Assessing the combinatorial chemo-photothermal therapy mediated by sulfobetaine methacrylate-functionalized nanoparticles in 2D and 3D in vitro cancer models

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**Abstract**

**Background:** Combinatorial cancer therapies mediated by nanomaterials can potentially overcome the limitations of conventional treatments. These therapies are generally investigated using 2D *in vitro* cancer models, leading to an inaccurate screening. Recently, 3D *in vitro* spheroids have emerged in the pre-clinical testing stage of nanomedicines due to their ability to mimic key features of the *in vivo* solid tumors. **Aim:** Investigate the chemo-photothermal therapy mediated by Doxorubicin and IR780 loaded sulfobetaine methacrylate functionalized nanoparticles, for the first time, using monolayers of cancer cells and spheroids. **Results:** In the 2D cancer models, the nanomaterials’ mediated photothermal therapy, chemotherapy, and chemo-photothermal therapy reduced cancer cells’ viability to about 58%, 29%, and 1%, respectively. Interestingly, when the nanomaterials’ mediated photothermal therapy was tested on 3D spheroids, no cytotoxic effect was noticed. In contrast, the nanostructures’ induced chemotherapy decreased spheroids’ viability to 42%. On the other hand, nanomaterials’ mediated chemo-photothermal therapy diminished spheroids’ viability to 16%, being the most promising therapeutic modality. **Conclusion:** These results demonstrate the importance of using 3D spheroids during the *in vitro* screening of single/combinatorial therapies mediated by nanomaterials.
3D in vitro spheroids are emerging in the pre-clinical testing stage of nanomedicines due to their ability to mimic key features of the in vivo solid tumors.

In this study, the photothermal therapy mediated by nanomaterials was unable to diminish the viability of spheroids despite having therapeutic efficacy towards the monolayers of cancer cells. The chemo-photothermal therapy mediated by nanomaterials was the most effective modality towards both 2D and 3D in vitro cancer models, hence confirming the importance of using spheroids during the screening of nanomaterial-based therapies.

Keywords: Cancer, Chemotherapy, Nanoparticles, Photothermal therapy, Spheroids.

Abbreviations: ANOVA, Analysis of Variance; BSA, Bovine Serum Albumin; DLS, Dynamic Light Scattering; DMEM-F12, Dulbecco's Modified Eagle's Medium-F12; DOX, Doxorubicin; DTT, DL-Dithiothreitol; EE, Encapsulation Efficiency; FBS, Fetal Bovine Serum; IR+DOX/SBMA-BSA NPs,
IR780 and DOX loaded SBMA-g-BSA nanoparticles; IR/SBMA-BSA NPs, IR780 loaded SBMA-g-BSA nanoparticles; MCF-7, Michigan Cancer Foundation-7; NHDF, Normal Human Dermal Fibroblasts; NIR, Near-Infrared; ns, Non-significant; PBS, Phosphate Buffered Saline; PDI, Polydispersity Index; PEG, Polyethylene Glycol; PI, Propidium Iodide; PTT, Photothermal Therapy; SBMA, [2-(Methacryloyloxy) ethyl] dimethyl-(3-sulfopropyl) ammonium hydroxide; SBMA-g-BSA, BSA grafted with SBMA; S.D., Standard Deviation; TEM, Transmission Electron Microscopy.

1 Introduction

Photothermal therapy (PTT) mediated by nanomaterials holds great potential for improving cancer treatment [1, 2]. This therapeutic approach generally employs photo-responsive nanostructures with a size ranging from 50 to 200 nm, that passively accumulate at the tumor site [1, 3]. The nanomaterials used in cancer PTT are mostly based on inorganic materials with near-infrared (NIR; 750-1000 nm) absorption (e.g. graphene derivatives, gold nanorods, transition metal dichalcogenides, black phosphorus nanosheets [4, 5]) or are formulated by encapsulating organic NIR absorbing dyes (Indocyanine Green, IR780, IR808, IR820, IR825 [6, 7]) in nanostructures. Once the nanomaterials achieve tumor uptake, the tumor zone is irradiated with NIR light, which displays a low interaction with biological components (e.g. water, melanin, hemoglobin) and a high penetration depth [1, 8]. Then, the tumor-homed nanostructures can absorb the NIR radiation and release it as heat, leading to a spatio-temporal controlled therapeutic effect [1, 9].

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In some cases, the sole application of nanomaterials’ mediated PTT may not lead to complete tumor eradication due to the heterogeneous heat distribution within tumor stroma [10, 11, 12]. To address this limitation, chemotherapeutic agents have been loaded into NIR-responsive nanomaterials [11, 13-15]. The temperature increase achieved upon NIR laser irradiation can trigger the release of the chemotherapeutic agents from the nanomaterials into the tumor zone, further contributing to an on-demand treatment [8, 16, 17]. In this way, the chemo-PTT mediated by nanomaterials can lead to an enhanced therapeutic effect when compared to the single therapies (nanomaterials’ mediated PTT or chemotherapy) [12, 17, 18].

