Engineered porphyrin loaded core-shell nanoparticles for selective sonodynamic anticancer treatment

Engineered porphyrin loaded core-shell nanoparticles for selective ultrasound mediated anticancer treatment

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Summary

Aims: Porphyrin-loaded core-shell nanoparticles are engineered as an *in vivo* ultrasound responsive system, a radio-tracer or a magnetic resonance (MR) imaging agent, suitable for the selective treatment of solid tumors and imaging analyses. **Materials & methods:** Poly methyl methacrylate nanoparticles (PMMANPs) are loaded with meso-tetrakis (4-sulphonatophenyl) porphyrin (TPPS) for anticancer sonodynamic treatment, or with ^{64}Cu-TPPS for positron emission tomography biodistribution studies or with Mn(III)-TPPS for MR tumor accumulation evaluation. **Results:** PMMANPs exhibit ease of functionalization with negatively charged molecules and favorable biodistribution. *In vivo* TPPS-PMMANPs demonstrate ultrasound responsiveness in a syngeneic rat breast cancer model by MR analyses of pre- and post-treatment tumor volumes. **Conclusion:** TPPS-PMMANPs is a multimodal system able to efficiently induce *in vivo* anticancer sonodynamic activity.

Introduction

In the last decades, one of the disciplines that mostly benefited from nanotechnology is oncology. Nanoparticle systems have been used in several studies aimed to investigate their potential as diagnosis and therapy tools [1]. In fact, one of the most fascinating aspect of nanoparticles relies on the possibility to manipulate molecules and supramolecular structures for producing objects with programmed functions, such as the ability to preferentially accumulate in the tumor vessels, to convey higher drug concentration at the target site and to provide a multi-purpose loading capabilities [2,3]. In addition, nanoparticles have been investigated as therapeutic drug-delivery systems, as contrast agents for magnetic resonance imaging (MRI) and as tumor visualizing agents during surgery [4,5]. Several types of nanoparticle platforms have also been evaluated for theranostic applications combining the modalities of therapy and diagnosis to deliver the drug and to allow performing diagnostic imaging at the same time and within the same macro-molecular system [6]. This approach allows to potentially overcome undesirable differences in biodistribution that currently exist between imaging and therapeutic agents, with the consequent advantage to allow the long-term hope of gaining the ability to tune therapy with heretofore unattainable control. In the field of cancer diagnosis, MRI and positron emission tomography (PET) are the most widespread imaging techniques able to provide a three-dimensional image of the body functional processes (MRI) and morphological/physiological information on body's organs (PET) [7,8]. In this regard, nanotechnology might provide a more sensitive and accurate approach able to manage diagnosis, staging, treatment and monitoring of cancer with the same hybrid technology [9,10].

Thus, further significant progresses in anticancer therapeutic options will also depend on efforts devoted to the development of novel nanotechnological-based anticancer treatments. In this regard in a previous study we developed poly-methyl methacrylate nanoparticles (PMMANPs) carrying the meso-tetrakis (4-sulfonatophenyl) porphyrin (TPPS) that were able

to improve the porphyrin sonodynamic activity in an *in vitro* two- and three-dimensional model of cancer [11]. The sonodynamic anticancer approach refers to the use of ultrasound, i.e, mechanical vibration above the threshold of human hearing (20 kHz), as an external stimulus to trigger the cytotoxicity of chemical agents, i.e., sonosensitizers. From a clinical point of view, ultrasound can be distinguished in diagnostic ultrasound generally with a frequency range between 3.0-30.0 MHz and therapeutic ultrasound with a frequency range between 0.5-3.0 MHz [12]. The latter is usually generated by applying an alternating voltage across a piezoelectric material and a focused ultrasound beam is directed, harmlessly, across the skin and intervening tissues towards the target. In the so-called high intensity focused ultrasound (HIFU), a combination of mechanical stresses and thermal insult at microscopic level induce cell necrosis [13]. Remarkably, the energy level sufficient to cause a temperature rise able to produce instantaneous cell death is reached only at the center of the beam. A wide range of tumor types can be targeted with HIFU. In fact, this technique has been taken into account for palliative treatment of symptoms or for local tumor control of patients with poor prognoses [13]. HIFU is now being used clinically to treat solid tumors, both malignant and benign, including prostate, liver, breast, kidney, bone, pancreas and soft-tissue sarcoma [14–16]. In addition to HIFU, a different anticancer ultrasound-based approach, namely sonodynamic therapy (SDT), is under investigation to promote, deeply in the tissues, the activation of sonosensitizers to induce a more selective cancer cell death [17]. The feasibility of this approach has been already demonstrated at preclinical level on some experimental tumor models [18–23].

In SDT, the sensitizer is believed to be activated by inertial cavitation, a process induced by ultrasounds that induces the formation of microscopic air bubbles within the tissues' liquids, which ultimately oscillate, expand and violently collapse. The cellular membrane is intrinsically capable of absorbing mechanical energy from the ultrasound field and to transform it into expansions and contractions of the intramembranous space [24]. When the

acoustic intensity is sufficient to induce microscopic air bubbles to collapse quickly, a great release of energy might occur, inducing a series of chemical reactions around the bubbles and the sensitizer. Accordingly, an excess amounts of reactive oxygen species (ROS) such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-), can be formed. These species are than able to damage cells by peroxidizing lipids and disrupting DNA and proteins, but also to exert signaling functions and modulate gene transcription, thereby resulting in cellular dysfunction and apoptosis [25]. On this topic, we first introduced the use of shock wave (SW) to trigger sonosensitizer's cytotoxicity, since pulsed ultrasound generates significantly less thermal effect than continuous ultrasound [26].

