Article

Hybrid Nanoparticles as Theranostics Platforms for Glioblastoma Treatment: Phototherapeutic and X-ray Phase Contrast Tomography Investigations

Loredana Ricciardi 1,* , Sharmistha Chatterjee 2 , Giovanna Palermo 1,2 , Elisabeta I. Szerb 3 , Alessia Sanna 4 , Francesca Palermo 2,4 , Nicola Pieroni 4 , Michela Fratini 4 , Roberto Bartolino 1 , Alessia Cedola 4 , Massimo La Deda 1,5,6,* and Giuseppe Strangi 1,2,7,*

1 CNR NANOTEC-Institute of Nanotechnology U.O.S. Cosenza, 87036 Rende, Italy; giovanna.palermo@fis.unical.it (G.P.); bartolino.fis@gmail.com (R.B.)
2 Department of Physics, University of Calabria, 87036 Rende, Italy; chatterjee.bwd@gmail.com (S.C.);
3 Coriolan Dragulescu Institute of Chemistry, Romanian Academy, 24 Mihai Viteazu Bvd., 300223 Timisoara, Romania; szella73@gmail.com
4 CNR NANOTEC-Institute of Nanotechnology U.O.S. Roma, Piazzale Aldo Moro 5, 00185 Roma, Italy; sanna.1469673@studenti.uniroma1.it (A.S.); nicola.pieroni.ph@gmail.com (N.P.);
michela.fratini@gmail.com (M.F.); alessia.cedola@cnr.it (A.C.)
5 Department of Chemistry and Chemical Technologies, University of Calabria, 87036 Rende, Italy
6 Calabria Unit, National Interuniversity Consortium of Materials Science and Technology, LASCAMM, 87036 Rende, Italy
7 Department of Physics and Case Comprehensive Cancer Center, Case Western Reserve University, 10600 Euclid Avenue, Cleveland, OH 44106, USA
* Correspondence: loredana.ricciardi@cnr.it (L.R.); massimo.ladeda@unical.it (M.L.D.); giuseppe.strangi@case.edu (G.S.)

Abstract: Glioblastoma multiforme (GBM) is one of the deadliest and most aggressive cancers, remarkably resilient to current therapeutic treatments. Here, we report preliminary in vivo studies of GBM treatments based on photo-nanotherapeutics to activate synergistic killing mechanisms. Core-shell nanoparticles have been weaponized by combining photophysical properties of a new generation PDT agent (Ir(III) complex) with the thermoplasmonic effects of resonant gold nanospheres. In order to investigate the damages induced in GBM treated with these photoactivable nanosystems, we employed X-ray phase-contrast tomography (XPCT). This high-resolution three-dimensional imaging technique highlighted a vast devascularization process by micro-vessels disruption, which is indicative of tumor elimination without relapse.

Keywords: gold–silica nanoparticles; phototherapy; glioblastoma multiforme; X-ray phase-contrast tomography

1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive and prevalent brain cancer, accounting for over 70% of high-grade gliomas diagnosed [1–4]. Characterized by necrotic primary tumor centers with abundant and aberrant neovascularization [5], current therapeutic treatments include surgical resection followed by adjuvant chemotherapy and radiotherapy [6]. Despite the recent advances in conventional therapeutic strategies, the GBM prognosis remains poor, with a median survival of 12–14 months [7–10]. One of the important features of GBM cells is their infiltrative nature which leads to indistinguishable margins between normal and malignant brain tissue [11]. Consequently, complete resection is rarely feasible. In order to identify and eliminate residual tumor cells at the boundaries of the resection area and at the same time minimize the damage to the surrounding healthy brain tissue, currently a new therapeutic strategy—undergoing phase I clinical
Photodynamic Therapy (PDT) is a minimally invasive cancer treatment approach that employs light to trigger a photodynamic mechanism mediated by a drug, the photosensitizer (PS), able to absorb and transfer the radiant energy to the ubiquitous molecular oxygen, generating reactive oxygen species (ROS), i.e., free radicals (type I reaction) and singlet oxygen ($^1$O$_2$; type II reaction) [13,14]. Recently, extraordinary efforts have been focused on investigating transition metal complexes (TMCs) as PSs for PDT applications [15]. Because of their photophysical properties, TMCs meet several essential requirements as PDT agents [16]. Furthermore, the remarkable luminescence associated with long emission lifetimes makes TMCs a useful probe for imaging [17,18]. Currently, the application of these complexes in PDT is in its infancy, with the first TMC entered into human clinical trials in early 2017 [19].

In the last decades, the development of nanotechnologies has opened new promising avenues in the biomedical field, improving the clinical outcomes of many lethal diseases, such as cancer [20,21]. Among the wide variety of nanomaterials reported in the literature, inorganic nanoparticles possess very interesting properties for clinical application [22,23]. In this frame, gold-core silica shell nanoparticles can be used to vehiculate treatment agents in specific sites by enhancing their efficacy significantly, thereby proving to be extremely promising for theranostic purposes [24]. Gold nanoparticles exhibit interesting size-dependent optical properties associated with the localized surface plasmon resonance phenomenon (LSPR) [25,26]. The interaction with light at a specific resonance wavelength (which depends on the particle size and shape) leads to the absorption of the radiant energy and its conversion both into heat and scattered radiation. Accordingly, if properly localized inside or in close proximity of tumor cells, light-activated metal nanoparticles induce temperature increase with consequent photothermal-mediated cell ablation (Photothermal Therapy, PTT) [27–29]. The inclusion of a silica shell confers stability to the gold nucleus and provides a reservoir for the encapsulation of PSs molecules acting as PDT therapeutic agents and imaging probes, simultaneously leading to the implementation of multifunctional nanoplatforms [30]. Recently, we reported the design and the development of a de novo platform for simultaneous cellular imaging, photodynamic and photothermal therapies [31]. The nanostructure is based on a highly luminescent water-soluble Ir(III) complex (Ir$_1$) [32] embedded into gold-core silica shell nanoparticles (Ir$_1$-AuSiO$_2$). In vitro photo-cytotoxicity tests on GBM cells clearly demonstrated the potential of this nanoplatform to play a key role in the imaging and treatment of GBM [31]. These results emphasized striking synergistic photodynamic and photothermal effects as a result of the coupling between the photophysical properties of the TMC with the thermoplasmonic effects of the gold nanospheres [31].

