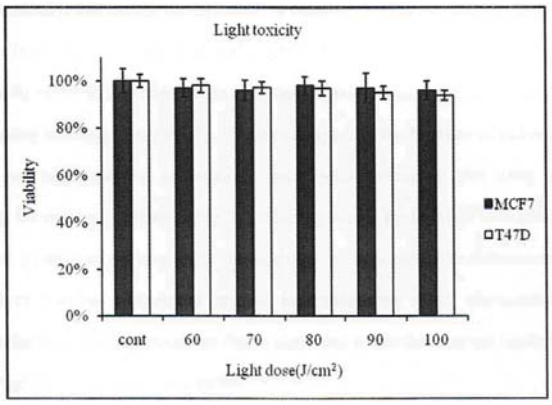

Fig. 5. Viability of MCF7 and T47D cancer cells at different light doses without photosensitizer.

4.2. Validation of the developed model

To validate the developed model, two confirmation experiments were carried out with viability conditions chosen randomly from the optimization results. The results of selected combinations of incubation time and photosensitizer concentration are given in Table 2, including actual and predicted values of viability. The validation results demonstrated that the model developed is quite accurate as the percentages of error in prediction was in a good agreement.

Table 2: Optimum factors

Cancer cell	Time	Concentration	Predicted	Actual
MCF7	6.96	33.07	46.21	47.37
T47D	6.84	35.00	42.69	43.28

5. Conclusions

Based on the above results and discussion, it can be said that Photodynamic treatment is a modality of treatment that rapidly improved. Photosensitizer showed low dark toxicity and might be used in photodynamic. The maximum absorption for the photosensitizer at 635 nm allows good penetration through tissues. The investigation of the best incubation time and Sn(IV) chlorine e6 photosensitizer concentration in vitro was also included. The results showed that this photosensitizer can be more effective on the viability of MCF7 and T47D cancer cells at 6 hours of incubation time and 30 µg/ml of photosensitizer concentration under a fixed light dose of 70 J/cm^2.

At these conditions the results also showed that the photosensitizer can destroy about 55% of cancer cells within 24 hours. This photosensitizer Sn(IV) chlorine e6 still need more studies and investigation for their photophysical and photochemical properties and its effect on other cancer cells.

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