TEMOPORFIN-CONJUGATED UPCONVERSION NANOPARTICLES FOR NIR-INDUCED PHOTODYNAMIC THERAPY OF PANCREATIC CANCER

[1]Oleksandr SHAPOVAL, [2]David VĚTVIČKA, [2]Martina KABEŠOVÁ, [3]Hana ENGSTOVÁ, [1]Daniel HORÁK

[1]Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague, Czech Republic, EU, shapoval@imc.cas.cz

[2]First Faculty of Medicine, Charles University, Prague, Czech Republic, EU

[3]Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic, EU

https://doi.org/10.37904/nanocon.2023.4803

Abstract

Photodynamic therapy (PDT), a clinically approved cancer treatment strategy, has the potential to cure pancreatic cancer with minimal side effects. PDT primarily uses visible wavelengths to directly activate hydrophobic photosensitizers, which may be insufficient for deep-seated cancer cells in clinical practice due to poor penetration. Upconversion nanoparticles (UCNPs) serve as an indirect excitation source to activate photosensitizers (PSs) in the NIR region, overcoming the limitations of molecular PSs such as hydrophobicity, non-specificity, and excitation in the UV/Vis region. Here, monodisperse upconversion $NaYF_4$:Yb^{3+}, Er^{3+}, Fe^{2+} nanoparticles (UCNPs) have been surface-engineered with poly(methyl vinyl ether-*alt*-maleic acid) (PMVEMA) and temoporfin (mTHPC), a clinically used PDT prodrug, for near-infrared (NIR) light-triggered PDT of pancreatic cancer. The incorporation of Fe^{2+} ions into the particles increased the fluorescence intensity in the red region matching the activation wavelength of mTHPC. Covalent binding of mTHPC to the surface of UCNP@PMVEMA particles provided colloidally stable conjugates enabling generation of singlet oxygen. *In vitro* cytotoxicity and photodynamic activity of the particles were evaluated using INS-1E rat insulinoma and Capan-2 and PANC-01 human pancreatic adenocarcinoma cell lines. The PDT efficacy of UCNP@PMVEMA-mTHPC conjugates after irradiation with 980 nm NIR light was tested *in vivo* in a pilot study on Capan-2 human pancreatic adenocarcinoma growing subcutaneously in athymic nude mice. The intratumoral administration of the nanoconjugates significantly hindered tumor growth and demonstrated promising PDT efficacy against human pancreatic cancer.

Keywords: Photodynamic therapy, temoporfin, upconversion nanoparticles, pancreatic cancer

1. INTRODUCTION

Photodynamic therapy (PDT) is an effective clinical tumor treatment that involves the interaction of photosensitizer (PS) with safe visible light and oxygen to produce reactive oxygen species (ROS) that lead to damage biomolecules and cell death [1]. PDT has been clinically used to treat different types of various diseases (e.g., acne, age-related macular degeneration, and early-stage cancers, including skin, esophageal, mouth, and lung cancer). Because PDT is noninvasive and minimally drug-resistant, it has found clinical application in the treatment of a wide variety of conditions, including acne, age-related macular degeneration, and early-stage cancer, including skin, esophagus, oral, and lung cancer [2]. The porphyrin-based PSs for PDT treatment of pancreatic cancer appeared to be effective, feasible and safe, resulting in tumor reduction with low morbidity [3]. Their efficacy depends on its physicochemical properties, photostability, absorption coefficient, minimal dark toxicity and delayed phototoxicity, preferential tumor localization, and efficiency of singlet oxygen ($^1O_2$) production.

Among conventional porphyrin-based PSs, temoporfin (Foscan®; 5,10,15, 20-Tetra(m-hydroxyphenyl)chlorin; mTHPC) has unique biopharmaceutical properties that define its high PDT efficacy [4]. Temoporfin's enhanced absorption of light in the red region (λ_{max} = 652 nm; high molar extinction coefficient) has been shown to be more specific than conventional porphyrins, which have been reported to reduce skin photosensitization, usually a side effect of PDT, due to the low photon energy in this region to produce $^1O_2$. However, mTHPC does not make use of the optimal spectral biological window for tissue penetration in the 700–1,000 nm range. The direct excitation of mTHPC with high-energy at this wavelength would eliminate the problem of low photon energy; however, direct excitation with high-energy light limits the use in biological applications due to its low tissue penetration depth.

