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Zinc Phthalocyanines Attached to Gold Nanorods for Simultaneous Hyperthermic and Photodynamic Therapies Against Melanoma *In Vitro*

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Abstract

Studies indicate that hyperthermic therapy using gold nanorods and photodynamic activity with many photosensitizers can present a synergistic effect, and offer a great therapeutic potential, although more investigation needs to be performed before such approach could be implemented. We proposed to investigate the effect of the attachment of phthalocyanines on the surface of gold nanorods (well-characterized devices for hyperthermia generation) for the elimination of melanoma, one of the most important skin cancers due to its high lethality. Following the synthesis of nanorods through a seed-mediated method, the efficacy of photodynamic therapy (PDT) and hyperthermia was assessed separately. We chose to coat the nanorods with two tetracarboxylated zinc phthalocyanines - with or without methyl-glucamine groups. After the coating process, the phthalocyanines formed ionic complexes with the cetyltrimethylammonium bromide (CTAB) that was previously covering the nanoparticles. The nanorod-phthalocyanines complexes were analyzed by transmission electron microscopy (TEM), and their singlet oxygen and hydroxyl radical generation yields were assessed. Furthermore, they were tested in vitro with melanotic B16F10 and amelanotic B16G4F melanoma cells. The cells with nanoparticles were irradiated with laser (at 635 nm), and the cell viability was assessed. The results indicate that the photodynamic properties of the phthalocyanines tested are enhanced when they are attached on the nanorods surface, and the combination of PDT and hyperthermia was able to eliminate over 90% of melanoma cells. This is a novel study because two tetracarboxylated phthalocyanines were used and because the same wavelength was irradiated to activate both the nanorods and the photosensitizers.

Keywords: Cancer, Nanorods, Gold, Porphyrin, Phthalocyanines, PDT, Hyperthermia

1. INTRODUCTION

Malignant melanoma is one of the most lethal types of cancer, and its incidence has been growing lately: according to World Health Organization, around 132,000 new melanoma cases are diagnosed every year. Although melanoma represents the minority of skin cancer cases, it is the main responsible for cancer mortality.

Classic treatments for cancer include the surgical removal of solid tumors, radiotherapy, chemotherapy, immunotherapy, hormone-therapy, and others. However, due to the high regrowth rates, as well as to the organic impairment caused by those interventions, there has been an increasing search for new and more efficient therapeutic interventions, especially less invasive ones (1). Over the last decades, an effort has been made towards the development of light-based therapies, most of them based on the effects of light interaction with tissues (2) such as hyperthermia. Studies indicate that temperatures between 42º and 47º C can induce cell death, proportionally to the time of irradiation, either by necrosis or by apoptosis (3). Apoptosis can be activated after hyperthermia due to the increased expression of p53 protein, or due to membrane blebbing (4).

The hyperthermic effect can be achieved directly by the interaction of light with the tissues, but can also be enhanced if some devices are used, i.e. plasmonic nanoparticles. One of the most common type of nanoparticles used in the last decades is colloidal gold, usually covered with a polymer like polyethylene glycol (PEG). Those nanoparticles can consist of pure gold or they can have a silica core in case of nanoshells (5). Gold nanoparticles are promising because of their low toxicity, conformational flexibility and intense plasmonic surface resonance with visible light and near infrared light (6). This last property allows the generation of heat via the interaction with light (7,8).

Another effect caused by light in biological systems is the photodynamic effect, as long as a photosensitizer compound is present on the target tissue. Photosensitizers are able to induce phototoxic effects, i.e. reactive oxygen species (ROS) and singlet oxygen generation, after their activation by light in a specific wavelength and when molecular oxygen is present. Basically, light takes the photosensitizer molecule up to a short-lived, excited, singlet state. Then, if the photosensitizer undergoes an intersystem transition, in which the excited singlet state becomes a triplet state, it can exchange energy with molecular oxygen, whose ground state is a triplet one. This triplet-triplet annihilation restores the ground singlet state to the photosensitizer and turns triplet oxygen into singlet oxygen. Singlet oxygen can, in turn, react with many biological molecules in a very localized manner, therefore photodynamic therapy (PDT) consists of a safe and efficient treatment for cancer (9,10).

Since hyperthermia and the photodynamic effect can be achieved with light, both therapeutic interventions could be combined in order to diminish the concentration of photosensitizers and nanoparticles required for the treatment. Some lines of evidence show that hyperthermia and photodynamic therapy can act synergistically, with deeper damage being caused specifically to malignant tissues and, consequently, higher success rates being achieved (11). If hyperthermia is applied right after PDT, the synergistic effect is optimized, but if there is an interval between the procedures, this synergism tends to disappear. Besides, if PDT is applied after hyperthermia, the efficacy is lost, since the higher temperatures can turn the tissue darker due to hemorrhage, and this would prevent further light penetration and diminish oxygen supply for the photosensitizer (12).