Among the sonosensitizers, porphyrin-type molecules have been extensively applied in SDT [27]. In addition, the use of nanoparticles as carriers of the sonosensitizer might provide an additional advantage with respect to nanoparticle-mediated drug delivery. Indeed, in the case of SDT, nanoparticles can represent the nucleation sites that participate in the formation of bubbles thus reducing the threshold of intensity required for cavitation, the phenomenon underlying the sonodynamic activity [28,29]. Moreover, moving our experiments in *in vivo* model, biodistribution studies are also needed to determine the *in vivo* TPPS-PMMANPs fate. So, in the present study, we aim to investigate our nanoparticle system, properly decorated with TPPS, ^{64}Cu-TPPS- and Mn(III)-TPPS to distinctly functions *in vivo* as sonosensitizing system, radio-tracer and MR imaging agent, respectively (Figure 1).

Materials and Methods

- Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA), 1-bromooctane, 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AIBA), methyl methacrylate (MMA, 99.0%) (distilled before use), TPPS, Mn(III)-TPPS, $Cu(CH_3CO_2)_2$, McCoy's 5A modified medium, fetal bovine serum (FBS), penicillin, streptomycin, Hank's-balanced salt solution (HBSS), formalin, xylene and hematoxylin-eosin were purchased from Sigma-Aldrich (Schnelldorf, DE) and used without further purification unless otherwise stated. Allprotect Tissue Reagent, AllPrep® DNA/RNA/protein Kit, QuantiTect® Reverse Transcription Kit and QuantiTect Primer Assay were purchased from Qiagen (Milano, Italy). Quant-iT™ RNA Assay Kit was purchased from Invitrogen (Milano, Italy). RNA 6000 Nano Kit was purchased from Agilent Technologies (Milano, Italy). SsoFast™ EvaGreen was purchased from Bio-Rad (Segrate, Italy).

- TPPS, Mn(III)-TPPS or ^{64}CuTPPS-PMMANPs synthesis and characterization

TPPS-PMMANPs were obtained as previously described.[11] Mn(III)-TPPS-PMMANPs synthesis and characterization was performed according to the same procedure used for the synthesis of TPPS-PMMANPs by replacing TPPS with commercially available Mn(III)-TPPS. Our cyclotron facility provides ^{64}Cu isotope on a routine basis for research purposes, using a target system developed in collaboration with Tema Sinergie (Faenza, Italy). ^{64}Cu was produced on a GE PETTrace cyclotron by the reaction ^{64}Ni(p,n) ^{64}Cu using an enriched ^{64}Ni target electroplated on a gold disk. $^{64}CuCl_2$ was recovered from the target and converted to ^{64}Cu-acetate by dissolving the $^{64}CuCl_2$ in ammonium acetate (0.1 M; pH 5.5), followed by evaporation to dryness. TPPS was labelled with ^{64}Cu following conditions optimized with "cold" $Cu(CH_3CO_2)_2$ [30].

^{64}Cu-TPPS preparation was performed as follows: 40 μl of a 3M solution of sodium acetate (NaOAc) were added to 160 μl of ^{64}Cu mother solution (5.46 mCi) followed by 25 μl TPPS water solution (2mg/mL) and 103 μl of physiological solution. The mixture was then vortexed for 10 sec and analysed by TLC (SiO_2; eluent: CH_2Cl_2/MeOH=1/2) and ^{64}Cu was detected on Instant Imager system (98% of ^{64}Cu chelation; radioactivity of the solution 1.66 mCi). ^{64}Cu amount was counted in a Capintec CRC15 PET Radioisotope Calibrator to calculate the activity of the product.

^{64}Cu-TPPS-PMMANPs preparation was performed as follows: 60 μl of a 3M solution of sodium acetate (NaOAc) were added to 240 μl of ^{64}Cu mother solution (5.3 mCi) followed by 25 μl TPPS water solution (2 mg/mL), 33.1 μl of PMMANPs (5 mg/mL) and 141.9 μl of physiological solution. The resulting solution was vortexed for 20 sec, filtered on a 0.1 μm filter for centrifuge and radioactivity was measured (1.72 mCi). The particles solution was then centrifuged (3000xG) for 15 min and filtered. The supernatant did not display any radioactivity. ^{64}Cu amount was counted in a Capintec Radioisotope Calibrator to calculate the specific activity of the product.

- Positron emission tomography studies

Female Balb/c mice 8 weeks-old (Charles River laboratories, Milano, Italy) were intravenously (iv) injected with 8.3 MBq ^{64}Cu-TPPS or ^{64}Cu-TPPS-PMMANPs [corresponding to 10 mg/Kg body weight (bw) TPPS] and at 1, 4 and 18 h static scans were acquired for 20 min using a small-animal PET system (GE, eXplore Vista DR; General Electric, Milwaukee, WI, USA) with the animals under isoflurane anesthesia. The images were reconstructed by a 2-dimensional ordered-subsets expectation maximum (OSEM) algorithm and read in three planes (axial, sagittal, and coronal). For each microPET scan, regions of interest were drawn over the major organs on the decay-corrected whole-body

coronal images. The radioactivity concentration (accumulation) within the brain, liver, spleen and kidneys were obtained from the mean value within the multiple regions of interest and then converted to standardized uptake value (SUV, corrected for body weight and injected radioactivity).