Here, we report preliminary in vivo studies based on the phototherapeutic treatment of human GBM xenograft mouse model after Ir$_1$ or Ir$_1$-AuSiO$_2$ intratumoral injection. High-resolution X-ray phase-contrast tomography (XPCT) [33,34] was employed to image the three-dimensional tumor vascular network with microscale resolution of ex vivo samples. In Figure 1a, a schematic illustration shows the treatment diagram and the effects of the nanotherapeutic agent, whereas Figure 1b shows a table of a direct comparison of Ir$_1$ and Ir$_1$-AuSiO$_2$ properties and resulting advantages and disadvantages for in vivo photo-theranostics application.
Figure 1. Overview of the therapeutic approach for human GBM treatment in xenograft mouse model. (a) Intratumoral injection of Ir1-AuSiO2, distribution and accumulation next to blood vessel walls followed by light-activation of photodynamic and photothermal processes. The light exposure excites the photosensitizer (Ir1) loaded in the silica shell of AuSiO2 nanoparticles, from the ground state to the short-lived excited singlet state. Following intersystem crossing (ISC), the resulting triplet state can directly interact with molecular oxygen (O2) and, via an energy transfer mechanism, generate cytotoxic singlet oxygen (1O2). Competitively, Ir1 from the triplet state drops to the ground level radiatively, giving rise to phosphorescence (P). Ir1 molecules in the shell and in close proximity to gold core nanospheres lead to a resonant energy transfer process from Ir1 to the plasmonic nanostructure, with consequent conversion of the radiant energy into heat. The synergistic combination of photodynamic and photothermal effects induces fragmentation and reduction in the tumor vasculature network, preventing the delivery of oxygen and nutrients. The starved tumor regresses because of a massive devascularization leading to the complete tumor elimination; (b) Schematic illustration of Ir1 and Ir1-AuSiO2 properties and resulting advantages (green) and disadvantages (red) for in vivo photo-theranostics application.

2. Materials and Methods
2.1. Nanoplatform Synthesis and Characterization

Ir1 and Ir1-AuSiO2 were prepared according to the previously reported procedures [31,32], and all the reported studies were performed using the same nanoparticles sample batch.
The obtained Ir$_1$-AuSiO$_2$ nanostructures were purified and concentrated by ultrafiltration method (Vivaspin R 20 equipped with a 100 KDa membrane), dispersed in pure water and finally filtered by using a 200 nm nylon membrane. The nanoparticles morphology was observed using a JEOL 2010F TEM at an operating voltage of 80 kV. The size distribution measurements were performed in triplicate at 25°C by using the Dynamic Light Scattering (DLS) technique on a Zetasizer Nano ZS system from Malvern Instruments (632.8 nm, 4 mW HeNe gas laser, avalanche photodiode detector, 175° detection). Extinction spectra were recorded on a PerkinElmer Lambda 900 spectrophotometer, while steady-state excitation/emission spectra were collected with a HORIBA Jojobin-Yvon Fluorolog-3 FL3-211 fluorometer equipped with a 450 W xenon arc lamp, double-grating excitation and single-grating emission monochromators (2.1 nm mm$^{-1}$ dispersion; 1200 grooves per mm), and a Hamamatsu R928 photomultiplier tube.

The studies reported below involving Ir$_1$ and Ir$_1$-AuSiO$_2$ (singlet oxygen generation and in vivo experiments) were performed by irradiating with a lamp at 365 nm (Thermo Fisher Scientific, Waltham, MA, USA). In all cases, the treatment consisted of a single optical radiation dose (20 mW/cm$^2$ for 15 min, equivalent to 18 J/cm$^2$).

2.2. Detection of $^{1}$O$_2$ Generation

Singlet oxygen generation was monitored by chemical oxidation of 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) (Sigma-Aldrich, St. Louis, MO, USA) in an aqueous solution. The anthracene derivative reacts specifically and irreversibly with $^{1}$O$_2$, leading to the corresponding endoperoxide (Scheme S1); the reaction can be monitored spectrophotometrically by measuring the decrease in the optical density at 378 nm [35]. Briefly, 15 µL of ABDA solution (2 mg/mL DMSO) was mixed, respectively, with 300 µL of Ir$_1$ and Ir$_1$-AuSiO$_2$ water solutions ([Ir$_1$] = 2 × 10$^{-5}$ M) and placed in a quartz cuvette with an optical path length of 0.2 cm. The absorption spectra were recorded upon irradiation at 365 nm for 15 min (20 mW/cm$^2$) at 3 min intervals. For comparative purposes, a similar ABDA solution was dispersed in water and irradiated under the same conditions. The reaction stoichiometric ratio (Scheme S1), leading to the formation of endoperoxides, implies the involvement of an equal number of moles of ABDA as well $^{1}$O$_2$. Monitoring the ABDA absorbance as a function of the number of moles present in solution (Figure S1) and the amount of reacted ABDA molecules was calculated, and conversely of $^{1}$O$_2$, after defined irradiation times. The absorption spectra were recorded using a Cary 300 UV-Vis spectrophotometer.