During the development of nanomaterials aimed for application in chemo-PTT, their efficacy is initially screened using monolayers of cancer cells (2D in vitro models) [12-14, 19]. Based on the data gathered using conventional cell cultures, the best formulation is then selected for in vivo validation [11, 19, 20]. However, the 2D in vitro models are unable to mimic the main features of the in vivo solid tumors, contributing to an inaccurate screening of the therapeutics. Due to this fact, numerous nanostructures that presented a good therapeutic effect towards monolayers of cancer cells have a shallow effect in vivo [21, 22, 23]. For instance, the chemo-PTT induced by targeted polydopamine-based nanoparticles incorporating doxorubicin (DOX) led to a decrease of cancer cells’ viability to about 9%, while in the in vivo studies only caused tumor’s regression [23]. In another work, the chemo-PTT mediated by PEGylated dendrimer-doxorubicin-gold nanorods conjugates could reduce the viability of cancer cell monolayers to about 2%, while in vivo only produced a reduction of the tumor growth [22].

To overcome this problem, the use of spheroids (3D in vitro models) has recently emerged in the pre-clinical testing stage of nanomedicines [14, 24, 25, 26]. Spheroids can mimic key features of the in vivo solid tumors by displaying: i) a 3D layered organization composed of proliferative, quiescent, and necrotic zones, ii) a gradient distribution of nutrients, pH, and
gases, and iii) a composition containing different cellular elements (cellular heterogeneity) and acellular matter (e.g. extracellular matrix, signaling factors) [27, 28]. In this way, spheroids present resistance and penetration patterns quite similar to those occurring in in vivo solid tumors, contributing to a more accurate in vitro screening of the nanomedicines (reviewed in detail in [26, 29, 30]).

In this work, the combinatorial chemo-PTT mediated by IR780 (photothermal agent) and DOX (a chemotherapeutic agent) loaded sulfobetaine methacrylate functionalized bovine serum albumin nanoparticles (IR+DOX/SBMA-BSA NPs) was investigated, for the first time, using monolayers of cancer cells and spheroids. In 2D cancer models, the PTT mediated by the NPs could reduce cells' viability to about 58 ± 2 %, while the NPs' induced chemotherapy was able to decrease the viability to 29 ± 4 %. On the other hand, the combinatorial chemo-PTT mediated by IR+DOX/SBMA-BSA NPs generated a better outcome by reducing cells' viability to 1 ± 0.2 %. Interestingly, when the NPs were tested in 3D spheroids, the NPs' mediated PTT did not induce any cytotoxicity. In contrast, the NPs' induced chemotherapy decreased spheroids' viability to 42 ± 3 %, while the combinatorial chemo-PTT mediated by the NPs could further diminish the viability of spheroids to 16 ± 1 %.

2 Materials and methods

2.1 Materials

Michigan Cancer Foundation-7 (MCF-7) and Normal Human Dermal Fibroblast (NHDF) cell lines were obtained from ATCC (Middlesex, UK) and Promocell (Heidelberg, Germany), respectively. Acetone, methanol, Triton X-100, and Tween 80™ were acquired from Fisher Scientific (Oeiras, Portugal). Fetal Bovine Serum (FBS) was purchased from Biochrom AG (Berlin, Germany). DOX
was obtained from Carbosynth (Berkshire, UK). DL-Dithiothreitol (DTT), Dulbecco's Modified Eagle’s Medium F12 (DMEM-F12), resazurin, [2-(Methacryloyloxy) ethyl] dimethyl-(3-sulfopropyl) ammonium hydroxide (SBMA), and IR780 iodide were supplied by Sigma-Aldrich (Sintra, Portugal). Agarose was obtained from Grisp (Porto, Portugal). Bovine Serum Albumin (BSA) was purchased from Amresco (Pennsylvania, EUA). T-Flasks, cell culture plates, Hoechst 33342®, Calcein-AM, and Propidium Iodide (PI) were acquired from Thermo Fisher Scientific (Porto, Portugal). BSA grafted with SBMA (SBMA-g-BSA) was synthesized as we have previously reported [7]. Double deionized water was used in all the required assays (0.22 μm filtered, 18.2 MΩ cm).

2.2 Methods

2.2.1 Preparation of IR+DOX/SBMA-BSA NPs and IR/SBMA-BSA NPs

IR+DOX/SBMA-BSA NPs were formulated using the nanoprecipitation method according to a previous publication of our group [7]. Briefly, 5 mL of a PBS solution containing SBMA-g-BSA (5 mg) and DTT (386 µg) was allowed to react for 20 min under stirring. Then, DOX (100 µg) and IR780 (100 µg) dissolved in acetone (1 mL) were added dropwise to the polymer-DTT solution for 2 h (room temperature, under stirring). Afterward, the solution was recovered and dialyzed against water (500-1000 Da molecular weight cut-off membrane), yielding IR+DOX/SBMA-BSA NPs. The same procedure was employed to formulate IR780 loaded SBMA-BSA NPs using 250 µg of IR780 (IR/SBMA-BSA NPs).

