

# Comparative study of some nano- and micro - sensitizers in photodynamic inactivation of microorganisms

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The development of new virus inactivation procedures became an area of growing interest mainly due to increased demands concerning the safety of biological products. Photochemical processes represent the most promising methods for the future to inactivate viruses. In these methods, dyes are the most widely used photosensitising reagents. The current article covers a new interesting compound microsensitizer as 5,10,15,20-tetra-sulfonato-phenyl porphyrin (TSPP), and nanosensitizers, as fullerenes (C<sub>60</sub>) –coated TSPP suspension (C<sub>60</sub>/TSPP). There was studied in this paper the photodynamic inactivation of herpes simplex virus type I (HSV-1) provided from rat, by the above macro and nanosensitizers. The concentration and temperature effects were evaluated. These sensitizers demonstrate a remarkable virucidal activity upon light activation, more pronounced for (C<sub>60</sub>/TSPP) than for TSPP, and after an optimum time of 300 minutes.

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## 1. Introduction

Photodynamic inactivation represents a new antimicrobial method against different microorganisms. This method is based on the photosensitizing substances (photosensitizers) administration, which in preferentially localized in the cell and then it is excited by the optical radiation with the wavelength appropriate with the photosensitizer absorbance maximum. The excitation of the photosensitizers by optical radiation results in the production of cytotoxic species as singlet oxygen and free radicals. Due to the highly reactive nature of these species, they will lead to a certain biological effects resulting in the microorganism death by a selective action on the target cells.

Viral inactivation properties have been described for a wide variety of dyes such as phthalocyanines, merocyanines, porphyrin derivatives, hypericin and rose bengal, and methylene blue was tested, until now, for photodynamic inactivation of microorganisms as bacteria, fungi, yeast, virus and algae using a large variety of both coherent and non-coherent light sources [1-4]. Most of them present an inherent disadvantage regarding their poorly water solubility. They are hydrophobic and therefore difficult to administer as they are, and by intravenous injection, especially.

This issue calls for the use of advanced delivery systems and different strategies have been investigated, which mainly include polymer-photosensitizer conjugation as well as encapsulation of the photosensitizer in colloidal carriers such as liposomes, oil dispersions, and polymeric particles [5].

Recently, nanoparticles have received increasing attention as potential delivery systems for photodynamic therapy agents. Quantum dots offer several advantages as potential delivery systems for photosensitizers. The optical properties of this nanomaterial can be tuned to absorb and emit in the near-infrared region of the spectrum by changing their size and composition. Light of low intensity can be used to penetrate tissue several centimetres allowing the access to deep-seated tumours. Importantly, the surface coating of quantum dots can be functionalised to make them more water soluble and biocompatible [6], which facilitates systemic delivery.

Quantum dots can act as photosensitizer alone generating reactive singlet oxygen as well as promote the effect of classical photosensitizers linked to quantum dots (Fig. 1).

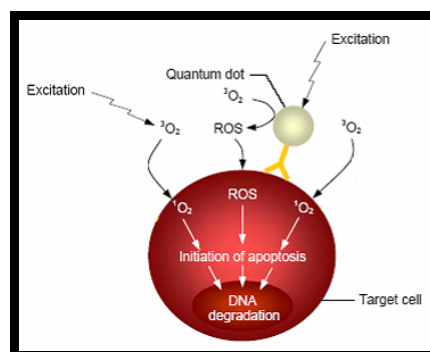


Fig. 1. Schematic illustration of possible mechanisms for quantum dot-induced photodynamic therapy.