Twenty-four hours after the intravenous administration of 8.3 MBq ^{64}Cu-TPPS or ^{64}Cu-TPPS-PMMANPs the animals were sacrificed and the organs of interest were collected, weighed, and processed for radioactivity counting using a c-counter with decay correction. The radioactivity uptake was expressed as radioactivity per gram of tissue (kBq/g).

- Breast cancer model

The rat mammary adenocarcinoma cell line, Mat B III (ATCC, Rockville, MD, USA), was maintained in McCoy's 5A modified medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at +37 °C in a humidified atmosphere containing 5% CO_2. Before inoculation, Mat B III cells were washed with HBSS and centrifuged at 1500 rpm for 5 min. Cell pellets (1×10^6 cells) were suspended in 0.5 mL saline and injected using 1 mL insulin syringes orthotopically into the abdominal mammary fat pad of inbred female 12 weeks-old Fisher 344 rats (Charles River laboratories) under isoflurane anesthesia. Following tumor-cell implantation, all animals were examined daily for the development of tumors for up to 12 days. The animals were randomly assigned to treatment groups, with at least four animals per group.

- Magnetic resonance imaging evaluations

Manganese-enhanced MRI was performed at day 9 after tumor cell inoculation to evaluate Mn(III)-TPPS or Mn(III)-TPPS-PMMANPs tumor accumulation to pick the right time for ultrasound exposure while for evaluating the sonodynamic treatment efficacy tumor bearing

rats were imaged at day 8 (24 h pre-treatment) and day 11 (48 h post-treatment) from Mat B III cell inoculation.

MRI was conducted using a dedicate whole body rat coil in a high field (7T) MRI scanner (Bruker, PharmScan, Germany) and rats were supplied with 1-2% isofluorane in air and O_2 for anesthetization (animals' respiration rate was monitored throughout the entire imaging analysis with a sensor connected to an ECG/respiratory unit). Spin echo (RARE) imaging (TR/TE/NEX = 3000/8.5/2, slices thick 1.5 mm, FOV 50 mm, matrix 256 x 256; 15 slices, imaging time = 2.5 min) was used to calculate tumor volume and to analyse tumor morphology.

Serial T1-weighted MR scans with multislice spin-echo sequence (TR/TE =500/12 ms, number of averages NA = 2), were collected before Mn(III)-TPPS as free or nanoparticle formulation were intravenously injected and after 1, 4 and 24 h.

The percentage enhanced signal on tumor, liver, spleen and kidneys at 1, 4 and 24 h from the iv administration of 10 mg/kg bw of Mn-TPPS as free or nanoparticle formulation were obtained by control to noise ratio percentage [CNR(T1)%] calculations which correlates CNR(T1) post-contrast to the corresponding pre-contrast value. CNR(T1) is defined as the difference between the averaged imaging coefficient within the region of interest (ROI) and the differences within the background region, divided by the averaged imaging coefficient variation in the background.

- Sonodynamic treatment

The tumor volumes of both control and experimental groups were monitored at day 8 (24 h pre-treatment) and at day 11 (48 h post-treatment) by MRI. The sonodynamic treatment was performed when the subcutaneous tumors reaching 300-500 mm^3 in volume typically in nine days. Control and experimental animals were treated on day 9 with one single iv injection into the tail vein of saline or 10 mg/kg bw TPPS or TPPS-PMMANPs, SW alone (0.88 mJ/mm^2,

500 impulses, 4 impulse/sec), or a combination of TPPS or TPPS-PMMANPs and SW (10 mg/kg bw iv 4 h before SW exposure at 0.88 mJ/mm^2, 500 impulses, 4 impulse/sec). All control and experimental animals were sacrificed at the end of the study (day 12), examined and scored for the development of macroscopic tumor metastases in various tissues. Primary tumor tissues were removed in 10% buffered formalin for histological examination and in Allprotect Tissue Reagent for mRNA gene expression evaluation. The piezoelectric shock wave generator Piezoson 100 (Wolf, Germany) was used for the sonodynamic treatment and the energy at the focal point is defined as energy flux density (EFD) per impulse, recorded as mJ/mm^2. It is assumed an elliptical focus cigar with a length of 10 mm in the direction of the axis of the shock wave propagation and a diameter of 2.5 mm perpendicular to this axis. Tumor bearing rats were anesthetized with 1-2% isofluorane in air and O_2, fixed to a board in a supine position with the tumor upwards and the hair over the tumor was shaved and ultrasound gel applied to the naked skin. The transducer was placed in close contact with the tumor. The position and the angle of the rat were adjusted to locate the tumor at the focal spot and, thus, allow the focused ultrasound to propagate throughout the tumor mass. All animals were then placed on a warm blanket and observed until their complete recovery before putting them back into their cages.

- Histopathological analysis

Tumor samples 12 days after Mat B III cell inoculation (72 h post-treatment) were fixed in 10% formalin, blocked in paraffin resin, cut in 4 μm, deparaffinized in xylene and rehydrated with alcohol following standard protocols. Sections were then stained with hematoxylin-eosin for histological examination and visualized by light microscopy (Leica DM600, Wetzlar, Germany). The morphological features of apoptosis and necrosis such as the presence of edema, inflammation, red blood cells extravasation in at least ten random fields per slide at

40x magnification were evaluated. All examinations were accomplished in a blind test without prior knowledge of the group sample.