The synergy between both therapeutic interventions can be explained, for instance, by the fact that individual therapies are insufficient to induce a thorough malignant tissue death, but if they are applied together, the extent of cell death is augmented. Besides, the thermal energy generated by the irradiated nanoparticles is able to accelerate the generation of reactive oxygen species and singlet oxygen (13). These features are important for the treatment of melanotic melanomas, since melanin tends to absorb the light required for the photodynamic effect, decreasing the effectiveness of PDT. Only a more potent therapeutic intervention could overcome the resistance of melanomas against photonic therapies.

In this study, we proposed to investigate the synergistic effect and the efficacy of both photodynamic and hyperthermic effect using gold nanorods *in vitro* against melanomas, as well as the likelihood of synthesizing gold nanorods covered with phthalocyanines.

2. METHODS

2.1. Nanorods synthesis

A seed-mediated method was used. Basically, 5 mL of cetyltrimethylammonium bromide (CTAB) (0.2 mol L^{-1}) were mixed with 5 mL of chloroauric acid ($HAuCl_4$) ($5x10^{-4}$ mol L^{-1}). After stirring, 600 μL of sodium borohydride ($1x10^{-2}$ mol L^{-1}) were added. The solution was vigorously stirred for 2 minutes, and then it was left standing for 6 hours in the dark. This procedure led to the formation of small nanospheres called seeds. On a second step, 200 μL of silver nitrate (0.004 mol L^{-1}) were added to 5 mL of CTAB (0.2 mol L^{-1}) and 5 mL of $HAuCl_4$ (0.001 mol L^{-1}). Then, 70 μL of ascorbic acid (0,0788 mol L^{-1}) were added to the yellow solution while stirring, turning the solution colorless. Finally, 12 μL of the seeds suspension were added to this solution, and the mixture was left standing for 15 minutes, the time when the color of colloidal gold is able to be seen (14).

2.3. Nanorods coating with phthalocyanines

Two zinc phthalocyanines were used: a tetracarboxylated zinc phthalocyanine (ZnTcPc) and a methylglucaminated tetracarboxylated zinc phthalocyanine (ZnTcPc-G). They were chosen because of their negative charge and their efficiency in PDT. 10 μmol L^{-1} of each photosensitizer were added separately to 5 mL of the nanorods suspension, and both mixtures were left under stirring overnight. The positively-charged CTAB from the nanorods forms an ionic complex with the negatively-charged phthalocyanine. The extinction coefficients of CTAB-coated nanorods and phthalocyanine-coated nanorods were used to calculate the amount of photosensitizer complexed with the nanorods (15). As a further characterization, the morphology of the nanoparticles was assessed by transmission electron microscopy (TEM), with magnifications of 18,000X and 46,000X.

2.4. Singlet Oxygen and Reactive Oxygen Species (ROS) generation assessment

The generation of singlet oxygen and hydroxyl radicals was assessed using a commercial kit. For singlet oxygen, the probe *Singlet Oxygen Sensor Green* was added to the nanorods suspensions in a final concentration of 5 μmol L^{-1} (with and without photosensitizer in a concentration of 4 μmol L^{-1}). The mixture was irradiated with light at 635 nm, 1 J cm^{-2}, and the singlet oxygen generation yield was compared among the samples using a microplate reader (excitation wavelength of 504 nm; emission wavelength of 525 nm). For hydroxyl radical, the probe 3'-(p-hydroxyphenyl) fluorescein was added to the nanorods suspensions in a final concentration of 5 μmol L^{-1} (with and without photosensitizer in a concentration of 4 μmol L^{-1}). The mixture was irradiated with light at 635 nm, 1 J cm^{-2}, and the hydroxyl radical generation yield was compared among the samples using a microplate reader (excitation wavelength of 490 nm; emission wavelength of 515 nm).

2.5. *In vitro* photodynamic and hyperthermic procedure

The efficacy of the gold nanorods-phthalocyanine complexes regarding cancer ablation was assessed *in vitro* using melanotic melanoma cells (B16F10 cell line) and non-melanotic melanoma cells (B16G4F cell line). The same wavelength was used for PDT and hyperthermia induction, and in the case of gold nanorods with attached phthalocyanines, these two therapeutic interventions happened simultaneously and with the same parameters.

