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Development of near-infrared photoactivable phthalocyanine-loaded nanoparticles to kill tumor cells: An improved tool for photodynamic therapy of solid cancers

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Abstract

Conventional photodynamic therapy has shown to be beneficial in the treatment of a variety of tumors. However, one of its major limitations is the inadequate penetration depth of visible light. In order to overcome this constraint, we developed 80 nm polymethylmethacrylate core-shell fluorescent nanoparticles (FNP) loaded with the photosensitizer tetrasulfonated aluminum phthalocyanine (Ptl). To demonstrate the efficacy of our Ptl@FNP we performed *in vitro* and *in vivo* studies using a human prostate tumor model. Our data reveal that Ptl@FNP are internalized by tumor cells, favour Ptl intracellular accumulation, and efficiently trigger cell death through the generation of ROS upon irradiation with 680 nm light. When directly injected into tumors intramuscularly induced in SCID mice, Ptl@FNP upon irradiation significantly reduce tumor growth with higher efficiency than the bare Ptl. Collectively, these results demonstrate that the newly developed nanoparticles may be utilized as a delivery system for antitumor phototherapy in solid cancers.

Key words: Nanoparticles; Tetrasulfonated aluminum phthalocyanine; Photodynamic therapy; Solid tumors; Prostate cancer; Bioluminescence imaging

Photodynamic therapy (PDT) is a successful, clinically approved, and minimally invasive alternative/co-adjuvant therapeutic option to conventional chemotherapy for the treatment of various tumors.[1,2]

PDT eliminates tumor cells by the combined action of nontoxic photosensitizers (PS), oxygen, and light at an appropriate wavelength.[3] Light activation of the PS results in an energy transfer cascade that ultimately yields the formation of cytotoxic reactive oxygen species (ROS), which are the effectors of apoptotic and necrotic cell death. These series of events lead to permanent cell destruction often associated with vasculature damage and upregulation of the immune system.[4]

Most of the current PDT applications are limited to PS photoactivable in the visible light range, with poor penetration capacity, restricting the treatment to tumors that grow on or right

Abbreviations: NIR, near-infrared; IRR, irradiation; NP, nanoparticles; FNP, fluorescent nanoparticles; PDT, photodynamic therapy; PS, photosensitizers; Ptl, tetrasulfonated aluminum phthalocyanine; Ptl@FNP, fluorescent nanoparticles loaded with Ptl; ROS, reactive oxygen species; BLI, in vivo bioluminescence imaging.

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under the skin, and along the lining of internal organs. In PDT, the use of PS activable in the near-infrared (NIR) region of the visible spectrum (>700 nm), allows to penetrate further through tissues and treat cancers located in internal regions of the human body such as muscles and bones.[5,6] Among NIR-PS, phthalocyanines (light absorption region between 600 and 800 nm) are very promising molecules as they allow effective treatment of deeper tissues.[7] In particular, the water-soluble tetrasulfonate aluminium phthalocyanine (Ptl) has appropriate photobiological characteristics given its high accumulation in tumors and cell death induction by photostimulation at NIR wavelengths.[8,9] However, self-aggregation resulting from its large hydrophobic skeleton[10] and its inability to efficiently permeate the lipid bilayer of target cells[11] contribute to a reduction of its efficacy.

Therefore, delivery/carrier systems that improve Ptl cell and tissue permeability are required. Nanomedicine has recently drawn strong interest due to its potential to improve cancer therapies by selectively targeting tumors, sparing healthy tissue.[12] In the last decade, a large number of studies have described nanoparticles (NP) as effective drug carriers, allowing a considerable reduction in drug dosages and toxic effects associated with the administration of systemic chemotherapeutic agents.[13] Alternatively, mesenchymal stromal/stem cells (MSC) thanks to their tumor homing ability are interesting vehicles to specifically deliver cytotoxic therapeutic agents to tumors.[14] We recently demonstrated that human MSC can be used as effective vehicles to deliver chemotherapeutics[15] and photosensitizer-loaded NP to kill osteosarcoma tumor cells.[16]

While our results pave the way for a three-component therapeutic strategy against solid tumors based on the use of MSC carriers bearing photosensitizer-loaded NP, a preclinical proof of principle for such strategy requires successful resolution of several issues. Photoactivation ability/efficacy, PS nanoparticles loading optimization, optimum activation wavelength, irradiation time and delivery routes should be carefully investigated.[17,18]

In the current work, we generated positively charged, core-shell poly-methyl methacrylate (PMMA) nanoparticles that incorporate a fluorescein derivative (FITC) in their inner hydrophobic core (FNP) and are electrostatically decorated with Ptl, and we evaluated their effectiveness *in vitro* and against a human prostate cancer model intramuscularly induced in SCID mice.