A type of such sensitizer is a fullerene,  $C_{60}$  which was reported to be highly stable (high kinetic stability), [7] i.e. it does not decay under biological conditions to the thermodynamically more stable graphite. For photochemical applications it is important that  $C_{60}$  does not show any photodegradation under the chosen conditions. The new form of carbon, the fullerene, enriched the family of carbon allotropes, because it represents a new variety with interesting properties. Buckminsterfullerene in hexane shows several strong absorption bands in the UV range and some weak bands in the visible part of the spectrum [8]. The symmetry-forbidden transitions between 410 and 620 nm are responsible for its purple colour and for the efficient generation of singlet oxygen.  $C_{60}$  can be excited by visible light to its singlet state.  $^1O_2$  is a highly reactive form of excited  $O_2$  and is known to damage biomolecules such as DNA and proteins.

Therefore, the effective generation of  $^1O_2$  by photoexcited buckminsterfullerene and its stability makes  $C_{60}$  a candidate for photodynamic processes in biological applications. The highly quantum efficiency into the photodynamic inactivation of different microorganisms, is dependent on some external factors, as e.g. concentration.

This relationship is analysed in this study for the photodynamic inactivation of Herpes Simplex Viruses, by using the nano-sensitizer  $C_{60}$ /TSPP in parallel with the sensitizer TSPP.

## 2. Experimental part

### 2.1. Photosensitizers

*5,10,15,20-tetra-sulfonato-phenyl porphyrin (TSPP)*, Figure 2, was synthesized and purified in the lab. after literature method [12].

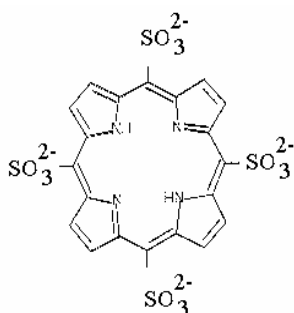


Fig. 2. Chemical structure of TSPP.

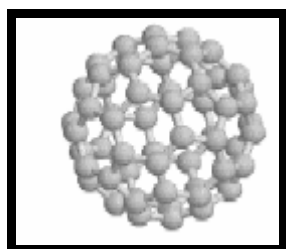


Fig. 3. The configuration of  $C_{60}$ .

TSPP was stored at 4 °C as  $5 \times 10^{-4}$  M stock solutions in PBS and sterilized by filtration. Imidazole, as an  $^1O_2$  acceptor, and *N,N*-Dimethyl-4-nitrosoaniline (RNO; Merck, Darmstadt, Germany) were used in parallel exposures for indirect measurement of the formation rate of  $^1O_2$  due to the secondary bleaching reaction.

The photosensitizer  $C_{60}$ /TSPP was tested for its ability to reduce virus infectivity. A suspension of  $C_{60}$  in buffer (MBS) was prepared by sonification with a sonifier converter tip. In order to coat photosensitizer on the  $C_{60}$  particles,  $5 \times 10^{-4}$  mol of TSPP was introduced into the solution, and the adsorption reaction was conducted at 60, 70, and 80 °C. The concentration of TSPP was obtained from the optical density of UV spectra and IR spectra. The amount of TSPP reacting with the dispersed fine particles increased with increasing temperature. Therefore, TSPP adhered to the  $C_{60}$  particles, and its coating efficiency reached about 50% in the prepared sensitizer.

### Viral photoinactivation

All sera and derma tissue culture media were purchased from Merck, and the cell lines were grown with Bacto- Difco Normal Serum provided from the same company.

The cells were infected with 200 ml of cell-free viral suspension at a concentration of  $2.6-2.9 \times 10^5$  (two suspension type A type with a cells concentration  $2.6-2.8 \times 10^5$  per cell standard, and B type with a cells concentration of  $2.7-2.9 \times 10^5$  per cell standard, for various time intervals at 37 °C (and 30 % humidity in the first 5 days and 5 %  $CO_2$  in the last two days). The sensitizer was added and the plates were exposed to light. The plates were incubated at 37 °C for 5 days in 30 % humidized incubator and in 5%  $CO_2$  humidified incubator in the last two days. Plaques were scored and the titers calculated based on the mean count of three replicate cells.