The cells were cultured in 96-well plates, 9000 cells per well, containing DMEM media supplemented with 10% fetal bovine serum (FBS). After 24 hours of incubation, the medium

was replaced by DMEM without FBS and containing: 1- ZnTcPc-coated nanorods; 2- ZnTcPc-G-coated nanorods; 3- nanorods without photosensitizer; 4- ZnTcPc solution; 5- ZnTcPc-G solution; 6- Phosphate Buffered Saline (PBS). 5 hours later, the wells were washed with fresh medium and irradiated with light at 635 nm, 5 J cm^{-2}. The temperature of the medium was monitored with a laser thermometer during the whole procedure. After the irradiation, the cells were incubated again for 24 hours with the supplemented medium, and then the cell viability was assessed using Presto Blue dye. The data were compared among the groups by a variance analysis.

3. RESULTS

3.1. Nanorods coating with photosensitizers

The coating of the nanorods with phthalocyanines was more successful for ZnTcPc (80% of the photosensitizer remained attached) compared to ZnTcPc-G (20% of the photosensitizer remained attached). This might have occurred because of the negatively-charged carboxyl groups from ZnTcPc, which tend to form ionic complexes with CTAB easier than the methylglucamine radicals. Figures 1 and 2 show the absorption spectrum of the complexes, and the absorption values in 680 nm (the peak of the phthalocyanines) were the following: 0.516 for the supernatant obtained after centrifugation of ZnTcPc-G-coated nanorods, 0.650 for ZnTcPc-G solution, 0.124 for the supernatant obtained after centrifugation of ZnTcPc-coated nanorods, and 0.650 for ZnTcPc solution.

The morphology of the nanorods did not change significantly after the addition of the photosensitizers, but we could observe lower aggregation in the presence of photosensitizers. Using ImageJ software, we could assess the dimensions of the nanoparticles. The mean length of gold nanorods was 51±9.4 nm, and the mean diameter was 6±0.8 nm. For ZnTcPc-coated nanorods, the mean length was 57.5±6.0 nm and the diameter was 5±1.5 nm. Finally, for ZnTcPc-G-coated nanorods, the mean length was 56.4±10.6 nm, and the mean diameter was 6±1.6 nm. Figure 3 shows the TEM images obtained.

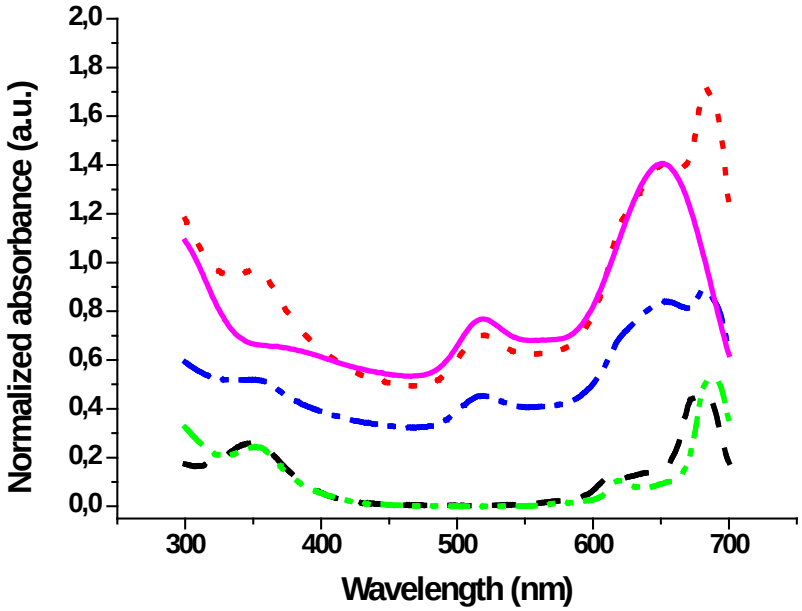

Figure 1. Absorption spectra of gold nanorods, covered or not with ZnTcPc-G, before and after centrifugation. (- -) ZnTcPc-G solution; (...) non-centrifuged ZnTcPc-G-coated nanorods; (-.-) centrifuged ZnTcPc-G-coated nanorods; (-..-) supernatant of ZnTcPc-G-coated nanorods; (---) Nanorods.