2.3. In Vivo and Ex Vivo Experiments

All animal studies were performed according to the guidelines of the Institutional Animal Care and Use Committee of Case Western Reserve University (Cleveland, OH, USA). Six to eight-week-old male athymic mice were injected subcutaneously into the right flank with 1 × 10$^6$ of human glioblastoma Gli36Δ5 cells (kindly provided by Prof. James P. Basilion, Case Western Reserve University, USA). Three to four weeks after cells implantation, when the tumors reached 120–170 mm$^3$ in volume, the mice were randomly divided into groups with equivalent starting tumor sizes and used to perform in vivo and ex vivo experiments.

2.3.1. Imaging

For in vivo imaging, mice bearing Gli36Δ5 tumors were anesthetized with isoflurane and injected intratumorally with Ir$_1$ or Ir$_1$-AuSiO$_2$ (200 µL, [Ir$_1$] = 2 × 10$^{-5}$ M in water). Fluorescent images were obtained using the IVIS Spectrum in vivo imaging system with the appropriate filters for Ir(III) complex (excitation = 430 nm, emission = 520 nm, acquisition settings = 520–620 in 20 nm steps). In order to provide autofluorescence spectra, background images were carried out before the Ir$_1$ or Ir$_1$-AuSiO$_2$ intratumoral injection. After samples administration, fluorescence images were acquired at regular intervals for 24 h. The region-of-interest (ROI) was selected over the tumor, and pixel values for the Ir(III) complex
signal were determined within these regions. The obtained values were normalized to the pre-injection ROI and then plotted.

2.3.2. Therapeutic Treatment

The in vivo phototherapy efficacy of Ir$_1$ and Ir$_1$-AuSiO$_2$ was preliminarily investigated using five experimental groups of Gli36Δ5-bearing mice (two treatment groups and three controls; $n=2$ mice per group). In the treatment groups (Ir$_1$ + light illumination or Ir$_1$-AuSiO$_2$ + light illumination), an aliquot of Ir$_1$ or Ir$_1$-AuSiO$_2$ ([Ir$_1$] = 2 × 10$^{-5}$ M in water) was directly injected into the tumor by five injection points, arranged according to a starry symmetry. The total volume injected was 50 µL, equally distributed over the five injection points (10 µL per injection point). After 2 min post-injection, the entire tumor region was irradiated at 365 nm for 15 min (20 mW/cm$^2$). One control group received an equivalent optical radiation dose and one an aliquot of Ir$_1$ or Ir$_1$-AuSiO$_2$ without light exposure. Tumor volume and body weight evolution were monitored for all mice for over 100 days after treatment. During the entire treatment period, no detectable signs of toxicity, side effects such as loss of body weight and activity, or neurological issues were observed in all the experimental groups. Tumor volumes were measured with a digital caliper every other day and were calculated according to the formula: volume = (tumor length × tumor width × tumor height)/2. When the tumor size exceeded 1500 mm$^3$, the mice were euthanized.

2.3.3. X-ray Phase-Contrast Tomography

In order to image the three-dimensional vasculature network and detect any abnormalities consequent to the phototherapy treatment, high-resolution X-ray phase-contrast tomography was performed on ex vivo Gli36Δ5 tumors. Mouse xenograft models were intratumorally injected with Ir$_1$ or Ir$_1$-AuSiO$_2$ (50 µL, [Ir$_1$] = 2 × 10$^{-5}$ M in water) and then irradiated at 365 nm for 15 min (20 mW/cm$^2$). A control mouse was treated only with the same optical radiation dose. Twenty-four hours after treatment, the mice were euthanized, then the flank tumors were excised, fixed in 4% paraformaldehyde for 24 h and stored in PBS at 4 °C until the phase-contrast tomography experiments were performed.

Phase-contrast tomography experiments were carried out at I13 Manchester Beamline at the Diamond Light Source in Didcot, UK. The measurements were performed in free space propagation mode [36] with a propagation distance of 15 cm and an incident filtered pink beam with X-ray energy peaked at 27 keV. Images were detected with a PCO.Edge 5.5 (sCMOS-technology, 2560 × 2160 pixels, 6.5 µm pixel size and a 16-bit nominal dynamic range) coupled with a scintillator screen and 1.25 × optics resulting in a total magnification of 2.5 × due to the setup configuration. The effective pixel size was 2.6 µm and had a field of view of 6.65 mm × 5.616 mm. Tomography was performed by acquiring 4001 projections, with an acquisition time of 0.06 s per projection. During acquisition, samples were kept in the air.

Data pre-processing, phase retrieval [37] and filter back-projection reconstruction were performed with the open-source software toolkit SYRMEP Tomo Project (STP) [38], implementing the ASTRA toolbox for efficient tomographic reconstruction on GPU. Post-processing and three-dimensional rendering procedures of the vasculature and nanoparticles were carried out using the image processing program ImageJ 1.8 and VGstudio Max 3.2 software, respectively.

3. Results

3.1. Nanoplatform Synthesis and Characterization

Ir$_1$ and Ir$_1$-AuSiO$_2$ were synthesized according to the reported procedures [31,32], and the characterization results (Figures S2–S4) confirmed the successful preparations. In particular, TEM images reveal a homogeneous population of spherical gold-core silica-shell nanostructures with an average size of 35 ± 2 nm and a metal core of 5 ± 1 nm (Figure S2a,b). The hydrodynamic diameter measured by the DLS technique is equal to 52 ± 1 nm (Polydispersity Index: PdI = 0.035), in line with the geometric one (Figure S3).
The synthesized nanoparticles are water-soluble and exhibit long-term colloidal stability with no agglomeration even after 18 months. The extinction spectrum of Ir$_1$-AuSiO$_2$ displays a plasmon resonant frequency at 520 nm, whereas excitation and emission spectra confirm the Ir$_1$ loading into the polysiloxane matrix (Figure S4a,b). The total amount of Ir(III) complex loaded into a nanoparticle was calculated as described elsewhere [31].