### Inactivation assay

The  $C_{60}$ -suspension (1 mg/ml) in MES-buffered saline (pH 7.4) was spiked with stock virus. The virus- $C_{60}$  suspension was stirred constantly throughout the duration of the inactivation assay.  $O_2$  saturation of this suspension was achieved by a constant flow of  $O_2$  (bubbling) which started 5 min prior to, and continued during the illumination.

### Survival tests and numerical analysis

After illumination, aliquots were taken from the suspension, diluted and plated on nutrient broth agar (two or three replicates). After incubating HSV at 37 °C for 48 h at 30 °C, the numbers of visible colonies were determined as described previously [13]. Survival  $S = N_F/N_0$  was determined as the average of the quotients of the number of colonies  $N_F$  at fluence  $F$  and of nonilluminated controls  $N_0$ . Uncertainties are given by the standard error of the mean. Survival curves are plotted on a semilogarithmic plot showing shouldered or exponential

survival curves of the form  $S = 1 - (1 - \exp(-k_s F))^m$  or  $S = \exp(-k_s F)$ . These equations were fitted to the respective experimental data. The fit parameter  $k_s$  reflects the sensitivity of the cells to be inactivated under the tested conditions and is represented by the (final) slope of the survival curve. The parameter  $m$  is the extrapolation number and marks the point of intersection of the final slope with the ordinate at  $F = 0$  for a shouldered survival curve. The shoulder width,  $F_q = \ln(m)/k_s$ , marks the point of intersection of the final slope with the abscissa. Fit parameters and their standard errors were determined by weighted least-square fits.

#### Measurement of $^1O_2$ production

The formation of  $^1O_2$  in the homogeneous aqueous solutions of 1–5 ppm TSPP was measured by a bleaching reaction method [14] at different indices of pH and temperature. The  $^1O_2$  production rate is proportional to the 'secondary' bleaching of RNO which can be assessed spectroscopically at  $\lambda = 440$  nm. The concentration of RNO between 0.1 and 0.01 mmol  $L^{-1}$  was previously calibrated. The linear regression of the decay of the absorbance at  $\lambda = 440$  nm with the concentration of RNO leads to a value of:  $\Delta E/\Delta c = 34120 \pm 7034$  L  $mol^{-1}$ . To initiate the secondary bleaching reaction of RNO, imidazole is used as a  $^1O_2$  acceptor to give a transannular peroxide. This peroxide oxidizes the yellow-colored RNO to colorless oxidation products. The kinetic of bleaching is independent of the dye concentration used. A 'primary' bleaching of RNO by the used sensitizers was not observed in the dark. The decay of the absorbance  $A$  of RNO is correlated with the  $^1O_2$  production of the sensitizer. The relative absorbance  $R_a = A_i/A_0$  was determined where  $A_i$  means absorbance at illumination  $i$  and  $A_0$  indicates no illumination. These data were fitted either by an exponential ( $Y = a \exp(-k_a F)$ ) or by a dual-exponential function ( $Y = a \exp(-k_a F) + b \exp(-k_b F)$ ). The slope  $k_a$  describing the initial  $^1O_2$  production rate is used to compare the influence of the different investigation parameters (temperature, pH, concentration) on the inactivation of HSV cells. The other term,  $k_b$ , reflects the consumption (bleaching) of TSPP during the illumination resulting in a reduced  $^1O_2$  production rate.

#### Light exposure

Illumination of the virus suspensions (2.3 ml, on ice) in a 1 cm glass cuvette was performed using a 350 W short arc mercury lamp (HBO 350 W; Osram, Germany) positioned at a distance of 28 cm from the sample and equipped with a 495 nm long pass filter (Carl Zeiss Jena, Germany). The light beam was focused on the cuvette, producing a light intensity of approximately  $2 \times 10^5$  lux (measured with a MX4 lux-meter (ROMLUX, Romania)). Therefore, Pyrex glass (2 mm thickness) was used as a cut-off filter to suppress UV radiation at wavelengths below about 320 nm. The incident fluence rate was 200  $W/m^2$ . Exposure of viral particles and cells was done in 2 ml of phosphate buffered saline (PBS) containing 1.5 %

fetal bovine serum (FBS) in 5 ml polystyrene plates at different temperature ( $35^\circ C$ ,  $37.5^\circ C$ , and  $42^\circ C$ ) for 30 minutes, resulting in a dose of 18  $J/cm^2$ .