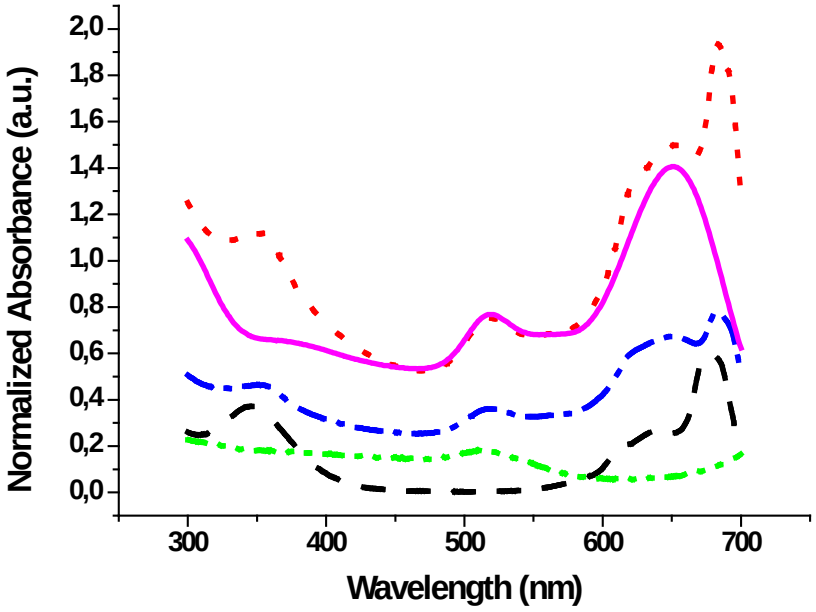

Figure 2. Absorption spectra of gold nanorods covered or not with ZnTcPc, before and after centrifugation. (- -) ZnTcPc solution; (...) non-centrifuged ZnTcPc-coated nanorods; (-.-) centrifuged ZnTcPc-coated nanorods; (-..-) supernatant of ZnTcPc-coated nanorods; (---) Nanorods.