To overcome this drawback, NIR-excitable upconversion nanoparticles (UCNPs) as a photosensitizer carrier convert NIR light to visible one at 650 nm, enabling light penetration up to 5 cm into biological tissue, typically in the 700-1,000 nm spectral range [5]. It is the ideal light source for PDT because of its low phototoxicity to normal cells and tissues. NIR-excitable UCNPs offer many advantages, including sharp emission bandwidth, long lifetime, tunable emission, photostability, cytotoxicity, and most important, low background autofluorescence. UCNPs, which exhibit the highest upconversion efficiency in NIR-induced PDT of cancer, have been successfully used as good candidates for *in vitro* and *in vivo* applications [6]. Numerous platforms based on a variety of inorganic nanomaterials (carbon nanotubes, iron oxides, upconverting α-$NaYF_4$) have been already investigated for efficient targeted drug delivery in pre-clinical models and/or treatment of glioblastoma [6,7]. The nanoplatforms effectively prevented aggregation of temoporfin, provided its administration, decreased dark toxicity (in the absence of light), and improved selectivity and bioavailability [8]. The main limitations of these temoporfin-based nanoparticles consisted of a rather poor drug release at the target site and low tissue penetration.

In this work, we have focused on the surface-design of PMVEMA-coated UCNPs with covalently conjugated clinically approved mTHPC as an alternative to currently used PDT photosensitizers for pancreatic cancers. The conjugates were employed to determine their cytotoxicity and photodynamic activity *in vitro* and in a pilot *in vivo* therapeutic experiment on an animal model of pancreatic adenocarcinoma.

2. MATERIAL AND METHODS

2.1. Synthesis of PMVEMA-coated $NaYF_4$:Yb^{3+}, Er^{3+}, Fe^{2+} nanoparticles (UCNP@PMVEMA)

PMVEMA-coated UCNPs were prepared according to the earlier reported procedure with some modification [9]. Briefly, 2 mmol of yttrium(III), ytterbium(III), erbium(III) and iron(II) chlorides (Sigma-Aldrich) and oleic acid (24 ml) were dissolved in octadec-1-ene (30 ml) at 170 °C for 30 min under an argon atmosphere. Afterward, the solution was cooled down to room temperature (RT) and 16 ml of methanolic solution of NaOH (5 mmol) and NH_4F (8 mmol) was added. The temperature was then slowly increased to 300 °C for 1.5 h with stirring under an argon atmosphere. The resulting UCNPs were separated by centrifugation, washed in hexane/ethanol mixture and water. The aqueous UCNP dispersion (10 mg/ml; 1 ml) was added to aqueous PMVEMA (Scientific Polymer Products) solution (M_w = 60 kDa; 50 mg/ml; 15 ml; pH 7.4). The mixture was stirred at 70 °C for 16 h. The resulting UCNP@PMVEMA particles were separated by centrifugation and washed with water to remove unbound polymer.

2.2. Conjugation of mTHPC to UCNP@PMVEMA particles

THPC (MedChemExpress) was bound to UCNP@PMVEMA particles by NaOH-catalyzed esterification of hydroxyl groups. Before the conjugation, the particle medium was exchanged from water to dimethylsulfoxid (DMSO). The aqueous particle dispersion (2 mg/ml; 500 µl) was transferred to DMSO, centrifuged and washed with DMSO. NaOH pellets (20 mg) were added to a solution of mTHPC (1 mg) in DMSO (1 ml), the mixture was stirred for 30 min. UCNP@PMVEMA dispersion in DMSO (1 mg/ml; 2 ml) was added dropwise over 10

min and stirred under an argon atmosphere in the dark at RT for 48 h. Finally, the NaOH pellets were removed and UCNP@PMVEMA-mTHPC conjugate was separated by centrifugation, washed several times with ethanol and water and redispersed there to the desired concentration.