3.2. Singlet Oxygen Studies

Since PDT efficacy mostly depends on the PS ability to produce $^{1}$O$_2$, the amount generated by Ir$_1$ and Ir$_1$-AuSiO$_2$ was evaluated using ABDA as a detection probe. The selected experimental conditions (PS concentration, fluence rate and distance between the excitation source and the sample solution) were the same used to carry out in vivo studies so as to mimic the $^{1}$O$_2$ generation occurring in the treated tumor tissue. Figure 2a shows the absorption spectra of ABDA in an aqueous solution (control) in the presence of Ir$_1$ and Ir$_1$-AuSiO$_2$ at different irradiation times. Although for the control sample, no change in the ABDA optical density was observed, in the presence of Ir$_1$ or Ir$_1$-AuSiO$_2$, the ABDA absorption peaks decrease in intensity as the exposure time increases, highlighting a generation of $^{1}$O$_2$ in both cases but with different efficiencies. In fact, as clearly shown in Figure 2b, the plots of ABDA absorption at 378 nm as a function of exposure time exhibit a linear trend with a slope for Ir$_1$ sharper than that obtained for Ir$_1$-AuSiO$_2$. The $^{1}$O$_2$ generation was quantitatively estimated by calculating the total number of moles produced upon photoirradiation, obtaining values of $5.4 \times 10^{-8}$ for Ir$_1$ and $2.6 \times 10^{-8}$ mol for Ir$_1$-AuSiO$_2$ (Table 1).

![Figure 2](image_url)
Table 1. Moles of $^{1}$O$_2$ generated after light exposure of Ir$_1$, Ir$_1$-AuSiO$_2$ and Pc4 ($\lambda_{\text{ex}}$ 365 nm—20 mW/cm$^2$—15 min).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration/mol L$^{-1}$</th>
<th>mol $^{1}$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ir$_1$</td>
<td>$2 \times 10^{-5}$</td>
<td>$5.4 \times 10^{-8}$</td>
</tr>
<tr>
<td>Ir$_1$-AuSiO$_2$</td>
<td>$2 \times 10^{-5}$</td>
<td>$2.6 \times 10^{-8}$</td>
</tr>
<tr>
<td>Pc4</td>
<td>$2 \times 10^{-5}$</td>
<td>$4.3 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

For comparison, the $^{1}$O$_2$ generation was measured in the same experimental conditions (PS concentration and radiation dose) for one of the most efficient phthalocyanine-based photosensitizers, the silicon phthalocyanine 4 (Pc4) [39] (Figure S5). Since Pc4 is insoluble in water, the sample was prepared by dissolving the compound in a small amount of ethanol (25 µL) and then dispersed in water (275 µL), reaching a final concentration of $2 \times 10^{-5}$ M. As reported in Table 1, the Pc4 exhibits a molar amount of $^{1}$O$_2$ generated of $4.3 \times 10^{-8}$ moles, a value lower than that obtained for Ir$_1$.

3.3. In Vivo Imaging

To demonstrate the efficiency of Ir$_1$ as a luminescent probe for in vivo imaging, we first tested the emission intensity, under 430 nm irradiation, immediately after subcutaneous injection. As shown in Figure 3a, the acquired fluorescence image exhibits a huge emission in the region of interest. Then, we injected Ir$_1$ or Ir$_1$-AuSiO$_2$ into the tumor of the human GBM xenograft mouse model for real-time imaging at different time points post-injection in order to follow their fate in terms of biodistribution, accumulation and permanence in the diseased region. Representative images pre- and post-injection of Ir$_1$ are displayed in Figure 3b; in particular, 30 min after the injection of Ir$_1$, a bright fluorescence was highlighted in the whole body of the animal, widely spread, with a considerable intensity detected in the tumor region (ROI Figure 3b). The fluorescence images acquired before the time course and after injection of both samples, Ir$_1$ and Ir$_1$-AuSiO$_2$, are reported in Figures S6 and S7, whereas the light signal intensity within the ROI as a function of time (0–24 h) is shown in Figure 3c. Here, two distinct trends were observed. After injection of Ir$_1$, the fluorescence intensity in the tumor region sharply increases, reaching a maximum after 30 min and then rapidly decreasing within a few hours (Figures 3c and S6). Conversely, the luminescent signal of Ir$_1$-AuSiO$_2$ increases gradually up to 90 min, then remaining mostly constant even at 24 h post-injection (Figures Figure 3c and S7), suggesting a higher permanence of the luminescent nanostructures in the tumor tissue compared to the free PS.