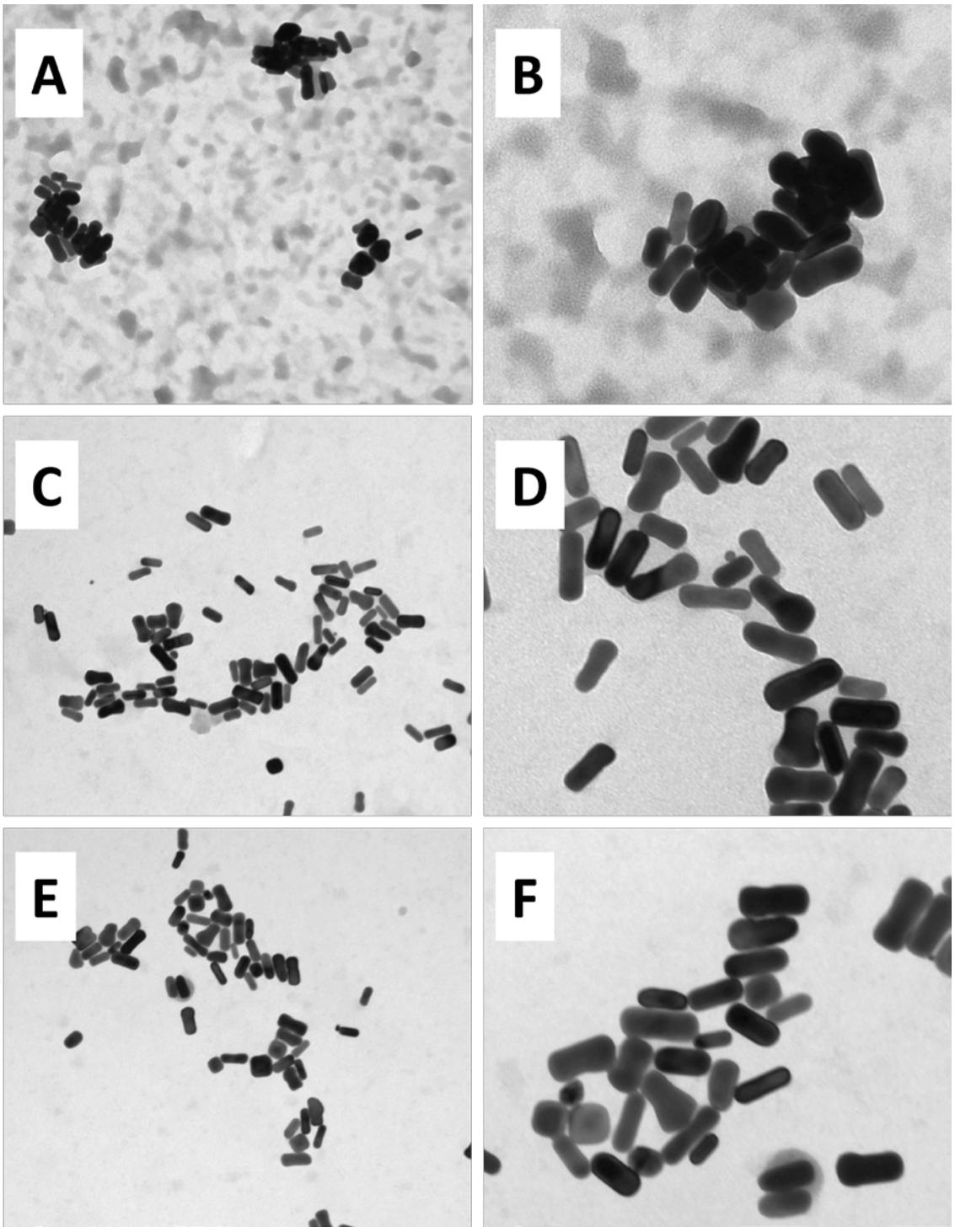

Figure 3. TEM images of the nanorods. (A) Gold nanorods, 18,000X; (B) Gold nanorods, 46,000X; (C) ZnTcPc-G-coated nanorods, 18,000X; (D) ZnTcPc-G-coated nanorods, 46,000X; (E) ZnTcPc-coated nanorods, 18,000X; (F) ZnTcPc-coated nanorods, 46,000X.

3.2. Singlet oxygen and hydroxyl radical generation

The probe for singlet oxygen revealed that the phthalocyanines alone generated low amounts of this reactive species in aqueous solution, and the results were similar for both photosensitizers. However, after being attached on the nanorods surface, the singlet oxygen yield increased 3-fold, therefore, the type II photodynamic mechanism is augmented. As expected, the nanorods alone did not generate singlet oxygen. Figure 4 shows the results for singlet oxygen.

The results for hydroxyl radicals generation revealed a decrease in HPF fluorescence for ZnTcPc, but an increase in HPF fluorescence for ZnTcPc-G. Briefly, ZnTcPc attached to nanorods generated 30% less hydroxyl radicals than the free photosensitizer, and ZnTcPc-G attached to nanorods generated 34% more hydroxyl radicals than the free photosensitizer. As expected, the nanorods alone did not produce hydroxyl radicals with the irradiation. The results for hydroxyl radicals are shown in figure 5.

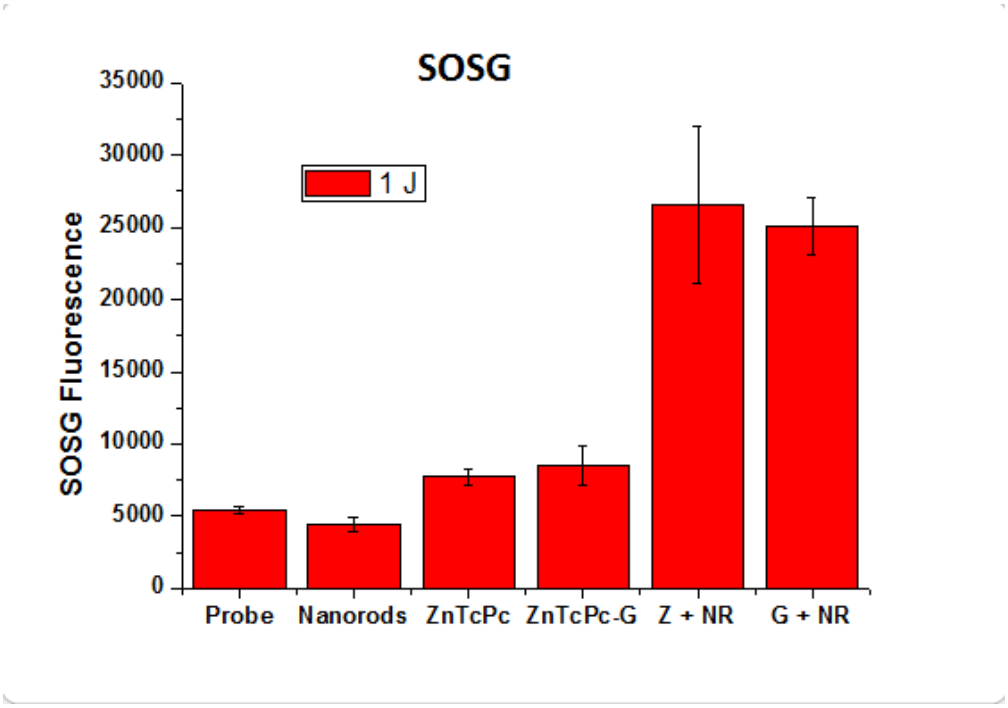

Figure 4. Results for singlet oxygen generation in solution, corresponding to the fluorescence of SOSG probe after irradiation with light in 660 nm (light dose: 1 J cm^{-2}). G refers to ZnTcPc-G, Z refers to ZnTcPc, and NR refers to the gold nanorods. The excitation and emission wavelengths of the probe were 504 nm and 525 nm, respectively.