To determine the kinetics of inactivation, some samples were taken after different illumination times.  $C_{60}$  was removed by centrifugation and residual virus determined by endpoint titration. In order to study the influence of temperature, the vessel containing the cell suspension or chemical solution was equipped with a thermostat to compensate the heating of the suspension by the intense infrared component of the spectrum of the lamp. The cell suspension was mixed mechanically with a magnetic stirrer to assure adequate mixing and  $O_2$  saturation during illumination.

### 3. Results and discussion

Rate studies of photodynamic inactivation of viruses as a function of time at constant light intensity show like in Fig. 2, expressed by the survival ration ( $N/N_0$ ) plotted against time. Fig. 2 shows the survival curves of dermal HSV from rats during photosensitization with TSPP. Could be observed that only TSPP with  $1.377 \times 10^{-5}$  M concentration is the most efficient during the inactivation process of HSV. The other concentrations are not proper for this inactivation.

#### Influence of dye concentrations

For TSPP the optimum concentration is  $1.377 \times 10^{-5}$  M because at higher concentrations, TSPP is toxic by itself for the HSV cells. At  $1.377 \times 10^{-5}$  M the cell survival is reduced to 0.60 if suspended over 2 h in the dark. Hence, the useful range of concentrations was between 1 and  $1.377 \times 10^{-5}$  M TSPP. Within this range we choose an uncritical concentration of  $1.377 \times 10^{-5}$  M TSPP in order to test the influence of temperature and pH index on PDT.

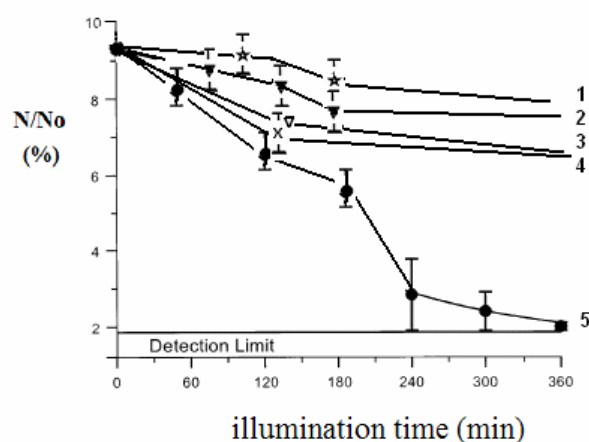


Fig. 2. The survival curves of HSV -derma from rats at different TSPP concentrations at  $35^\circ C$  ( $1 = 0.274 \times 10^{-4}$  M;  $2 = 0.6885 \times 10^{-4}$  M;  $3 = 2.754 \times 10^{-6}$  M;  $4 = 5.508 \times 10^{-6}$  M;  $5 = 1.377 \times 10^{-5}$  M).

### Influence of pH index

The modification of the photodynamic effect on cells of HSV and the variation of  $^1\text{O}_2$  production with changes in the pH index are shown in Fig. 3.