2.2.2 Characterization of the physicochemical, optical, and photothermal properties of the IR+DOX/SBMA-BSA NPs and IR/SBMA-BSA NPs

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IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs size distribution (in water) and zeta potential (in PBS (10 mM of Na₂HPO₄)) were evaluated on a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The morphology of both nanoformulations was confirmed by transmission electron microscopy (TEM). For this purpose, the nanoformulations were stained with phosphotungstic acid (2% (w/v)) and then imaged on a Hitachi-HT7700 transmission electron microscope (Hitachi Ltd., Tokyo, Japan), operated at an accelerating voltage of 80-100 kV. The NIR absorption of the nanoformulations was determined in an Evolution 201 spectrophotometer (Thermo Scientific Inc.). For such, the absorption spectra of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs in water (1.25 µg mL⁻¹ of IR780 equivalents) and of free IR780 in methanol (1.25 µg mL⁻¹) were acquired.

The photothermal capacity of IR+DOX/SBMA-BSA NPs was determined by placing aqueous solutions of these nanostructures (at different concentrations of IR780 equivalents; 200 µL) in 96-well plates [5]. Afterward, the samples were irradiated with NIR light (808 nm, 1.7 W cm⁻²) for 1, 2, 3, 4, or 5 minutes, and the temperature changes after each irradiation were determined using a thermocouple thermometer (each sample was only irradiated once) [5].

To determine the IR780 encapsulation efficiency (EE), the samples’ absorbance at 780 nm in a water:methanol (1:1 (v/v)) solution was analyzed, as we have previously described [7]. In turn, to analyze the EE of DOX, the fluorescence emitted by the samples (λₑₓ = 488 nm, λₑ𝐦 = 590 nm) was analyzed in a Spectramax Gemini EM spectrofluorometer (Molecular Devices LLC, California, USA) [14].

The DOX cumulative release from IR+DOX/SBMA-BSA NPs was investigated as previously described by us [14]. Briefly, IR+DOX/SBMA-BSA NPs dispersed in release medium (PBS containing Tween 80 (0.1% (v/v)); pH of 7.4) were placed into a dialysis tube (500–1000 Da molecular weight cut-off). Then, the tube was immersed in 15 mL of release medium, and
samples were withdrawn at predetermined time points to quantify the DOX released. IR+DOX/SBMA-BSA NPs were also exposed to NIR light (808 nm, 1.7 W cm⁻², 5 min), 4 h after the beginning of the release study, to determine the influence of the NIR exposure in the release of DOX.

2.2.3 Evaluation of the therapeutic effect of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs towards 2D cancer models

The therapeutic effect mediated by IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs towards MCF-7 cells was determined using the resazurin assay, as we have previously described [14]. For such, cells were cultured in DMEM-F12 medium supplemented with 10% (v/v) of FBS and 1% (v/v) of streptomycin/gentamycin in a humidified incubator (37 °C, 5% CO₂). In brief, MCF-7 cells were seeded in 96-well plates at a density of 1×10⁴ cells per well. After 24 h, cells were incubated with a fresh culture medium containing different concentrations of IR/SBMA-BSA NPs (IR780: 1.00 or 3.00 µg mL⁻¹), IR+DOX/SBMA-BSA NPs (IR780/DOX: 1.00/0.66 µg mL⁻¹ or 3.00/1.98 µg mL⁻¹), or free DOX (1.98 µg mL⁻¹). After 4 h, the cells were irradiated with NIR light (808 nm, 1.7 W cm⁻², 5 min). After totaling 24 h of incubation, the culture medium was replaced with a fresh medium containing resazurin (10% (v/v)) and the cells were incubated for 4 h in the dark (37 °C, 5% CO₂). Subsequently, the cells’ viability was determined by measuring the fluorescence of resorufin (λ_ex = 560 nm, λ_em = 590 nm; Spectramax Gemini EM spectrofluorometer (Molecular Devices LLC, California, USA)). Cells incubated with ethanol (70% (v/v)) and cells incubated solely with culture medium were used as a positive control (K⁺) and negative control (K⁻), respectively.

Confocal Laser Scanning Microscopy (CLSM) was employed to visualize the different therapeutic effects using the Calcein-AM/PI staining. For such, MCF-7 cells (seeded as described above)
were incubated with IR/SBMA-BSA NPs (IR780 concentration of 3.00 µg mL⁻¹) or IR+DOX/SBMA-BSA NPs (IR780/DOX concentration of 3.00/1.98 µg mL⁻¹). After 4 h of incubation, cells were exposed to NIR light (808 nm, 1.7 W cm⁻², 5 min). Subsequently, the cells were stained with Calcein-AM and PI (according to the manufacturer's protocol) and a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) was used to acquire the fluorescence images. The control for live cells was cells solely incubated with a culture medium.