Methods

Preparation of Ptl@FNP

Tetrasulfonate aluminium phthalocyanine (Ptl), in quantities ranging from 50 μg/mL to 600 μg/mL in mQ H_2O, was added to an aqueous solution of positively charged poly-methylmethacrylate (PMMA) core-shell fluorescent nanoparticles (FNP) (0.5 mg/mL), prepared and characterized as previously described,[16] and then stirred at room temperature in a Vortex apparatus for 20 sec. Each sample was subsequently centrifuged (4 min, 4722RCF) through a 100 kDa filter and the filtrate removed. The residual nanoparticles were then re-suspended in mQ H_2O to 1 mL and the amount of loaded Plt was determined by adsorption at 670 nm using the calibration curve of free Ptl. Samples for the atomic force microscopy (AFM) measurements were prepared by spin-coating deposition of 1 mL of FNP or Ptl@FNP (1 mg/mL) on atomic flat silicon substrate at 1500 rpm, to prevent the aggregation of the nanoparticles and obtain a very uniform deposition over 1 cm^2 of isolated nanoparticles, which can be clearly distinguished from the substrate. AFM topographical images were collected using an NT-MDT (Sondrio, Italy) solver scanning probe microscope in tapping mode.

For *in vitro* assays we produced a Ptl@FNP stock solution by adding 50 μl of Ptl (1 mg/mL in mQ water) to 50 μl of FNP (10 mg/mL) and mQ water to a final 1 mL volume; 360 μl were then added to tissue culture plates containing 1 mL of complete cell culture medium, resulting in a 18 μg/mL-Ptl@FNP final concentration (18 μg-Ptl over 180 μg-FNP or 100 $\mu g_{Ptl}/mg_{FNP}$).

Cell cultures

The androgen-independent human prostate carcinoma PC3 cell line (1:1 ratio HAM'S F12:DMEM-hg, 10% FBS, GlutaMAX™ 1% and 50 U ml^{-1} penicillin/streptomycin) was purchased from ATCC (CRL-1435). PC3 cells implanted in SCID mice were transfected as described.[19]

Cytocompatibility and uptake of Ptl@FNP

PC3 cells were incubated for 10 or 60 min with i) FNP, ii) Ptl and iii) Ptl@FNP, washed with PBS and processed for analyses. *In vitro* cytotoxicity and cell viability were evaluated using the WST-1 metabolic assay, according to manufacturer's instructions, and methylene blue assay.[20] Quantitative evaluation of Ptl@FNP uptake was performed using a FACScanto II cytometer Becton-Dickinson (Franklin Lakes, NJ, USA).

In vitro PDT parameters

The 2D and 3D *in vitro* data were generated using a LED light (λ_{max} = 668 ± 3 nm) during a 5 min (fluence 263 J/cm^2 - 876.6 mW/cm^2) and 30 min (fluence 1581 J/cm^2 - 878.3 mW/cm^2) period, respectively, at room temperature, with the light source directly under the same tissue culture plates where cells were previously plated and loaded with Ptl@FNP. The 405 nm diode laser integrated in the confocal microscope was used to induce photoactivation in the ROS detection experiment, taking advantage of Ptl Soret band (λ_{max} = 350 nm).

Cell viability assays

Loaded and irradiated cells were incubated with green-fluorescent calcein-AM and red-fluorescent ethidium homodimer-1 (EthD-1) (LIVE/DEAD® Viability/Cytotoxicity Kit) solutions, according to the manufacturer's protocol. Images were acquired with an inverted Nikon Eclipse TE2000-U microscope (Nikon, Amsterdam, Netherlands), equipped with a Nikon DS-Vi1-U3 CCD color digital camera. For quantitative evaluation, Alexa Fluor® 488 annexin V/PI Dead Cell Apoptosis Kit was used according to the manufacturer's protocol and analyzed with cytometer. Three independent experiments have been performed for each analysis.

ROS detection

Ptl@NP-loaded cells (instead of FNP to avoid crosstalk between the ROS probe and the FITC fluorophore) were incubated with 1 mL of 10 μM H_2DCFDA at 37 °C for