3.4. In Vivo Phototherapeutic Treatment

In order to evaluate the efficacy of Ir$_1$ and Ir$_1$-AuSiO$_2$ as phototherapeutic agents in vivo, Gli36Δ5 tumors were established into the right flank of nude mice. Ir$_1$ or Ir$_1$-AuSiO$_2$ were injected into the tumor site, then irradiated at 365 nm for 15 min (20 mW/cm$^2$), delivering radiant exposure of 18 J/cm$^2$. As controls, one group received only the radiation dose and one group the intratumoral injection of Ir$_1$ or Ir$_1$-AuSiO$_2$ without light exposure. Then, the phototherapeutic effect was assessed by monitoring the tumor volumes of the different groups over a period of 14 weeks. As clearly shown in Figures 4a and S8, the control groups’ tumor sizes increased dramatically up to 1500 mm$^3$ in less than 18 days. For the group treated with Ir$_1$ + light exposure, the tumor growth showed some fluctuations with spikes and slight inflections in the early days post-treatment, and then a rapid increase, reaching the ethical limit (1500 mm$^3$) in 18 days (Figure 4a). Of note, for this treatment group, in the initial stage, the growth of the tumor size is more significant than that observed for the control groups (Figures 4a and S8). Regarding the Ir$_1$-AuSiO$_2$ treatment group, Figure 4a,b clearly shows a volume increase in the tumor region in the phase immediately following the treatment, properly attributable to a swelling process, with a maximum value reached in 10 days. Beyond this stage, a gradual reduction in the tumor size was observed,
leading to surprisingly complete tumor regression in 24–26 days post-treatment without highlighting relapses for over 100 days (Figure 4a).

![Figure 3](image_url)  
**Figure 3.** In vivo imaging of mice treated with Ir$_1$ or Ir$_1$-AuSiO$_2$. (a) Fluorescence image of Ir$_1$ injected subcutaneously into the mouse. The fluorescence signal intensity, measured as photon counts, is shown as color scale bar (radiant efficiency from $5.27 \times 10^9$ (blue) to $4.74 \times 10^{10}$ (red)); (b) representative real-time in vivo fluorescence images pre and 30 min post-injection of Ir$_1$ into the GBM mass; (c) normalized fluorescence signals acquired before time course and after injection of Ir$_1$ (black) or Ir$_1$-AuSiO$_2$ (red) into the GBM mass. Inset: bright field image of the flank tumor. The blue circle in all the images identifies the region-of-interest (ROI).

### 3.5. X-ray Phase-Contrast Tomography Investigations

Figure 5a shows tomographic images of ex vivo GBM samples, both control and treated, which were illuminated with the same light dose (365 nm for 15 min—20 mW/cm$^2$) delivered during the in vivo studies. The XPCT image of the control sample is reported as the top left panel of Figure 5a, whereas the treated sample after intratumoral injection of Ir$_1$ or Ir$_1$-AuSiO$_2$ is shown in the top-mid panel and top-right panel, respectively. Anatomical structures of GBM, such as blood vessels (black areas) and cancer cells (gray areas), are clearly recognized at micrometer-scale resolution in all the samples, whereas the sample treated with Ir$_1$-AuSiO$_2$ shows clusters of nanoparticles (white areas) easily identified mostly in the proximity of the microvessels. For better visualization, the same images are shown by using bright colors. The color gradient reflects the tissue density ranging from high (blue) to low (red) (Figure 5a, bottom). A quantitative analysis of vessel cross-section ($\mu$m$^2$) and diameter ($\mu$m) was performed, and the average distribution is reported in Figure 5b,c, respectively. Both histograms are obtained by calculating a weighted average of the datasets displayed in Figures S9–S11. Regarding the control sample, an average lumen area and diameter of 94 $\mu$m$^2$ and 17 $\mu$m were, respectively, measured, in agreement with the values reported for GBM vessels [5]. By comparing the control sample with the tumor sample treated with Ir$_1$, a massive increase in the vascular network is clearly present, with a significantly larger vessel lumen area (276 $\mu$m$^2$) and diameter (34 $\mu$m) compared with the control. On the contrary, the tumor treated with Ir$_1$-AuSiO$_2$ shows a remarkable devascularization in terms of reduction in the number of microvessels. However, in this
tumor sample, we only observed a moderate increase in both the average vessel diameter (29 µm) and the average lumen area (169 µm²).
easily identified mostly in the proximity of the microvessels. For better visualization, the same images are shown by using bright colors. The color gradient reflects the tissue density ranging from high (blue) to low (red) (Figure 5a, bottom). A quantitative analysis of vessel cross-section (µm²) and diameter (µm) was performed, and the average distribution is reported in Figure 5b,c, respectively. Both histograms are obtained by calculating a weighted average of the datasets displayed in Figures S9–11. Regarding the control sample, an average lumen area and diameter of 94 µm² and 17 µm were, respectively, measured, in agreement with the values reported for GBM vessels [5]. By comparing the control sample with the tumor sample treated with Ir1, a massive increase in the vascular network is clearly present, with a significantly larger vessel lumen area (276 µm²) and diameter (34 µm) compared with the control. On the contrary, the tumor treated with Ir1-AuSiO₂ shows a remarkable devascularization in terms of reduction in the number of microvessels. However, in this tumor sample, we only observed a moderate increase in both the average vessel diameter (29 µm) and the average lumen area (169 µm²).

Figure 5. X-ray phase-contrast tomography images and quantitative analysis of the vascular network of ex vivo GBM samples. (a) Grayscale (top) tomography images of GBM samples treated (from left to right) only with the radiation dose (control), or after intratumoral injection of Ir₁ or Ir₁-AuSiO₂ followed by light exposure. At the bottom, the same images are reported by using different colors for better visualization (red: blood vessels; green: GBM cells; blue: Ir₁-AuSiO₂ clusters). Scale bar: 300 µm; box: 800 × 800 pixels. (b) Average distribution of vessels at different sizes and (c) diameter range in GBM samples treated only with the radiation dose (control) or after intratumoral injection of Ir₁ or Ir₁-AuSiO₂ followed by light exposure. Quantitative analysis was performed on a working area of 400 × 400 pixels.