2.2.4 Evaluation of the therapeutic effect of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs towards 3D cancer models

3D tumor spheroids were produced as we have previously described [14, 31]. Briefly, agarose structures (2% (w/v)) with spherical microwells were formed by using a micro-mold (3D Petri Dish®, Microtissues Inc., Providence RI, USA). Afterward, MCF-7 cells and NHDF at 1:1 cell ratio were seeded in the agarose structures (1×10⁶ cells per structure), leading to the formation of 81 spheroids per agarose structure. Then, the spheroids were grown for 10 days in DMEM-F12 medium supplemented with 10% (v/v) of FBS and 1% (v/v) of streptomycin/gentamicin, in an incubator with a humidified atmosphere (37 °C, 5% CO₂). Every 2 days, the culture medium was changed.

After the generation of the 3D spheroids, the therapeutic effect mediated by IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs was determined using the resazurin method as described above. Briefly, spheroids were incubated with a culture medium containing IR/SBMA-BSA NPs (IR780: 3.00 µg mL⁻¹ or 9.00 µg mL⁻¹), IR+DOX/SBMA-BSA NPs (IR780/DOX: 3.00/1.98 µg mL⁻¹ or 9.00/5.93 µg mL⁻¹), or free DOX (5.93 µg mL⁻¹) for 24 h. Each experimental condition was evaluated using 30 spheroids. Then, the spheroids were irradiated with NIR light (808 nm, 1.7 W cm⁻², 5 min) and the fluorescence images were acquired.
W cm\(^{-2}\)) for 5 min. After 48 h, the culture medium was replaced with a fresh culture medium containing resazurin (10% (v/v)). Finally, the viability of the cells within spheroids was determined as described in section 2.2.3.

For visualizing the different therapeutic effects, spheroids (assembled as described above) were incubated with IR/SBMA-BSA NPs (IR780 concentration of 9.00 µg mL\(^{-1}\)) or IR+DOX/SBMA-BSA NPs (IR780/DOX concentration of 9.00/5.93 µg mL\(^{-1}\)). After 24 h of incubation, spheroids were exposed to NIR light (808 nm, 1.7 W cm\(^{-2}\), 5 min). Subsequently, spheroids were stained with Calcein-AM/PI, and the fluorescence images were acquired as described in section 2.2.3.

### 2.2.5 Uptake of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs by MCF-7 cells and spheroids

The uptake of both SBMA-BSA NPs by MCF-7 cells was determined by fluorescence spectroscopy as previously described by our research group [7, 32]. In brief, MCF-7 cells (seeded as described in section 2.2.3) were incubated with IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs (1 µg mL\(^{-1}\) of IR780 equivalents) during 4 h. Subsequently, non-internalized SBMA-BSA NPs were removed by washing the cells with ice-cold Krebs Ringer Buffer. Then, cells were lysed by using 1% (v/v) of Triton X-100 in Krebs Buffer under orbital stirring for 30 min. Finally, the IR780 fluorescence in the cell lysate was quantified in a spectrofluorometer (\(\lambda_{\text{ex}} = 780\) nm; \(\lambda_{\text{em}} = 800\) nm). The control was performed by incubating cells solely with Krebs Buffer.

For analyzing the uptake of SBMA-BSA NPs in the spheroids, the 3D cell culture systems (as described in section 2.2.4) were incubated with IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs (2.5 µg mL\(^{-1}\) of IR780 equivalents) during 24 h. Subsequently, spheroids were harvested using
trypsin to attain single-cell suspensions. Then, the washing, lysis, and IR780 quantification steps were performed as described above.

The uptake of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs by MCF-7 cells and their penetration into spheroids were also visualized by CLSM. For this purpose, the IR780 fluorescence was explored. For the spheroids’ analysis, Z-stacks were collected with 13 µm intervals, and the fluorescence intensity plots along the spheroids’ diameter (at different penetration depths) were analyzed [14].

2.2.6 Statistical analysis

All data are presented as the mean ± standard deviation (S.D.). The unpaired t-student test and the one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test were used to compare two or multiple (≥ 3) groups, respectively (GraphPad Prism 6 software). A value of p lower than 0.05 (*p < 0.05) was considered statistically significant.

3 Results and Discussion

3.1 Preparation and characterization of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs

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To compare the efficacy of the nanomaterials’ mediated chemotherapy, PTT, and chemo-PTT towards 2D and 3D in vitro cancer models, IR780 loaded SBMA-BSA NPs (IR/SBMA-BSA NPs), and IR780 and DOX co-loaded SBMA-BSA NPs (IR+DOX/SBMA-BSA NPs) were prepared (Fig. 1A). In a previous study, our team has already performed an extensive characterization of the IR/SBMA-BSA NPs in 2D in vitro cancer models, demonstrating their cytocompatibility and that the SBMA functionalization improves the nanoformulations’ colloidal stability and cellular uptake [7].

The IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs were produced by the nanoprecipitation method due to its reliability and simplicity [5, 7]. The DLS analysis revealed that IR/SBMA-BSA NPs have an average size of 97.2 ± 0.8 nm, while the IR+DOX/SBMA-BSA NPs display an average size of 92.3 ± 1.2 nm (n = 3; batch triplicates; Fig. 1B). The slightly lower size of the IR+DOX/SBMA-BSA NPs may be correlated to the different hydrophobic interactions occurring at the core of this nanoformulation. As importantly, the size of both nanoformulations is within the ideal range for tumor accumulation through the enhanced permeability and retention effect [1, 3, 33]. The polydispersity index (PDI) of IR/SBMA-BSA NPs (0.196 ± 0.007) and IR+DOX/SBMA-BSA NPs (0.330 ± 0.019) is in agreement with that reported in other studies using nanoformulations loaded with IR780 and/or DOX [5, 7, 11, 14]. The zeta potential of the two nanoformulations was also measured. The results revealed that IR/SBMA-BSA NPs (-9.57 ± 0.81 mV) and IR+DOX/SBMA-BSA NPs (-9.55 ± 0.67 mV) have a similar surface charge. These charge values are in agreement with those reported in other studies using zwitterionic-based nanostructures and are also within the ideal range (-10 mV to 10 mV) for application in cancer therapy [7, 34, 35].

The IR/SBMA-BSA NPs displayed a 53% efficiency in encapsulating IR780 (Fig. 1C). Interestingly, the IR+DOX/SBMA-BSA NPs had an IR780 and DOX EE of 68% and 45%,
respectively (Fig. 1C). Such may be correlated to stronger hydrophobic interactions occurring in the core of IR+DOX/SBMA-BSA NPs, leading to a higher drug entrapment. Similar findings have been previously reported for other nanoformulations co-encapsulating multiple agents [14, 36, 37].

Finally, the morphology of IR/SBMA-BSA NPs (Fig. 1D) and IR+DOX/SBMA-BSA NPs (Fig. 1E) was analyzed by TEM, suggesting that both formulations have a spherical shape. This spherical morphology has also been observed in other formulations prepared by the nanoprecipitation technique [7, 14, 36]. Furthermore, this shape has also been associated with a better internalization by cancer cells and higher tumor uptake [5, 35, 38].

### 3.2 NIR absorption and photothermal capacity of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs

To characterize the capacity of the nanoformulations to interact with the NIR light, their absorption spectra were acquired (Fig. 2A). As expected, free IR780 (dissolved in methanol) demonstrated a high absorption in the NIR region, with a maximum absorption peak at 780 nm (Fig. 2A). When the IR780 was encapsulated in IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs, it suffered a red-shift (Fig. 2A). Due to this shift into the NIR range, the absorption of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs at 808 nm was 1.03-fold and 1.35-fold greater than that of free IR780, respectively. This deviation of the IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs absorptions towards the NIR zone has been attributed to changes in solvents’ polarity and/or to different hydrophobic interactions occurring in the nanoparticles’ core [7, 14, 15, 36]. Considering that 808 nm light will be used in the photothermal experiments, the higher absorption of the nanoformulations at this wavelength may lead to a greater therapeutic outcome.

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Subsequently, the photothermal capacity of IR+DOX/SBMA-BSA NPs was evaluated (Fig. 2B). For such, the IR+DOX/SBMA-BSA NPs (at different concentrations of IR780) were irradiated with NIR light (808 nm, 1.7 W cm⁻²) for 5 min and the temperature changes were recorded (Fig. 2B). At the maximum concentration tested (12.00 µg mL⁻¹ of IR780 equivalents), the IR+DOX/SBMA-BSA NPs could generate a temperature increase of 11.0 °C, which is sufficiently high to cause damage to cancer cells [1]. After 4 minutes of laser irradiation, there is a small decrease in the photoinduced heat generated by IR+DOX/SBMA-BSA NPs (Fig. 2B). This phenomenon is related to the photodegradability of the IR780 incorporated on these nanomaterials [11, 14, 39]. The photothermal capacity of IR/SBMA-BSA NPs was described by us in a previous publication [7]. At a concentration of 8.00 µg mL⁻¹ of IR780 equivalents, the IR/SBMA-BSA NPs were able to produce a maximum temperature increase of about 7 °C (808 nm, 1.7 W cm⁻²) [7]. Herein, at the same concentration and laser intensity, the IR+DOX/SBMA-BSA NPs produced a maximum temperature increase of 9.0 °C (Fig. 2B). The slightly higher photothermal capacity of IR+DOX/SBMA-BSA NPs may be related to their greater absorption at 808 nm (Fig. 2A). As importantly, water exposed to NIR light (control) did not suffer a meaningful temperature increase. Such is in agreement with the weak interaction of 808 nm radiation with water [14]. Furthermore, the photoinduced heat generated by IR+DOX/SBMA-BSA NPs also accelerated the release of DOX from this nanoformulation (Fig. S1). Together, these results indicate that IR+DOX/SBMA-BSA NPs may produce an on-demand therapeutic effect.