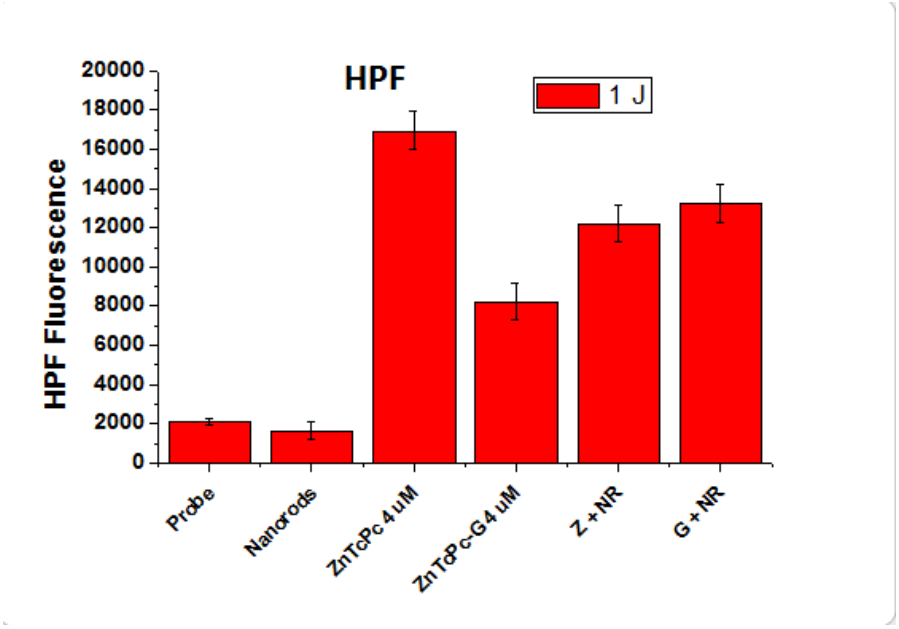

Figure 5. Results for hydroxyl radical generation in solution corresponding to the fluorescence of HPF probe after irradiation with light in 660 nm (light dose: 1 J cm^{-2}). G refers to ZnTcPc-G, Z refers to ZnTcPc, and NR refers to the gold nanorods. The excitation and emission wavelengths of the probe were 490 nm and 515 nm, respectively.

3.3. Photodynamic therapy *in vitro* on melanoma cells

The results *in vitro* were similar for both photosensitizers. There was a slight toxicity in the dark caused by the gold nanorods (with or without attached photosensitizers), probably because there was still some traces of free CTAB in the samples. The nanorods *per se* were able to eliminate 43% of non-melanotic melanoma cells (B16G4F) and 49% of melanotic melanoma cells (B16F10) due to hyperthermia after irradiating 20 J cm^{-2} of light in 635 nm (there was a 6ºC increase in the temperature). When the photosensitizers were attached to the nanorods, the cell damage increased significantly: 91% of B16G4F elimination, and 95% of B16F10 elimination. It is interesting to notice that the melanotic cells are more susceptible to the combined hyperthermic and photodynamic therapies, although melanin absorbs red light and could compete with the photosensitizers. One possible explanation relies on the effect of hyperthermia on cell membranes. It is possible that hyperthermia is able to disrupt the membrane of some organelles, including melanosomes, liberating cytotoxic melanin precursors and synthesis products to the cytosol. These compounds, such as some quinones, semiquinones, peroxide and superoxide, can lead the cells to necrosis and apoptosis, as observed in the cultures from our study (data not shown) (16). The figures 6 and 7 show the results *in vitro*.

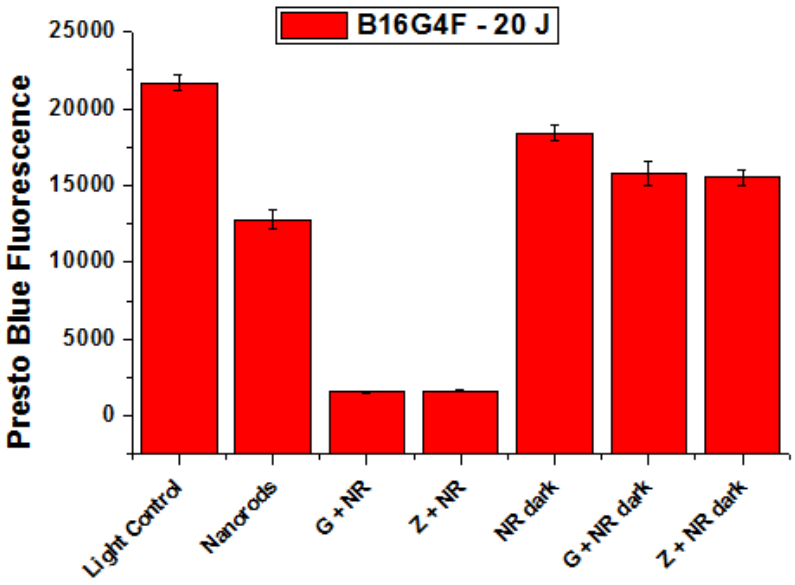

Figure 6. Fluorescence of Presto Blue as an indicative of cell viability after light irradiation (635 nm, 20 J cm^{-2}). G refers to ZnTcPc-G, and Z refers to ZnTcPc. NR refers to the gold nanorods without photosensitizer attachment.