Three-dimensional renderings of the segmented vascular network are reported in Figure 6. In particular, the control sample (Figure 6a) shows an enormous hypervascularization of the tumor, where the microvascular structure appears as a glomeruloid tuft [40] with a high degree of branching. A similar network with dense and proliferative vessels is observed in the case of the GBM treated with Ir₁ and exposed to light waves (Figure 6b). On the contrary, as clearly shown in Figure 6c,d and Supplementary Video S1, after Ir₁-
AuSiO\textsubscript{2}/light treatment, the tumor tissue appears vastly devascularized, and the residual vascular structure seems to be collapsed, discontinuous and highly fragmented.

![Image](image_url)

**Figure 6.** Three-dimensional images of the vasculature of ex vivo GBM samples. Three-dimensional volume (350 × 350 × 350 µm\textsuperscript{3}) of GBM samples treated (a) only with the optical radiation dose (control) and (b) after intratumoral injection of Ir\textsubscript{1} followed by light exposure. The microvasculature is rendered in red. The tissues were computationally removed from the three-dimensional rendering to highlight the vessel’s distribution. (c,d) Three-dimensional volume (350 × 350 × 350 µm\textsuperscript{3}) of GBM sample after intratumoral injection of Ir\textsubscript{1}-AuSiO\textsubscript{2} followed by light exposure (top view). Vessels are rendered in red, and the Ir\textsubscript{1}-AuSiO\textsubscript{2} clusters in yellow. Clusterized nanostructures appear distributed along some vascular channels highlighted by blue dashed lines. The inset highlights the details of nanoparticle clusters in the vascular structure and how their distribution correlates with the devascularized structure, which appears to be reduced and discontinued.

4. Discussion

The in vitro cytotoxic activity of Ir\textsubscript{1} and Ir\textsubscript{1}-AuSiO\textsubscript{2} on GBM cells was previously reported [31]. In particular, the incubation of GBM cells with Ir\textsubscript{1}-AuSiO\textsubscript{2} was followed by a radiant exposure of 1.44 J/cm\textsuperscript{2}. It revealed a 50% of the cell viability at the photosensitizer concentration of 0.3 µg/mL, reaching a value of 5% at 3 µg/mL [31]. As reported by Chakrabarti et al. [41], the light treatment of GBM cells by using the same fluence
(1.44 J/cm²), after incubation with Photofrin, one of the most used PDT agent, reduces the cell viability to 50% by using a photosensitizer concentration of 1 μg/mL. In vitro results, where remarkable light-induced cytotoxicity of the nanosystem Ir₁-AuSiO₂ was observed, led to performing further in vivo studies.

It is broadly reported that ROS plays an important role in cancer treatments [42,43]. Elevated oxidative signaling, contributing to mutation and growth, can be implicated in the promotion and progression of malignant diseases, including GBM [44], whereas a huge increase in ROS up to toxic intracellular levels may provide a unique opportunity to destroy cancer cells activating various ROS-induced cell death pathways [43].

The ROS generation, particularly ¹O₂, is the key event underlying the tumor destruction process promoted by the PDT agent and three distinct and interrelated mechanisms are responsible for the in vivo anti-tumor effects of the phototherapeutic treatment [45,46]. In particular, the generated ¹O₂ can (I) directly kill the tumor cells by apoptosis and/or necrosis, (II) damage the tumor-associated vasculature, resulting in tumor death via deprivation of oxygen and nutrients, (III) induces an acute inflammation with consequent activation of the immune response to recognize, track down and destroy tumor cells [47].

In our study, we performed a comparative analysis of the Ir₁ and Ir₁-AuSiO₂ ¹O₂ generation efficiency for the experimental conditions chosen to carry out preliminary in vivo studies. The high photosensitizing ability of the organometallic complex is observed by measuring the molar amount of ¹O₂ generated after irradiation. Considering the solubility of molecular oxygen in water at room temperature (0.27 mmol/L) [48], we observed that after 15 min of light exposure in the presence of Ir₁, most of the molecular oxygen present in the solution was converted into ¹O₂ (5.4 × 10⁻⁸ moles of 8.1 × 10⁻⁹, Table 1). By comparing Ir₁ with the well-known photosensitizer Pc4, Ir₁ displays a higher ¹O₂ generation efficiency, as well as a better solubility in an aqueous solution.

The Ir₁-AuSiO₂ ¹O₂ generation ability turns out to be lower than that of Ir₁ at the same TMC concentration. The spectral overlap between the emission band of the cyclometallated complex and the gold nanostructure plasmon resonance leads to a competitive energy transfer process from the molecule to the metal core [31]. Then, in the implemented nanosystem Ir₁-AuSiO₂, the radiant energy absorbed by Ir₁ is partly transferred to molecular oxygen-generating ¹O₂ and partly to the metal core, with consequent conversion into heat. The energy transfer towards the gold sphere is also highlighted by the quenching of the nanoplatform luminescence intensity compared with that of the bare compound [31].

In vivo fluorescence imaging of the GBM xenograft mouse model after intratumoral injection of Ir₁ or Ir₁-AuSiO₂ allowed for tracking and localizing the distribution of the PDT agent over time, both as single molecules and those embedded in the nanostructure. After intratumoral injection of Ir₁ or Ir₁-AuSiO₂, the fluorescence emission intensity in the tumor region reaches the maximum level after 30 min for Ir₁ and after 90 min for Ir₁-AuSiO₂. This delay relative to the time of the injection could be attributed to phenomena that occur at high concentrations of an organometallic compound or plasmonic nanostructures. In particular, high concentrations of Ir₁ could lead to luminescence quenching processes due to intermolecular interactions, thus favoring non-radiative deactivation paths of the single-molecule excited state. In the case of Ir₁-AuSiO₂, the presence of high concentrations of plasmonic gold nanoparticles, with absorbing and scattering properties, could give rise to “Inner Filter Effect” phenomena, preventing the excitation light penetration and photo-activation of the Ir₁-AuSiO₂ nanoplatforms located into depth regions of the tumor. In order to confirm the above, the emission spectra of Ir₁ and Ir₁-AuSiO₂ water solution at different concentrations were acquired and reported in Figure S12. As clearly shown, the luminescence intensity of both Ir₁ and Ir₁-AuSiO₂ in the investigated concentration range increases as the sample concentration decreases.