- Real Time RT-PCR analyses

Total RNA was isolated from tumor samples 12 days after Mat B III cell inoculation (72 h post- treatment). Briefly, tumor samples were collected in Allprotect Tissue Reagent and stored at -80 °C. Total RNA was then obtained by the AllPrep® DNA/RNA/protein Kit according to the manufacture instructions. The total RNA concentration (µg/mL) was determined by the fluorometer Qubit (Invitrogen, Milano, Italy) and the Quant-iT™ RNA Assay Kit; calibration was done applying a two-point standard curve, according to the manufacturer's instructions. The integrity of the RNA samples was determined by the total RNA 6000 Nano Kit by the Agilent 2100 Bioanalyzer (Agilent Technologies). Real-time reverse transcriptase (RT)-PCR analysis was carried out using 500 ng of total RNA, which was reverse transcribed in a 20 µL cDNA (complementary DNA) reaction volume, using the QuantiTect® Reverse Transcription Kit, according to the manufacturer's instructions; 12.5 ng of cDNA was used for each 10 µL real-time RT-PCR reaction. Quantitative RT-PCR was performed using the SsoFast™ EvaGreen. QuantiTect Primer Assay was used as the gene-specific primer pair for *APAF1* (cat n° QT01611225), *BAD* (cat n° QT00190407), *BAX* (cat n° QT01081752), *HIF1A* (cat n° QT00182532), *MMP9* (cat n° QT00178290), *NFE2L2* (cat n° QT00183617), *NQO1* (cat n° QT00186802), *RNR1* (cat n° QT00199374), and *TP53* (cat n° QT00193522). The transcript of the reference gene ribosomal 18s and 28s RNA (RRN1) was used to normalize mRNA data, and real-time PCR was performed by a MiniOpticon™ Real Time PCR system (Bio-Rad). The PCR protocol conditions were as follows: HotStarTaq DNA polymerase activation step at +95 °C for 30 sec, followed by 40 cycles at +95 °C for 5 seconds and +55 °C for 10 sec. All runs were performed with at least three independent cDNA preparations per sample, and all samples were run in duplicate. At least two non-

template controls were included in all PCR runs. The quantification data analyses were performed using the Bio-Rad CFX Manager Software version 1.6 (Bio-Rad) according to the manufacturer's instructions. These analyses were performed in compliance with the MIQE (Minimum Information for Publication of Quantitative Real-time PCR Experiments) guidelines [31].

- Statistical analysis

Results are expressed throughout as the average value ± standard deviation (SD). Statistical analyses were performed with Graph-Pad Prism 5.0 software (La Jolla, CA, USA); one-way ANOVA and Bonferroni's post test were used to calculate the threshold of significance. Statistical significance was set at $p < 0.05$.

Results

- TPPS, Mn(III)-TPPS or ^{64}Cu-TPPS- PMMANPs

PMMANPs synthesis, characterization and loading with TPPS and Mn(III)-TPPS was performed as previously described [11]. PMMANPs were characterized by an average hydrodynamic diameter of 93 ± 1.4 nm, with 265 μ mol of quaternary ammonium bromides per gram of nanospheres and a ζ -potential of 38.8 $\pm$ 2.4 mV. The stability of the TPPS-PMMANPs system was evaluated by mimicking physiological conditions using further PBS treatment (150 mM) at + 37 °C and cell growth culture medium as the washing solutions. ^{64}Cu-TPPS-PMMANPs were straightforwardly obtained by simple mixing of the PMMANPs water suspension and freshly prepared ^{64}Cu-TPPS.

- Sonosensitizing system biodistribution

The PET analysis of the biodistribution of ^{64}Cu-TPPS or ^{64}Cu-TPPS-PMMANPs at different time point from iv administration in healthy mice, highlighted that the nanoparticle system was not able to cross the blood brain barrier (BBB). This was proved through the PET analysis of its biodistribution *in vivo*, as a negligible signal was detected in the brain (Figure 2A-B). On the other hand, a strong increase of ^{64}Cu-TPPS-PMMANPs uptake was found in the liver and spleen over time, suggesting a low rate of renal excretion associated to a high trapping in the reticulo-endothelial system (Figure 2A). The renal excretion of ^{64}Cu-TPPS was higher since the uptake in the kidney was significant over time whilst the renal excretion of ^{64}Cu-TPPS-PMMANPs was significant only 1 h after the iv administration. This was confirmed at the *ex-vivo* analysis performed 24 h after the iv administration. So a higher trapping of the ^{64}Cu-loaded nanoparticles was found in the liver and spleen as compared to ^{64}Cu-TPPS and a higher uptake of ^{64}Cu-TPPS in the kidney was found as well (Figure 2B).