Lu et al. showed that IR780 loaded PEGylated zwitterionic nanoparticles could produce a temperature increase of about 11 °C at an IR780 concentration of 27 µg mL⁻¹ and laser intensity of 1.0 W cm⁻² [40]. The IR+DOX/SBMA-BSA NPs produced herein, generated a similar
temperature increase (11.0 °C) using only 12.00 μg mL\(^{-1}\) and laser intensity of 1.7 W cm\(^{-2}\), confirming their good photothermal potential.

3.3 Therapeutic effect mediated by IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs in 2D cancer models

Initially, the uptake of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs by MCF-7 cells was analyzed. CLSM images revealed that both SBMA-BSA NPs can achieve internalization in cancer cells (Fig. S2). Further investigation revealed that the IR780 fluorescence intensity in the cells incubated with IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs was similar (Fig. S3A). These results imply that IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs have a similar internalization in the cancer cells, which can be justified by the fact that both formulations have identical surface chemistry, size distribution, and surface charge (Fig. 1). Importantly, the similar uptake of both formulations by MCF-7 cells ensures that any differences in the nanomaterials’ efficacy are related to the different therapeutic modalities.

Then, the effect of nanomaterials’ mediated PTT (IR/SBMA-BSA NPs + NIR light), chemotherapy (IR+DOX/SBMA-BSA NPs), and chemo-PTT (IR+DOX/SBMA-BSA NPs + NIR light) towards monolayers of MCF-7 cells (2D in vitro model) was then investigated (Fig. 3A).

As expected, MCF-7 cells treated with only IR/SBMA-BSA NPs (without NIR light; 3.00 μg mL\(^{-1}\) of IR780 equivalents) demonstrated high viability (> 87 ± 4 %) (Fig. 3B). This result is in agreement with the fact that IR780 loaded nanoformulations are generally non-cytotoxic when not interacting with NIR light [14, 41]. Moreover, cells solely exposed to NIR light did not suffer alterations in their viability, which is explained by the minimal/insignificant interactions of this radiation with biological components (Fig. 3B).
In turn, at the same IR780 dose (3.00 µg mL\(^{-1}\) of IR780 equivalents), cells treated with IR/SBMA-BSA NPs + NIR light (nanomaterials’ mediated PTT) had their viability diminished to about 58 ± 2 % (Fig. 3B). Such indicates that the photothermal heating mediated by IR/SBMA-BSA NPs is capable of generating a therapeutic effect.

On the other hand, when these breast cancer cells were incubated with IR+DOX/SBMA-BSA NPs (3.00 µg mL\(^{-1}\) of IR780; 1.98 µg mL\(^{-1}\) of DOX) without laser irradiation (nanomaterials’ mediated chemotherapy), there was a reduction of their viability to about 29 ± 4 % (Fig. 3B). The action of free DOX (conventional chemotherapy), at the same concentration (1.98 µg mL\(^{-1}\) of DOX), only reduced the breast cancer cells’ viability to 57 ± 3 % (Fig. S5A), an effect that is in line with that described in previous reports [42]. By combining IR+DOX/SBMA-BSA NPs with NIR radiation (nanomaterials mediated’ chemo-PTT), the cells’ viability was further reduced to about 1 ± 0.2 % (Fig. 3B). These results were further confirmed by the Calcein-AM (labels live cells) and PI (labels dead cells) staining of the MCF-7 cells after the different treatments (Fig. 3C). By screening these 3 different therapeutic modalities in 2D in vitro cancer models, it was possible to conclude that both the nanomaterials’ mediated PTT and chemotherapy can promote a therapeutic effect, being outperformed by the nanomaterials’ mediated chemo-PTT.

Alves et al. verified that the chemo-PTT mediated by DOX and IR780 loaded Hyaluronic acid-based micelles could reduce MCF-7 cell viability to about 20% (IR780/DOX: 3.5/1.9 µg mL\(^{-1}\); 808 nm, 1.7 W cm\(^{-2}\), 5 min) [14]. In another work, monolayers of cancer cells treated with IR780 and DOX loaded PEGylated micelles plus NIR radiation (IR780/DOX: 5.0/10.0 µg mL\(^{-1}\), 808 nm, 4.0 W cm\(^{-2}\), 5 min) suffered a decrease in their viability to about 22% [17]. Compared to these studies, the IR+DOX/SBMA-BSA NPs in combination with NIR light could diminish cells’ viability to 1 ± 0.2 %, at a lower dose of therapeutics (IR780/DOX dose: 3.00/1.98 µg mL\(^{-1}\)) and using a
similar or weaker intensity (808 nm, 1.7 W cm\(^{-2}\), 5 min), hence demonstrating their potential for cancer chemo-PTT.