After a time lapse which allows the distribution and dilution of the administered aliquot in the tumor tissue, the luminescence of Ir₁ or Ir₁-AuSiO₂ reach its highest level.
While the decrease in luminescence of Ir\textsubscript{1} within the tumor site proves to be rapid, the luminescence intensity of Ir\textsubscript{1}-AuSiO\textsubscript{2} reveals the nanostructure permanence in the ROI even 24 h post-injection.

Since both Ir\textsubscript{1} and Ir\textsubscript{1}-AuSiO\textsubscript{2} are water-soluble, their size could be the key parameter dominating the different kinetics, thereby accumulating nanoparticles in the tumor region via Enhanced Permeability and Retention (EPR) effect [49–51]. Strikingly, the in vivo treatment after Ir\textsubscript{1} and Ir\textsubscript{1}-AuSiO\textsubscript{2} intratumoral injection followed by a radiant exposure of 18 J/cm\textsuperscript{2} (365 nm for 15 min-20 mW/cm\textsuperscript{2}) revealed opposite clinical outcomes: Ir\textsubscript{1} treated tumors have shown a tumor growth similar to the control, whereas the cases treated with Ir\textsubscript{1}-AuSiO\textsubscript{2} have shown a slow regression, which leads to tumor elimination without recurrence. It is important to remark that in the latter cases, the tumor regression was achieved by administering a single intratumoral injection of Ir\textsubscript{1}-AuSiO\textsubscript{2} (Ir\textsubscript{1} = 24 \textmu g Kg\textsuperscript{-1} body weight) followed by a single optical radiation dose (18 J/cm\textsuperscript{2}). Chakrabarti et al. reported in vivo studies on GBM xenograft murine models using Photofrin (10 mg Kg\textsuperscript{-1} body weight, intravenous injection; radiant exposure 100 J/cm\textsuperscript{2}; fluence rate 50 mW/cm\textsuperscript{2}). The phototherapeutic treatment based on the porphyrin-based compound led to a limited regression of the tumor, and no elimination was observed [41]. A recent review by Alhandéry et al. focused on nanosystems with different compositions and mechanisms of action for GBM treatment. Concerning the in vivo efficacy, a decrease in the tumor growth and increased survival of mice were observed in several cases, but no tumor eradication without relapse was observed [52]. In particular, Seo et al. used gold nanostructures as efficient photothermal agents for GBM treatment, developing small gold nanorods, loaded hybrid albumin nanoparticles that, after irradiation, caused a decrease in tumor growth in the xenograft mouse model [53]. Likewise, Lee et al. observed a tumor growth delay in GBM tumor-bearing mice after treatment with virus-mimetic silica-coated gold nanorods able to induce a hyperthermal effect in response to NIR irradiation [54].

Three-dimensional XPCT investigations of ex vivo GBM tissues after Ir\textsubscript{1} or Ir\textsubscript{1}-AuSiO\textsubscript{2} treatment provided considerable insights regarding the tumor vascular network, highlighting large morphological and structural differences between the two samples with respect to the control sample. Upon analyzing the XPCT images after treatment with Ir\textsubscript{1}, neovascularization accompanied by a marked vessel dilatation is observed, whereas, after Ir\textsubscript{1}-AuSiO\textsubscript{2} treatment, we observed a significant microvascular collapse with only a slight increase in the average diameter of the vessels.

In order to elucidate the different effects caused by the bare Ir\textsubscript{1} photosensitizer and by the Ir\textsubscript{1}-AuSiO\textsubscript{2} nanosystem, in terms of mechanism of action and treatment outcome, it was important to focus on the peculiar features of the two samples. In both cases, the same light dose (365 nm for 15 min-20 mW/cm\textsuperscript{2}) was applied a few minutes after intratumoral injection. The short time frame between the administration and light-mediated activation excludes an efficient cell internalization of both phototherapeutic agents, Ir\textsubscript{1} and even more Ir\textsubscript{1}-AuSiO\textsubscript{2}. This suggests that the localization may predominantly be in the interstitial space and/or close to the blood vessels, limiting a possible anti-tumor effect through the direct cellular damage of bulk tumor cells.

Regarding the process of generation of \textsuperscript{1}O\textsubscript{2}, Ir\textsubscript{1} gives rise to an enormous amount of reactive oxygen species in a relatively short time (Figure 2 and Table 1). The rates of \textsuperscript{1}O\textsubscript{2} generation, and therefore the oxygen depletion within the tumor, are key factors in the efficacy of PDT treatment [46]. Paradoxically, a high \textsuperscript{1}O\textsubscript{2} generation rate achieved, for instance, with high photosensitizer concentrations and/or high light fluence rate, causes faster oxygen consumption than that replaced by the bloodstream leading to severe levels of tumor hypoxia. The latter is known as the main stimulator of tumor angiogenesis [55], especially in GBM [5,56,57], by promoting the secretion of growth factors.