- Sonosensitizing system tumor accumulation

In order to evaluate further application of our porphyrin-based nanoparticle system the time scheduling for ultrasound exposure was performed by a Mn(III)-enhanced magnetic resonance analysis of the tumor after the iv administration of Mn(III)-TPPS or Mn(III)-TPPS-loaded onto nanoparticles shell, Mn(III)-TPPS-PMMANPs. As reported in Figure 3 the MR signal expressed as the percentage of control to noise [CNR(T1)%] at the tumor level had a different trend depending to the Mn(III)-TPPS administration form. Indeed, the highest CNR(T1)% in the tumor was recorded at 24 h after the i.v. administration of Mn(III)-TPPS, (Figure 3). As it has been reported, TPPS is well accumulating in tumor tissue and localized mostly in tumor stroma [32]. Mn(III)-TPPS-PMMANPs determined a significant increase of the CNR(T1)% already at 1 h after the iv administration in the tumor and in spleen (Figure 3). The analysis of the animals treated with Mn(III)-TPPS-PMMANPs displayed an almost equal CNR(T1)% value at 1, 4 and 24 h upon iv administration (Figure 3). We observed persistent but lower CNR(T1)% values after the Mn(III)-TPPS-PMMANPs with respect to the Mn(III)-TPPS iv administration in the tumor and a higher signal in liver and spleen and the lower signal in kidneys after Mn(III)-TPPS-PMMANPs administration (Figure 3). We then decided to treat tumor bearing rats 4 h after the iv injection of TPPS or TPPS-loaded onto nanoparticles, TPPS-PMMANPs.

- Sonosensitizing system anticancer activity

To investigate the effect of the sonodynamic treatment with TPPS or TPPS-PMMANPs on the tumor growth, tumor volumes were determined by MR 24 h pre- and 48 h post-sonodynamic treatment. Ultrasound exposure as shock wave mode was performed at day 9 from tumor inoculation, i.e. when the tumors reached a volume of approximately 500 mm^3. Figure 4 shows a significant reduction of tumor volumes in the group treated with the

nanoparticles formulation and shock waves, confirming our hypothesis of an enhancement of the porphyrin sonodynamic activity when loaded onto the nanoparticles.

The histological examinations of tumor sections of animals treated with TPPS-PMMANPs and SW highlighted a strong increase of necrotic and apoptotic features, while no injury of the blood vessels with blood cells extravasation respect to untreated animals was observed (Figure 5A-B).

As the controlled and targeted specific ROS generation can be the effector of the sonodynamic anticancer activity, we looked at the mRNA levels in tumor tissue of the group treated with TPPS-PMMANPs plus SW. Indeed, with respect to untreated group, we observed an increased expression of oxidative stress related genes such as the transcription factor Hypoxia Inducible Factor 1 Alpha Subunit (*HIF1A*), the reductase NAD(P)H Dehydrogenase, Quinone 1 (*NQO1*) and the cytochrome c-dependent apoptosis activation mediator Apoptotic Peptidase Activating Factor 1 (*APAF1*). Moreover, a significant reduction of the apoptosis regulator BCL2-Associated X Protein (*BAX*) and of the extracellular matrix endopeptidases Matrix Metallopeptidase 9 (*MMP9*) gene expression was detected (Figure 5C).

Discussion

Nanotechnology works towards satisfying desired objectives using materials and device whose valuable properties owe to some specific nanometer-scale elements of their structures, making this discipline extremely dynamic and highly application oriented [33]. In this regard we considered an innovative nanoscale platform able to improve a less investigated but, in our opinion, promising therapeutic approach in oncology, i.e., sonodynamic therapy. Indeed recently, external stimuli mediated treatments, relying on the synergistic effects of two components of either chemical or physical nature, received considerable attention in cancer therapy due to their lower systemic toxicity and higher selectivity as they are based on the preferential tissues uptake and/or retention of a sensitizer which is subsequently activated by light (PDT) or ultrasound (SDT). Although the basic principle of STD may be related to PDT, since in both mechanisms a non-toxic chemical compounds must be first activated before becoming cytotoxic, the use of ultrasound as external stimulus (STD) might represent a great advantage in terms of tissues penetration ability. Indeed, ultrasound energy are able to enter the target site deeply within the tissues, thus overcoming one of the major drawbacks of PDT, i.e., light's relatively limited capacity to penetrate human tissue [34,35]. Noteworthy is the fact that attention is now increasingly focused towards the possibility of enhancing the cancer-specific immunity upon direct ultrasound-induced treatment [35,13].

As confirmed by the rapidly expanding knowledge on the fundamental mechanisms of SDT, the development of ultrasound selective sensitizers is one of the most essential factors in SDT. Discovery and development of novel sensitizers are becoming the major focus of recent investigation in this field [27]. In order to improve the sonodynamic cancer therapy, we developed a core-shell polymer-based nanoparticle, namely PMMANPs, carrying porphyrin to be *in vivo* triggered by ultrasound applied as shock wave mode. In fact, the nanoparticles mediated drug delivery concept is not the only factor that can enhance SDT [20]. Indeed, the existence of appropriate nanoparticles in the milieu might provide nucleation sites decreasing

the cavitation threshold that must be reached through ultrasound exposure [36,37]. Moreover, the ease of the manipulation and loading procedure of our PMMANPs, along with their high stability under forced release conditions, make these nanoparticles particularly attractive for theranostic applications. In principle, thanks to their high external shell charge density, the sequential post-loading of different negatively charged compounds, e.g. sulfonates, is easily achievable, thus allowing the preparation of nanocarriers either with sensitizers, imaging agents and radiotracers for theranostic applications. With regard to MRI and taking into account the increasing concerns of nephrogenic system fibrosis caused by gadolinium-based blood poll agents in patients with renal disease or with recent liver transplant, we selected Mn(III) as the T1 contrast agent for MR analysis [38,39].