3.4 Therapeutic effect mediated by IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs in 3D cancer models

After characterizing the performance of the different SBMA-BSA NPs in the monolayers of cancer cells (2D \textit{in vitro} model), their behavior in the spheroids (3D \textit{in vitro} model) was analyzed. For such, CLSM images of spheroids incubated with both nanoformulations were acquired. The IR780 fluorescence signals in the spheroids, at different penetration depths, indicated that IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs present a similar penetration capacity into the spheroids (Fig. S4A and B). Furthermore, the cross-section penetration capacity of both formulations was also similar, as observed by analyzing the plots of the IR780 fluorescence intensity along the diameter of the spheroids (Fig. S4A and B). The higher accumulation of both SBMA-BSA NPs in the spheroids’ outer layers is in agreement with previous reports [14, 43]. These observations were further confirmed by quantifying the IR780 fluorescence intensity in the spheroids (Fig. S3B). In this regard, spheroids incubated with IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs presented an equal IR780 fluorescence intensity, indicating a similar uptake of both formulations by the spheroids.

Then, the nanomaterials’ mediated PTT, chemotherapy, and chemo-PTT towards spheroids were analyzed (Fig. 4A). In general, the different therapeutic modalities mediated by the nanomaterials had a weaker effect on spheroids (Fig. 4B) when compared to their outcome in the conventional 2D cultures (Fig. 3B). This behavior has also been described in the literature for other nanostructures [14, 44, 45]. The nanoformulations’ weaker effect on the spheroids can
be justified by the ability of this 3D in vitro model to mimic key features of the in vivo solid tumors, including penetration and resistance patterns [27, 30].

Interestingly, spheroids treated with IR/SBMA-BSA NPs plus NIR light (nanomaterials' mediated PTT) did not have their viability affected, even at a high IR780 dose (9.00 µg mL⁻¹) - Fig. 4B. Such may be related to the fact that spheroids display higher resistance to temperature-mediated cell death [46]. Considering that nanomaterials' mediated PTT was effective on the cancer cell monolayers, this result highlights the value of using spheroids for more realistically determining the therapeutic capacity of nanomaterials in vitro.

In turn, spheroids treated with only IR+DOX/SBMA-BSA NPs (nanomaterials' mediated chemotherapy; IR780/DOX concentration of 9/5.93 µg mL⁻¹) suffered a reduction in their viability to about 42 ± 3 % (Fig. 4B). Interestingly, the action of free DOX (at 5.93 µg mL⁻¹) only diminished the spheroids' viability to 51 ± 5 % (Fig. S5B). By further combining IR+DOX/SBMA-BSA NPs with NIR light (nanomaterials' mediated chemo-PTT), a decrease of spheroids viability to about 16 ± 1 % was achieved (Fig. 4B), indicating that this is the most effective therapeutic modality.

Spheroids subjected to the different therapeutic modalities mediated by the SBMA-BSA NPs were also stained with Calcein-AM/PI and imaged by CLSM (Fig. 5). As expected, the non-treated spheroids (control) displayed Calcein-AM and PI fluorescence mostly on the spheroid's outer layer and inner core, respectively (Fig. 5). This Live/Dead pattern is in agreement with the spheroid's layered organization, composed of an outer layer of highly proliferative cells and an inner core of necrotic cells [25, 47]. As anticipated, spheroids incubated with IR/SBMA-BSA NPs or IR/SBMA-BSA NPs plus NIR light showed a Calcein-AM/PI staining quite similar to that of the non-treated spheroids (Fig. 5). In contrast, spheroids treated with IR+DOX/SBMA-BSA NPs presented a high amount of PI stained cells, being in line with the cell viability results (Fig. 5).
Moreover, the highest amount of PI stained cells was imaged on the spheroids treated with IR+DOX/SBMA-BSA NPs plus NIR light, further confirming that the nanomaterials’ mediated chemo-PTT is the most effective therapeutic approach.

Chen et al. produced PEGylated CuS based micelles loaded with aminoflavone, whose chemo-PTT was able to reduce spheroids’ viability to about 46% (980 nm, 0.75 W cm$^{-2}$, 10 min, 0.05 μg mL$^{-1}$ of aminoflavone) [45]. In another study, Su et al. verified that the chemo-PTT induced by transferrin functionalized PEGylated reduced graphene oxide-based hybrids loaded with docetaxel and perfluorohexane could reduce spheroids’ viability to about 2% (808 nm, 2.0 W cm$^{-2}$, 10 min, 20 μg mL$^{-1}$ of graphene derivatives) [48]. Herein, the chemo-PTT mediated by IR+DOX/SBMA-BSA NPs could reduce spheroids viability to $16 \pm 1\%$, with only $9.00/5.93$ μg mL$^{-1}$ of IR780/DOX and with only 5 min of irradiation (808 nm, $1.7$ W cm$^{-2}$). Together, these results demonstrate that IR+DOX/SBMA-BSA NPs are promising agents for breast cancer chemo-PTT.