2.3. *In vitro* cell viability and photodynamic activity

Cytotoxicity of PMVEMA-coated UCNPs with/without bound mTHPC was assessed by XTT assay (Sigma-Aldrich) on Capan-2 and PANC-01 pancreatic carcinoma cell lines (DSMZ-German Collection of Microorganisms and Cell Cultures) according to the manufacturer's protocol. Briefly, harvested cells were cultured in growth medium for 48 h under 5 % CO_2 humidified atmosphere and then incubated with the tested particles (0.001-0.3 mg/ml) for 48 h under the same atmosphere. After 48 h, 150 µl of supernatant was discarded and 25 µl of a mixture of XTT and phenazine methosulfate was added to the plates and incubated for 2 h. The absorbance of the samples was measured at 450nm with a reference wavelength of 620 nm using a Tecan Infinite® F50 plate reader (Schoeller).

The photodynamic activity *in vitro* was determined using rat insulinoma INS-1E cells. The cells were cultivated with 11 mM glucose in RPMI 1640 medium with *L*-glutamine supplemented with HEPES, pyruvate, fetal calf serum, mercaptoethanol, penicillin, and streptomycin and seeded on coverslips. Aqueous UCNP@PMVEMA-mTHPC particles (0.3 mg/ml) were incubated with the cells for 20 h, excited at 980 nm by a Coherent 170 fs pulsed Chameleon laser with power of 40 mW for 30 min, and observed using a Leica SP 8 confocal microscope (Leica Microsystems).

2.4. *In vivo* NIR-induced photodynamic therapy

Harvested Capan-2 cells (5×10^6) were administered subcutaneously cells as a mixture with BD Matrigel (VWR International) into the abdominal right flank of outbred nude mice. Mice were randomized into control and experimental groups (n = 4) when tumors were ~6 mm in diameter. Ketamine/xylasine anesthesia was used for all experimental animals. An aqueous dispersion of UCNP@PMVEMA and UCNP@PMVEMA-mTHPC particles (100 µl; 1.5 mg/ml) was applied intratumorally. The intratumorally administered PBS served as a control. After 10 min, the 2-cm^2 tumor area was irradiated for 3 min using a Quanta System IG980 excitation laser (Medicom) with a power of 1 W, a power density of 0.5 W/cm^2, and an energy density of 90 J/cm^2. The survival of the mice was monitored for 30 days, and tumor volume and mouse weight were assessed twice weekly.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of UCNP@PMVEMA particles

The conventional $NaYF_4$: Yb, Er upconversion nanoparticles have strong emission at 540 nm and negligible emission at 650 nm, which is where mTHPC absorbs light. Previously, doping the particles with various transition metal ions was investigated as an effective strategy to increase the upconversion emission intensity in the red region [10,11]. This high intensity at NIR excitation allows for deep light penetration within an optimal biological spectral window and thus direct excitation of mTHPC as a PDT transducer.

In this report, the uniformly sized $NaYF_4$:Yb^{3+} (20 mol. %), $Er3^+$ (15 mol. %), Fe (5 mol. %) UCNPs were prepared by high-temperature coprecipitation of lanthanide and iron chlorides in octadec-1-ene as a solvent in the presence of oleic acid as a stabilizer. According to TEM, the UCNPs were spherical in shape and monodisperse in size with D_n = 30 nm and $Đ$ = 1.01 (**Figure 1A**). The polydispersity of the particles measured by DLS in water was small (*PD* = 0.11) and the hydrodynamic diameter was larger than D_n, with D_h of 191 nm indicating their slight tendency to form aggregates. The positively charged metal ions on the surface of UCNPs resulted in a positive ζ-potential of 43 mV. Since the particles were hydrophobic due to the presence of OA stabilizer on the surface, their modification with PMVEMA is necessary to ensure colloidal stability in water,

functionality and conjugation with mTHPC. The successful modification of the UCNPs with PMVEMA was documented by DLS, TGA and FTIR spectroscopy. According to TGA, the amount of PMVEMA on the particle surface was 5.7 wt.%. The hydrodynamic diameter of UCNP@PMVEMA particles in water increased to 237 nm (*PD* = 0.21) and their ζ-potential became negative (-29 mV).