The sonodynamic treatment was performed by ultrasound applied as shock wave mode to improve the non-thermal interactions of ultrasound energy with biological tissue that are primarily mediated by cavitation. Moreover, the use of pulsed ultrasound as shock wave well-fit with the theory of the "bilayer sonophore" affirming that the bilayer membrane is capable (under appropriate conditions) of transforming the (millimetre wavelength) oscillating acoustic pressure wave into (nanometric and micrometric) intracellular deformations able to induce intracellular cavitation [24].

The ultrasound responsiveness of the TPPS-PMMANPs was confirmed by a significant decrease in the volume of the tumor masses, i.e., up to the fifty percent, for the sonodynamic treatment with TPPS-PMMANPs as shown in Figure 4. Moreover, the observed persistent TPPS-PMMANPs tumor accumulation recorded by MR analysis, might be highly beneficial for programming repeated ultrasound exposure, without need of multiple administrations (Figure 3). Finally, to our knowledge, this work highlighted for the first time the *in vivo* modulation of a panel of genes involved in the ROS mediated cell death by sonodynamic treatment. Indeed, in tumors treated with TPPS-PMMANPs and SW, it was observed a

significant ROS-induced overexpression of *APAF1, HIF1A* and *NQO1* genes that might lead to an oxidative stress and consequently to apoptosis as shown in Figure 5 [40].

Conclusion

In summary, a multifunctional nanoparticle system carrying meso-tetrakis (4-sulfonatophenyl) porphyrin was subsequently *in vivo* applied as sonosensitizing system in sonodynamic anticancer treatment, as radio-tracer in PET biodistriobution studies and as MR imaging agent in tumor accumulation studies. Our results clearly indicate that the *in vivo* sonosensitizing system was able to efficiently induce selective ultrasound mediated cancer cell death. Moreover, we provided an *in vivo* proof-of-concept of the viability of PMMANPs as valuable tool for theranostic applications.

Future perspective

Investigations on the contemporary multiple loading of TPPS, Mn(III)-TPPS and ^{64}Cu-TPPS on PMMANPs are actively underway in our laboratories for future theranostic applications. Such an innovative nanoscale platform would allow to set personalized treatment schedules for the anticancer sonodynamic therapy by imaging modalities that at the same time might be effective in monitoring the treatment response throughout the control of the induced sonodynamic activity.

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Ethical conduct of research

The authors state that all the animals were housed in temperature- and humidity-controlled facilities with a 12 h light/dark cycle and were allowed free access to food and water; they were handled according to European guidelines (Directive CEE 86/609). The experimental protocol was reviewed and approved for the Fischer 344 by the Ethics Committee of the University of Torino, Italy and for the Balb/c by the Ethics Committee of the University Hospital S. Orsola-Malpighi of Bologna, Italy.

Executive summary

- The use of a single nano-object, i.e. PMMANPs, as versatile and easily functionalizable platform for the multiple loading of the sonosensitizer, the imaging agent and the radiotracer
- The use of ultrasound applied as shock wave mode to induce the sonosensitizing system activation
- The evidence of *in vivo* anticancer activity of the sonosensitizing system triggered by ultrasound through sonodynamic activity promoting oxidative stress-mediated cell death in a rat syngeneic breast cancer model.

Bibliography

Papers of special note have been highlighted as:

* of interest ** of considerable interest

1 Sun T, Zhang YS, Pang B, Hyun DC, Yang M, Xia Y. Engineered Nanoparticles for Drug Delivery in Cancer Therapy. *Angew. Chem. Int. Ed Engl.* (2014).

** A very important review on the state-of-the-art of nanomedicince and on the critical need for novel nanocarriers for the delivery of different types of anticancer drugs.

2 Doane TL, Burda C. The unique role of nanoparticles in nanomedicine: imaging, drug delivery and therapy. *Chem. Soc. Rev.* 41(7), 2885 (2012).

3 Iyer AK, Khaled G, Fang J, Maeda H. Exploiting the enhanced permeability and retention effect for tumor targeting. *Drug Discov. Today*. 11(17-18), 812–818 (2006).

4 Jabir NR, Tabrez S, Ashraf GM, Shakil S, Damanhouri GA, Kamal MA. Nanotechnology-based approaches in anticancer research. *Int. J. Nanomedicine*. 7, 4391–4408 (2012).

5 Malam Y, Loizidou M, Seifalian AM. Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. *Trends Pharmacol. Sci.* 30(11), 592–599 (2009).

6 Kelkar SS, Reineke TM. Theranostics: combining imaging and therapy. *Bioconjug. Chem.* 22(10), 1879–1903 (2011).

7 Mansi L, Ciarmiello A, Cuccurullo V. PET/MRI and the revolution of the third eye. *Eur. J. Nucl. Med. Mol. Imaging*. 39(10), 1519–1524 (2012).

8 Partovi S, Kohan A, Rubbert C, *et al.* Clinical oncologic applications of PET/MRI: a new horizon. *Am. J. Nucl. Med. Mol. Imaging*. 4(2), 202–212 (2014).

* A recent survey on PET/MRI hybrid technology and ist potential in clinical oncology applications.

9 Hu Z, Yang W, Liu H, *et al.* From PET/CT to PET/MRI: Advances in Instrumentation and Clinical Applications. *Mol. Pharm.* (2014).