4 Conclusion

In this work, the effect of SBMA-BSA NPs’ mediated PTT (IR/SBMA-BSA NPs + NIR light), chemotherapy (IR+DOX/SBMA-BSA NPs), and chemo-PTT (IR+DOX/SBMA-BSA NPs + NIR light) towards monolayers of cancer cells and spheroids was investigated. In 2D cancer models, the NPs’ mediated PTT could reduce cells’ viability to about $58 \pm 2\%$, while the NPs’ induced chemotherapy was able to decrease the viability to $29 \pm 4\%$. The combinatorial chemo-PTT mediated by the NPs generated a better outcome by diminishing cells’ viability to $1 \pm 0.2\%$. Interestingly, when the NPs were tested in 3D spheroids, the NPs’ mediated PTT did not induce any cytotoxicity. In contrast, the NPs’ induced chemotherapy decreased spheroids’ viability to $42 \pm 3\%$. In turn, the combinatorial chemo-PTT mediated by the NPs could further diminish the viability of spheroids to $16 \pm 1\%$, hence being the most promising therapeutic modality.
Overall, these results highlight the relevance of using 3D spheroids during the *in vitro* screening of single and combinatorial therapies mediated by nanomaterials. Furthermore, this experimental approach may also contribute to reducing the number of animals used in experimentation.

**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Author Contributions**

Inês Mó: Investigation, Formal analysis, Writing – original draft.

Cátia G. Alves: Investigation, Writing - review & editing.

Duarte de Melo-Diogo: Conceptualization, Investigation, Supervision, Writing - review & editing.

Rita Lima-Sousa: Investigation, Supervision.

Ilídio J. Correia: Project administration, Funding acquisition, Supervision, Writing - review & editing.

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**Conflict of interest**

The authors declare no financial or commercial conflict of interest.
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Figure legends

Figure 1. Formulation and characterization of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs. Schematic representation of the nanoparticle assembly and application in cancer therapy (A). DLS size distribution of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs (B). EE of IR780 and DOX in IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs (C). Each bar represents mean ± S.D.
(n = 3; batch triplicates). TEM images of IR/SBMA-BSA NPs (D) and IR+DOX/SBMA-BSA NPs (E). Scale bars correspond to 200 nm.
Figure 2. Characterization of the optical properties and photothermal capacity of IR+DOX/SBMA-BSA NPs. Absorption spectra of free IR780 (1.25 µg mL⁻¹; in methanol), and of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs (1.25 µg mL⁻¹ of IR780 equivalents; in water) (A). Photoinduced heat produced by IR+DOX/SBMA-BSA NPs during 5 min of irradiation (808 nm, 1.7 W cm⁻²) (B).
Figure 3. Determination of the therapeutic capacity of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs using 2D in vitro cancer models (monolayers of cancer cells). Schematic representation of the procedure used to evaluate the nanoparticles’ singles and combinatorial therapies towards MCF-7 cells (A). Therapeutic effect mediated by SBMA-BSA NPs towards MCF-7 cells without (w/o NIR) and with NIR (w/ NIR) laser irradiation (808 nm, 1.7 W cm$^{-2}$, 5 min) (B). IR/SBMA-BSA NPs were incubated at an IR780 dose of 1 or 3 µg mL$^{-1}$. IR+DOX/SBMA-BSA NPs were incubated at an IR780/DOX dose of 1/0.66 or 3/1.98 µg mL$^{-1}$. K+ represents the positive...
control. K⁻ w/o NIR represents the negative control, while K⁺ w/ NIR represents cells solely treated with NIR light. Data represent mean ± S.D., n = 5, (*p < 0.0001), ns = non-significant.

CLSM images of MCF-7 cells stained with Calcein-AM/PI after incubation with SBMA-BSA NPs w/o NIR and w/ NIR laser irradiation. The control for live (Medium w/o NIR) cells was also performed (C). Cells solely treated with NIR light were also analyzed (Medium w/ NIR). Green channel: Calcein-AM; Red channel: PI. Scale bars correspond to 50 µm.

**Figure 4.** Determination of the therapeutic capacity of IR/SBMA-BSA NPs and IR+DOX/SBMA-BSA NPs using 3D in vitro cancer models (tumor spheroids). Schematic representation of the procedure used to evaluate the nanoparticles’ singles and combinatorial therapies towards spheroids (A). Therapeutic effect mediated by SBMA-BSA NPs towards spheroids without (w/o NIR) and with NIR (w/ NIR) laser irradiation (808 nm, 1.7 W cm⁻², 5 min) (B). IR/SBMA-BSA NPs were incubated at an IR780 dose of 3 or 9 µg mL⁻¹. IR+DOX/SBMA-BSA NPs were incubated at an IR780/DOX dose of 3/1.98 or 9/5.93 µg mL⁻¹. K⁺ represents the positive control. K⁻ w/o NIR represents the negative control, while K⁻ w/ NIR represents spheroids solely treated with NIR light. Data represent mean ± S.D., n = 30, (*p < 0.0001), ns = non-significant.
**Figure 5.** Live/Dead staining analysis of spheroids incubated with the nanoformulations. CLSM images of spheroids stained with Calcein-AM/PI after incubation with SBMA-BSA NPs w/o NIR and w/ NIR laser irradiation (C). Green channel: Calcein-AM; Red channel: PI. Scale bars correspond to 100 μm.