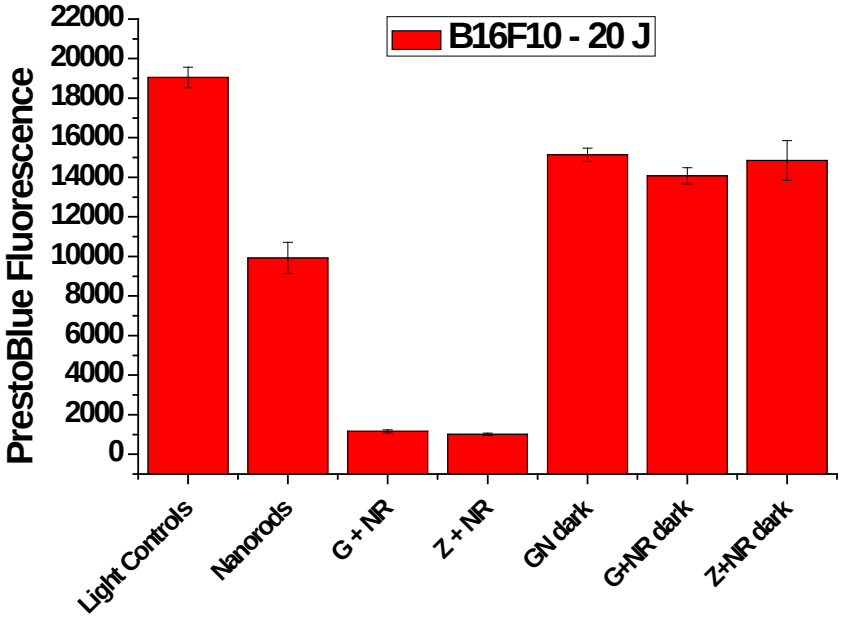

Figure 7. Fluorescence of Presto Blue as an indicative of cell viability after light irradiation (635 nm, 20 J cm^{-2}). G refers to ZnTcPc-G, and Z refers to ZnTcPc. NR refers to the gold nanorods without photosensitizer attachment.

4. DISCUSSION

The first choices for the treatment of cancer are usually chemotherapy, radiotherapy and surgical removal of solid tumors, but it is known that those interventions are very invasive and the prognosis is often poor. The high regrowth rates observed after classic cancer treatments, as well as the organic impairment caused by them, boosted the search for safer and more efficient techniques for tumor ablation. The urge for novel treatments is even higher for fast-growing and highly metastatic cancers, such as melanoma. In this matter, light-based therapeutic interventions offer a great potential to be applied for main and ajuvant cancer therapies, i.e. photothermic and photodynamic therapies (2).

Many studies demonstrate that hyperthermia immediately after PDT enhances the efficacy of both treatments. This happens probably because of an inhibition of the cellular repair system caused by PDT, or because hyperthermia also inhibits the repair response against PDT. There is a synergistic effect as well, since PDT induces mitochondrial damage while hyperthermia induces damages in other organelles, such as nucleus and plasma membrane which are not oxidized by the singlet oxygen produced in the mitochondria (17,18). In this study, we investigated if this synergism remains when both therapies are performed simultaneously.

Initially, the synthesis of nanorods was successful, and we could obtain nanorods with homogeneous dimensions after the addition of silver in the synthesis medium. The nanorods presented around 55 nm length and 6 nm diameter.

Regarding their photodynamic activity after the attachment of phthalocyanines, the singlet oxygen generation increased three times for both photosensitizers, suggesting that a modification on the photodynamic mechanism might have occurred. The results with HPF probe reinforced the idea that the photosensitizers undergo a modification in their photodynamic mechanism. Apparently, they can suffer a transition from a type I (hydroxyl, superoxide and peroxynitrite radicals generation due to electron transfer from the photosensitizer to molecular oxygen or other biomolecules) to a type II mechanism (singlet oxygen generation due to energy transfer from the photosensitizer to molecular oxygen). This is more evident for ZnTcPc, which acts markedly with a type I mechanism, but after the attachment on the nanorods surface, this mechanism is decreased and the type II mechanism is augmented significantly. Both mechanisms are increased for ZnTcPc-G after the attachment on the nanorods.