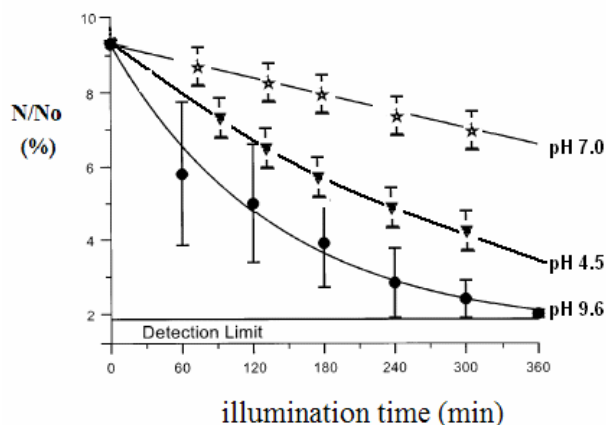


Fig. 3. The survival curves of HSV -derma from rats at different pH values.

Survival of HSV was drastically reduced under PDT at indices of pH = 4.5 and 9.6 in comparison to the survival at pH = 7.0 (25°C,  $1.377 \times 10^{-5}$  M TSPP) PDT with pH indices above and below pH = 7.0 lead to a reduction of surviving cells by many decades at much smaller radiant exposures, with cell killing at pH = 9.6 being more effective. Regarding the effect of pH alone, the survival curves without TSPP are identical for pH = 7.0 and 4.5, whereas a remarkable increase of inactivation was observed for pH = 9.6.

The explanation reside in the aggregation processes of TSPP, which depend on porphyrin concentration [16], pH [17], ionic strength [18], temperature [19]. This porphyrin is an anionic one with a very large disk-shaped molecule which possess four negative charges (the sulfonate groups from the four corners (Fig. 1). By increasing the medium acidity, complicated changes of the form of TSPP [20]. New aggregated forms of dicationic species ( $c > 10^{-4}$ M) (at pH < 5) and new anionic forms (at pH > 7) appear in system. Some J-aggregate (edge-to-edge interaction of porphyrin molecules [21]) appear in the system, responsible for the viral inactivation.

TSPP have a zwitterionic structure between the double charge from the macrocycle inside (central dicationic group) and one of the other negative charges provided by the sulfonic groups, all these being responsible for J-aggregation. In the studied case, TSPP at  $1.377 \times 10^{-5}$  M have a J-aggregated forms being a proof for the special efficient activity of this porphyrin. In other literature reports, similar data concluded that J-aggregates form due to their spiral geometric forms have a special ability to penetrate more efficient the cellular membrane [22]. After penetration of the cellular wall, the J-aggregation can be transformed into monomeric form, exhibiting a high efficiency for singlet oxygen generation. At indices of pH = 4.5 and 9.6 the increase of inactivation

rates by a factor 7.5 and 4.8, respectively, is still more pronounced, again in contrast to the minor variation of  $^1\text{O}_2$  production rates.

### Influence of temperature

The influence of temperature was investigated in a range from 10 to 37.5°C with  $1.377 \times 10^{-5}$  M TSPP. The results displayed in Fig. 3 show an increasing efficiency for cell inactivation of HSV with increasing temperature. The photosensitization reactions showed increases in the overall reaction rates with temperature. This suggests that the influence of hyperthermia on PDT may caused by in part to the temperature dependence of the basic chemical processes which play a role in photosensitization. The photosensitized HSV inactivation is presented in Fig. 4 for three temperatures: 35°, 37.5° and 42 °C.

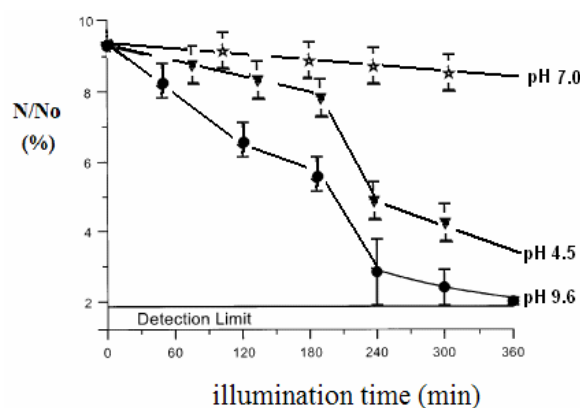


Fig. 4. The HSV-derma from rats inactivation kinetics at different temperatures.