In our case, although Ir\textsubscript{1} concentration and fluence rate are significantly low, the amount of \textsuperscript{1}O\textsubscript{2} generated in 15 min is high considering the total amount of molecular oxygen dissolved in the solution. Conversely, the treatment carried out with Ir\textsubscript{1}-AuSiO\textsubscript{2} exhibits
a lower $^1\text{O}_2$ generation rate, avoiding plausibly oxygen-depleted conditions. Moreover, the sample treated with Ir$_1$ highlights an average vessel dilation where approximately 60% of vessels double the lumen area. The PDT-induced oxidative stress is known to activate an acute inflammatory response with consequent vessel dilation, which promotes the infiltration of the treated tissue by cells of the immune system with the presentation of tumor-derived antigens [47].

As mentioned above, the anti-tumor immune development process is a potential tumor destruction mechanism mediated by PDT. However, unless to innate immune cell-related effects, we exclude an immune system response activated by these treatments since the in vivo studies were carried out on immunodeficient nude mice.

On the contrary, the higher blood supply, following the vessel dilation, could provide more nutrients and oxygen to the tumor, thus promoting its proliferation. These observations shed some light on our hypothesis regarding the failure of the in vivo treatment with Ir$_1$, as opposed to the results achieved with Ir$_1$-AuSiO$_2$. In the latter case, in fact, a lower generation of ROS determines a lower inflammatory response, as confirmed by the less pronounced vessel dilation. In this regard, it is broadly reported in the literature that the optimal curative treatment with PDT should produce minimal inflammation [47,58].

5. Conclusions

To summarize, we reported a comparative in vivo study of GBM treatments by using two phototherapeutic agents: an Ir(III) complex (Ir$_1$) and the same compound embedded in core-shell plasmonic nanoparticles (Ir$_1$-AuSiO$_2$), spectrally resonant with the luminescent TMC. GBM xenograft mice were exposed to a single light dose after treatment with the two therapeutics, inducing the generation of ROS (Ir$_1$) or transducing the radiant energy into ROS and heat (Ir$_1$-AuSiO$_2$). The regression and elimination of the tumor in mice treated with Ir$_1$-AuSiO$_2$ and exposed to VIS light required a more detailed analysis of the damages to understand the mechanism underlying the tumor elimination. Three-dimensional high-resolution XPCT reveals a massive devascularization of the region where clusters of particles were concentrated, first inducing a vascular shut down followed by a significant tumor mass regression. On the other hand, an XPCT study on mice treated with Ir$_1$ shows that the vasculature remains structurally unmodified, but a significant vessel dilation occurs as a consequence of local inflammatory processes. However, these results provide evidence that nanotherapeutics based on plasmonic nanoparticles and weaponized with PDT agents have the potential to operate an effective killing physical mechanism in solid tumors by harnessing synergistic effects.

The reported studies are preliminary investigations deserving—for the great value of the obtained results—further insights. In particular, accurate research involving a higher number of animals per group is currently underway. Moreover, in order to improve the tissue penetration of the excitation wavelength, the two-photon absorption abilities of the Ir(III) compound will be exploited to trigger the multifunctional nanosystem.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jnt3010001/s1, Scheme S1: Chemical reaction of ABDA with singlet oxygen. Figure S1: Correlation between optical density and molar concentration of ABDA. Figure S2: Morphological characterization of the photo-therapeutic nanoplatform. Figure S3: Nanoparticles analysis via Dynamic Light Scattering. Figure S4: Photophysical characterization of Ir$_1$-AuSiO$_2$. Figure S5: Detection of $^1\text{O}_2$ generation of Pc4 by ABDA method. Figure S6: Post-injection trafficking of Ir$_1$ analyzed via in vivo imaging. Figure S7: Post-injection trafficking of Ir$_1$-AuSiO$_2$ analyzed via in vivo imaging. Figure S8: Control groups—Follow-up of GBM xenograft mice treated with Ir$_1$ or Ir$_1$-AuSiO$_2$ in absence of optical radiation dose. Figure S9: X-ray phase-contrast tomography images and quantitative analysis of the vascular network of ex vivo GBM sample treated only with the radiation dose. Figure S10: X-ray phase-contrast tomography images and quantitative analysis of the vascular network of ex vivo GBM sample treated with Ir$_1$ + light exposure. Figure S11: X-ray phase-contrast tomography images and quantitative analysis of the vascular network of ex vivo GBM sample treated with Ir$_1$-AuSiO$_2$ + light exposure. Figure S12: Emission spectra of Ir$_1$ and Ir$_1$-AuSiO$_2$ in water
solution at different concentrations, Video S1: Three-dimensional volume \((350 \times 350 \times 350 \, \mu m^3)\) of GBM sample after intratumoral injection of Ir\(_1\)-AuSiO\(_2\) followed by light exposure.

**Author Contributions:** Conceptualization, L.R., M.L.D. and G.S.; formal analysis, L.R., S.C., G.P. and E.I.S.; investigation, L.R., S.C., A.S., F.P., N.P., M.F. and A.C.; resources, M.L.D., R.B. and G.S.; data curation, L.R., S.C., G.P., A.S., F.P., N.P., M.F. and A.C.; writing—original draft preparation, L.R.; writing—review and editing, L.R. and G.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by: Romanian Ministry of Education and Research, CNCS-UEFISCDI, project number PN-III-P4-ID-PCE-2020-1958, within PNCDI III; FISR Project “Tecnopolio di nanotecologia e fotonica per la medicina di precisione” (funded by MIUR/CNR, CUP B83B17000010001); TECNOMED project (funded by Regione Puglia, CUP B84I18000540002).

**Institutional Review Board Statement:** The animal study protocol was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University (Cleveland, OH, USA) (protocol code 2015-0033, 17 February 2021).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors thank Ramamurthy Gopalakrishnan and James P. Basilion (Case Western Reserve University, Cleveland, OH, USA) for technical support for in vivo studies. E.I.S. acknowledges the Romanian Academy, Program 4.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

**References**