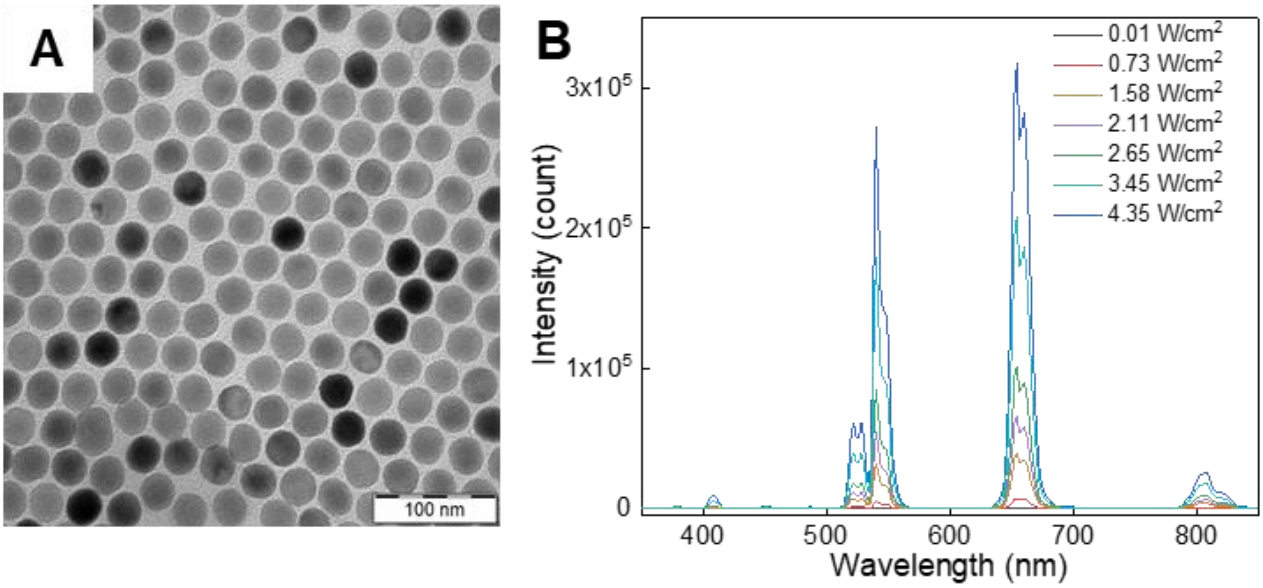

Figure 1 (A) TEM image of UCNPs. (B) Upconversion photoluminescence emission spectra of UCNP@PMVEMA particles in water (1 mg/ml) excited at 980 nm with different power densities.

The upconversion luminescence emission of UCNP@PMVEMA particles was measured under NIR excitation at 980 nm with a laser of different power density (**Figure 1B**). By incorporating Fe^{2+} ions (5 mol.%) into particles, upconversion emission became dominant in red region, making it suitable for direct excitation of mTHPC. The particles exhibited characteristic Er^{3+} emissions typical of upconverting nanomaterials. The PMVEMA coating of UCNPs did not affect the emission intensity. Both red and green emission intensities increased with increasing laser power and red/green ratio reached maximum at lower excitation intensities.