The photosensitization in vitro carried out under conditions of simultaneous hyperthermia (caused by heating of cell suspension) could involve a decrease of singlet oxygen quantum yield at high temperature [23], knowing that TSPP has a quantum yield of singlet oxygen of 0.64 [24].

Our studies have focused on the use of the singlet oxygen-generating agent  $\text{C}_{60}/\text{TSPP}$ , which has the additional benefit that it can be completely and rapidly removed from aqueous solutions.  $\text{C}_{60}/\text{TSPP}$  is a potent generator of singlet oxygen ( $\Phi=0.7$ ).

In this report, we studied the inactivation of enveloped viruses in the presence of  $\text{C}_{60}/\text{TSPP}$  when illuminated with visible light.

Fig. 5 shows the resultant time-dependent loss of infectivity of HSV. The points represent the mean values and standard errors from three independent experiments. As a control, HSV was illuminated in the absence of  $\text{C}_{60}/\text{TSPP}$ , or incubated with 1 mg  $\text{C}_{60}/\text{TSPP}$  per ml (optimum concentration) at 4°C without illumination. The observed maximal non-specific inactivation was after 5 h incubation. A further control was performed by chasing the oxygen from the solution. To reduce the oxygen available during the inactivation, the suspension was

flushed with argon 5 min prior to and during the illumination.

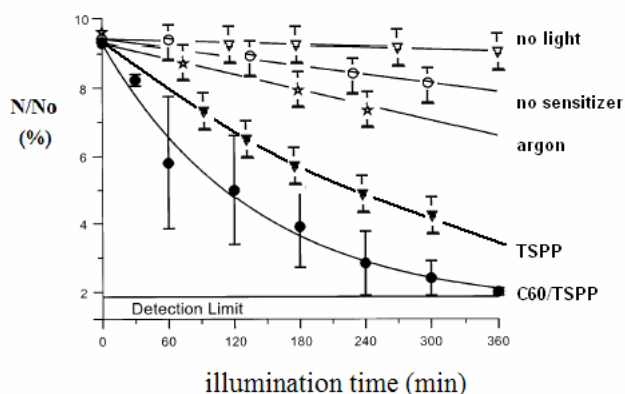


Fig. 5. Kinetics of the photodynamic inactivation of HSV by different experimental conditions.

This treatment resulted in a significant loss of the inactivation capacity of C<sub>60</sub>/TSPP, thus confirming that the photodynamic inactivation process is oxygen-dependent and is stimulated by suspension of nanoparticle sensitizer.

Neither pH value dependence, nor temperature dependence was observed in the case of this heterogeneous sensitizer C<sub>60</sub>/TSPP.

Also, the heterogeneous sensitizer C<sub>60</sub>/TSPP has few supplementary advantages, as follows:

- First of all, should be kept in mind that it is more efficient to use C<sub>60</sub>/TSPP than TSPP due to the absence of photodegradation reactions of TSPP in solution, reaction which is the most undesirable aspect of the sensitization processes.

- Secondly, the nanoparticle sensitizers can be rapidly removed from the cellular suspension, thus protecting the cells, and separating the processes responsible for photodynamic inactivation.

#### 4. Conclusions

The current article covers a new interesting compound, microsensitizer as 5,10,15,20-tetra-sulfonatophenyl porphyrin (TSPP), and nanosensitizers, as fullerenes (C<sub>60</sub>) -coated TSPP suspension (C<sub>60</sub>/TSPP). The photodynamic inactivation of herpes simplex virus type I (HSV-1) provided from rat, by the above macro and nano-sensitizers, demonstrated a remarkable virucidal activity upon light activation, more pronounced for (C<sub>60</sub>/TSPP) than for TSPP, and after an optimum time of 300 minutes. The concentration and temperature effects were evaluated, and also the time interval between dye treatment of cells and virus inoculation.

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