10 Kim BYS, Rutka JT, Chan WCW. Nanomedicine. *N. Engl. J. Med.* 363(25), 2434–2443 (2010).

11 Canaparo R, Varchi G, Ballestri M, *et al.* Polymeric nanoparticles enhance the sonodynamic activity of meso-tetrakis (4-sulfonatophenyl) porphyrin in an *in vitro* neuroblastoma model. *Int. J. Nanomedicine*. 8, 4247–4263 (2013).

12 Leighton TG. What is ultrasound? *Prog. Biophys. Mol. Biol.* 93(1-3), 3–83 (2007).

13 Kennedy JE. High-intensity focused ultrasound in the treatment of solid tumours. *Nat. Rev. Cancer*. 5(4), 321–327 (2005).

14 Al-Bataineh O, Jenne J, Huber P. Clinical and future applications of high intensity focused ultrasound in cancer. *Cancer Treat. Rev.* 38(5), 346–353 (2012).

15 Ganzer R, Fritsche H-M, Brandtner A, *et al.* Fourteen-year oncological and functional outcomes of high-intensity focused ultrasound in localized prostate cancer. *BJU Int.* 112(3), 322–329 (2013).

16 Malietzis G, Monzon L, Hand J, *et al.* High-intensity focused ultrasound: advances in technology and experimental trials support enhanced utility of focused ultrasound surgery in oncology. *Br. J. Radiol.* 86(1024), 20130044 (2013).

17 Tachibana K. Emerging technologies in therapeutic ultrasound: thermal ablation to gene delivery. *Hum. Cell*. 17(1), 7–15 (2004).

18 Gao Z, Zheng J, Yang B, *et al.* Sonodynamic therapy inhibits angiogenesis and tumor growth in a xenograft mouse model. *Cancer Lett.* 335(1), 93–99 (2013).

19 Jeong E-J, Seo S-J, Ahn Y-J, Choi K-H, Kim K-H, Kim J-K. Sonodynamically induced antitumor effects of 5-aminolevulinic acid and fractionated ultrasound irradiation in an orthotopic rat glioma model. *Ultrasound Med. Biol.* 38(12), 2143–2150 (2012).

20 Meng Y, Zou C, Madiyalakan R, *et al.* Water-soluble and biocompatible sono/photosensitizer nanoparticles for enhanced cancer therapy. *Nanomed.* 5(10), 1559–1569 (2010).

21 Sazgarnia A, Shanei A, Meibodi NT, Eshghi H, Nassirli H. A novel nanosonosensitizer for sonodynamic therapy: in vivo study on a colon tumor model. *J. Ultrasound Med. Off. J. Am. Inst. Ultrasound Med.* 30(10), 1321–1329 (2011).

22 Serpe L, Canaparo R, Berta L, Bargoni A, Zara GP, Frairia R. High energy shock waves and 5-aminolevulinic for sonodynamic therapy: effects in a syngeneic model of colon cancer. *Technol. Cancer Res. Treat.* 10(1), 85–93 (2011).

23 Tsuru H, Shibaguchi H, Kuroki M, Yamashita Y, Kuroki M. Tumor growth inhibition by sonodynamic therapy using a novel sonosensitizer. *Free Radic. Biol. Med.* 53(3), 464–472 (2012).

24 Krasovitski B, Frenkel V, Shoham S, Kimmel E. Intramembrane cavitation as a unifying mechanism for ultrasound-induced bioeffects. *Proc. Natl. Acad. Sci. U. S. A.* 108(8), 3258–3263 (2011).

** This work support for the first time the hypothesis that ultrasonically induced bilayer membrane motion may account for a variety of bioeffects elucidating mechanisms of ultrasound interaction with biological tissues.

25 Misík V, Riesz P. Free radical intermediates in sonodynamic therapy. *Ann. N. Y. Acad. Sci.* 899, 335–348 (2000).

** The first work which outlined the possible sonodynamic mechanisms underpinning the anticancer sonodynamic treatment.

26 Canaparo R, Serpe L, Catalano MG, *et al.* High energy shock waves (HESW) for sonodynamic therapy: effects on HT-29 human colon cancer cells. *Anticancer Res.* 26(5A), 3337–3342 (2006).

27 Chen H, Zhou X, Gao Y, Zheng B, Tang F, Huang J. Recent progress in development of new sonosensitizers for sonodynamic cancer therapy. *Drug Discov. Today*. 19(4), 502–509 (2014).

** An extensive review on the recent progress in the discovery of new sonosensitizers for SDT, discussing the future directions for one of the most essential factors in SDT.

28 Serpe L, Foglietta F, Canaparo R. Nanosonotechnology: the next challenge in cancer sonodynamic therapy. *Nanotechnol. Rev.* 1(2), 173-182 (2012).

29 Tuziuti T, Yasui K, Sivakumar M, Iida Y, Miyoshi N. Correlation between acoustic cavitation noise and yield enhancement of sonochemical reaction by particle addition. *J. Phys. Chem. A*. 109(21), 4869–4872 (2005).

30 Makarska M, Radzki S, Legendziewicz J. Spectroscopic characterization of the water-soluble cationic porphyrins and their complexes with Cu(II) in various solvents. *J. Alloys Compd.* 341(1-2), 233–238 (2002).