3.2. Conjugation of mTHPC to UCNP@PMVEMA particles

mTHPC was attached to UCNP@PMVEMA by carboxyl groups of PMVEMA, which partially coordinated with the surface rare-earth ions of UCNP and partially were available for conjugation with the hydroxyl groups of THPC via NaOH-catalyzed esterification in DMSO (**Figure 2**). When UCNP@PMVEMA particles were conjugated with THPC, their hydrodynamic diameter in water increased slightly from 237 to 273 nm and the polydispersity was moderate (*PD* = 0.33). The ξ-potential of UCNP@PMVEMA-THPC-2 conjugates in water decreased to -33 mV. In addition, THPC-conjugated UCNP@PMVEMA were stable in saline solution (0.9 % NaCl); their D_h decreased to 150±2 nm with *PD* <0.2. The amount of mTHPC on the shell of UCNPs was found to be 1 μg/mg of particles. The presence of mTHPC on the UCNP@PMVEMA particles was also confirmed by photoluminescence spectra. UCNP@PMVEMA-mTHPC conjugates demonstrated luminescence at 652 nm and corresponding excitation at 420 nm, which is typical for free mTHPC. Singlet oxygen generation by THPC-conjugated UCNPs was detected spectrophotometrically via the bleaching of 1,3-diphenylisobenzofuran (DPBF) as a function of irradiation time at 980 nm excitation. NIR light exposure of DPBF ethanol/water solution containing the mTHPC-conjugated particles over a period of 120 min demonstrated the photobleaching of DPBF. The decrease in DPBF absorbance at 415 nm with increasing irradiation time indicated increasing singlet oxygen production and the efficient energy transfer from the excited particles to the photosensitizer.

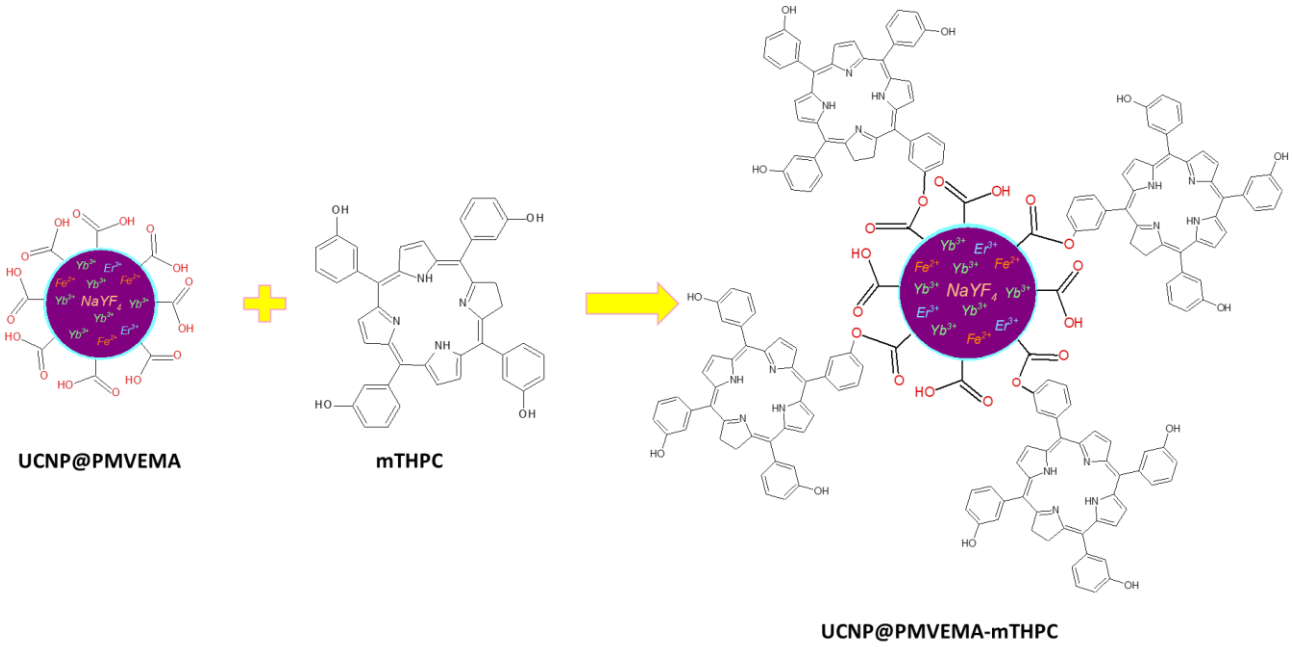

Figure 2 Schematic illustration of general strategies used for mTHPC conjugation to UCNP@PMVEMA