Our results with hyperthermia and photodynamic therapy corroborate with the data from the literature, where Kuo and co-workers used the photosensitizer Indocyanine Green (ICG) attached to nanorods and investigated its photodynamic action. Their results show that the

conjugated nanorods-ICG generate a higher amount of singlet oxygen than the photosensitizer alone, therefore being more effective. The authors hypothesize that this happens because of an increased intersystem crossing and a higher triplet yield of the photosensitizer, which guarantees a greater photostability. During the treatment in Kuo's study, the temperature rose to 46ºC, enough to induce a significant cell damage by hyperthermia. Since there was no apparent photosensitizer degradation, the conjugated nanorods-ICG have shown to be efficient and stable nanoparticles for *in vitro* and *in vivo* treatments (19).

The same mechanism of action hypothesized by Kuo et al. might be the responsible for the results observed in our study. Besides, there is evidence that the generation of singlet oxygen is increased if the photosensitizers are attached on metallic surfaces, since photobleaching is inhibited in this condition. Furthermore, the surface plasmons can somehow contribute by donating energy or electrons to the photosensitizer, leading to a more efficient ROS generation (13).

When tested *in vitro* against melanotic and non-melanotic melanoma cells, the gold nanorods were able to eliminate over 40% of the malignant cells hyperthermically, more specifically 43% of B16G4F and 49% of B16F10. With 20 J cm^{-2}, the temperature increased in 6ºC. The results *in vitro* for nanorods complexed with photosensitizers were similar for ZnTcPc and ZnTcPc-G, and showed a significant enhancement of the tumor elimination potential. The complexes were able to eliminate 91% of B16G4F cells and 95% of B16F10 cells, either by necrosis or apoptosis. The synergism between the two therapeutic interventions can be explained, for instance, by the fact that the treatments alone tend to be insufficient to induce a thorough cell death, but when combined, the tumor damage is increased significantly. Besides, the thermal energy generated by the nanoparticles is able to accelerate the generation of reactive species, especially singlet oxygen (13).

Our *in vitro* results are in accordance with other studies involving the combination of hyperthermia and PDT. For instance, Hirschberg et al. investigated the combination of 5-aminolevulinic acid-mediated PDT and hyperthermia induced by an incubator with controlled temperature. These two protocols were used aiming to eliminate two different cell line-derived spheroids: human grade IV GBM cell line and murine BT_4C cells. The treatments alone were able to inhibit the survival in some extent, but the combination of PDT with a 25 J cm^{-2} fluence and 49ºC was able to eliminate almost completely the spheroids (20). Another study, performed by Trinidad and co-workers in 2014, investigated the effects of PDT (mediated by a sulphonated phthalocyanine, activated by light in 670 nm) combined with hyperthermia induced by gold nanoshells (activated by light in 810 nm) on the elimination of human FaDu squamous cells *in vitro*. Although they could not eliminate the tumor cells completely, there was a significant growth inhibition when the treatments were performed together, compared to the treatments alone (21).

Furthermore, Di Corato and co-workers combined magnetic hyperthermia with PDT mediated by Foscan for the elimination of tumor cells *in vitro* (Human adenocarcinoma SKOV-3 cells) and *in vivo* (murine epidermoid carcinoma A431 cells grown in both flanks). The treatments alone were able to inhibit the tumor growth, but the combination resulted in complete tumor destruction *in vitro* as well as *in vivo* (22). Finally, Bolfarini et al. also investigated the elimination of melanoma cells with the combination of PDT and hyperthermia. Both therapies were applied by using magnetoliposomes loaded with a zinc phthalocyanine-based complex. The authors found that PDT was more effective than hyperthermia when applied separately, but the combination of treatments reduced the viability of B16F10 cells to about half that of PDT alone (23). Other authors observed similar results for the combination of both therapies, and their results can be found in Brodin's review study (24).

The fact that melanotic melanoma cells were more susceptible than amelanotic ones to hyperthermia and to the combined treatments suggests that melanin or the melanosomes might have played a role in the observed cell death. One explanation would be the disruption of the membrane of melanosomes caused by hyperthermia, liberating cytotoxic and genotoxic compounts to the cytosol, such as superoxide, peroxide, quinones and semiquinones. This additional cytotoxic effect would cause a greater elimination of melanotic cells (16,25). Other experiments must be performed in order to confirm this hypothesis.

5. CONCLUSION

The attachment of the two zinc phthalocyanines on the surface of gold nanorods was successful, and led to a better outcome regarding melanoma elimination *in vitro*. The results of combined and simultaneous hyperthermic and photodynamic therapies was better than the results for the treatments alone, showing that there is, indeed, a synergistic effect when the photosensitizers are attached to a metallic surface, even if the treatments are not performed one after the other.

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