31 Bustin SA, Benes V, Garson JA, *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55(4), 611–622 (2009).

32 Luksiene Z, Rutkovskiene G, Jurkonien S, Maksimov G, Griciute. Evaluation of the photobiological efficiency of TPPS4 in two different types of mice tumors. *Acta Medica Litu.* 9(1), 62–66 (2002).

33 Ferrari M. Biological and Biomedical Nanotechnology. In: BioMEMS and biomedical nanotechnology (Volume 1). Lee AP, Lee LJ (Ed.), Springer, New York, NY,USA, 520 (2006)

34 Agostinis P, Berg K, Cengel KA, *et al.* Photodynamic therapy of cancer: an update. *CA. Cancer J. Clin.* 61(4), 250–281 (2011).

35 Trendowski M. The promise of sonodynamic therapy. *Cancer Metastasis Rev.* (2013).

36 Sazgarnia A, Shanei A, Taheri AR, *et al.* Therapeutic effects of acoustic cavitation in the presence of gold nanoparticles on a colon tumor model. *J. Ultrasound Med. Off. J. Am. Inst. Ultrasound Med.* 32(3), 475–483 (2013).

37 Shanei A, Sazgarnia A, Tayyebi Meibodi N, *et al.* Sonodynamic Therapy Using Protoporphyrin IX Conjugated to Gold Nanoparticles: An In Vivo Study on a Colon Tumor Model. *Iran. J. Basic Med. Sci.* 15(2), 759–767 (2012).

38 Pan D, Caruthers SD, Hu G, *et al.* Ligand-directed nanobialys as theranostic agent for drug delivery and manganese-based magnetic resonance imaging of vascular targets. *J. Am. Chem. Soc.* 130(29), 9186–9187 (2008).

39 Shin J, Anisur RM, Ko MK, Im GH, Lee JH, Lee IS. Hollow manganese oxide nanoparticles as multifunctional agents for magnetic resonance imaging and drug delivery. *Angew. Chem. Int. Ed Engl.* 48(2), 321–324 (2009).

40 Liu B, Chen Y, St. Clair DK. ROS and p53: versatile partnership. *Free Radic. Biol. Med.* 44(8), 1529-1535 (2008).

Figure Legends

Figure 1. **Representation of PMMANPs, TPPS, Mn(III)-TPPS and ^{64}Cu-TPPS.**

Figure 2. PET analysis of ^{64}Cu-TPPS and ^{64}Cu-TPPS-PMMANPs biodistribution in healthy mice. The radioactivity uptake in brain, liver, spleen and kidneys was expressed as standardized uptake value (SUV) after 1, 4 and 18 h of iv administration of 10 mg/kg bw ^{64}Cu-TPPS as free or nanoparticle formulation (A). The ex-vivo tissue radioactivity was expressed as kBq per gram of tissue 24 h after the iv administration of 10 mg/kg bw ^{64}Cu-TPPS as free or nanoparticle formulation (B). Data are expressed as mean ± SD of at least two separate experiments each with at least four animals per group.

Figure 3. MRI analysis of Mn(III)-TPPS and Mn(III)-TPPS-PMMANPs biodistribution in Mat B III/ Fisher 344 breast cancer model. The manganese-enhanced MRI were determined at 1, 4 and 24 h from the iv administration of 10 mg/kg bw Mn(III)-TPPS as free or nanoparticle formulation at day 9 after tumor cell inoculation. The percentage enhanced signal of tumor, liver, spleen and kidneys were obtained by the contrast to noise ratios (CNR) calculations. Data are expressed as mean ± SD of at least two separate experiments each with at least four animals per group.

Figure 4. Effect of sonodynamic treatment on Mat B III/ Fisher 344 tumor growth. Rats with growing tumors were treated with TPPS or TPPS-PMMANPs plus SW (10 mg/kg b.w. i.v. and 0.88 mJ/cm^2 for 500 impulses, 4 impulses/sec, respectively) at day 9 from tumor cells inoculation and tumor volumes were determined by MR at day 8 and 11. Representative T_2-weighted images of control (A and B) and TPPS-PMMANPs plus SW treated (C and D) rats at day 8 (24 h pre-treatment, A and C) and 11 (48 h post-treatment, B and D). The relative

tumor volumes, i.e. the ratio between volumes at day 11 and at day 8, of each experimental groups are reported as mean ± SD for at least three separate experiments each with at least four animals per group (E). Statistical significance versus tumor volumes at day 8, *** $p < 0.001$.

Figure 5. Effect of sonodynamic treatment with TPPS-PMMANPs on Mat B III/Fisher 344 tumor features. Representative hematoxylin-eosin section images of control (A) and TPPS-PMMANPs plus SW treated (B) rats 72 h from the sonodynamic treatment (10x magnification). Rats with growing tumors were treated with TPPS or TPPS-PMMANPs plus SW (10 mg/kg bw iv and 0.88 mJ/cm^2 for 500 impulses, 4 impulses/sec, respectively) at day 9 from tumor cells inoculation. mRNA expression analysis 48 h after the sonodynamic treatment (C). *RNR1* (ribosomal RNA 18S and 28S) was used as a reference gene to normalize the data. The sonodynamic therapy-induced alterations in mRNA levels were compared with those of the control, ie untreated rats, stated as 1 and are shown by the dotted line. Data are reported as mean ± SD for at least three separate experiments each with at least four animals per group. Statistical significance versus control, * $p < 0.05$, ** $p < 0.01$.