3.3. *In vitro* and *in vivo* photodynamic efficiency

To evaluate the potential of UCNP@PMVEMA-mTHPC nanoparticles for PDT, it was essential to examine their toxicity. The cell viability of Capan-2 and PANC-01 pancreatic carcinoma cells incubated with the particles in the concentration range of 0-0.3 mg/ml demonstrated their safety without 980 nm irradiation. The photodynamic effect of mTHPC-conjugated particles on INS-1E rat insulinoma cells as a pancreatic β-cell tumor model was examined under NIR excitation at 980 nm. After 30 min of laser exposure, UCNP@PMVEMA-THPC conjugates were able to convert oxygen into $^1O_2$ in the cells, leading to their destruction and demonstrating PDT activity. The cells without conjugates were not affected by laser irradiation.

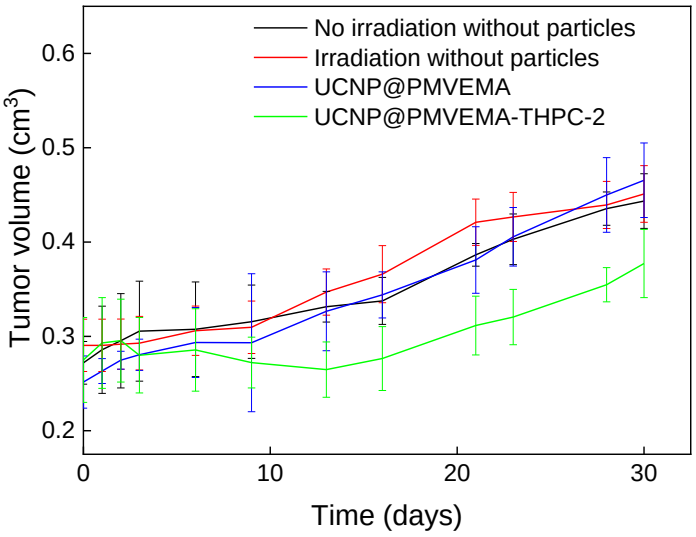

Figure 3 Time dependence of tumor volume in the nu/nu mice with growing Capan-2 human pancreatic adenocarcinoma after 980-nm NIR-induced PDT. Mice were intratumorally (i.t.) injected with 100 µl of PBS (two control groups) or UCNP@PMVEMA and UCNP@PMVEMA-mTHPC particles and irradiated 10 min after administration. Controls: untreated mice with i.t. injected PBS without and with irradiation. Statistical significance between UCNP@PMVEMA-mTHPC and other three groups * p <0.05.

The therapeutic efficiency of the mTHPC-conjugated UCNP@PMVEMA nanoparticles was tested in a pilot *in vivo* experiment lasting for 30 days on outbred athymic nude mice with subcutaneously growing Capan-2 pancreatic tumors. The mice were exposed to 980 nm radiation (0.5 W/cm^2) for 3 min only once, 10 min after intratumor application of the particles. There was no further irradiation during the entire 30-day follow-up period. All four mice in the UCNP@PMVEMA-THPC-2 particle-treated group had extensive necrosis on the first day after irradiation. No necrosis was observed in the control (without nanoparticles) and UCNP@PMVEMA-containing groups. No significant difference in body weight was observed between the two groups, which suggests that there were no side effects during the treatment period. Over a period of thirty days, tumors were measured twice weekly with calipers, and significant suppression of tumor growth rate was observed in the UCNP@PMVEMA-mTHPC group (**Figure 3**). Tumor volume at the end of the pilot study was ~0.35 cm^3. Thus, the potential of THPC-conjugated PMVEMA-coated UCNPs in the treatment of pancreatic adenocarcinoma was confirmed.

4. CONCLUSION

In this study, monodisperse upconversion $NaYF_4$:Yb^{3+},Er^{3+},Fe^{2+} nanoparticles were designed, coated with a PMVEMA, and finally conjugated with mTHPC, clinically approved photosensitizers, with the aim to treat pancreatic cancer cells by NIR-induced PDT. The incorporation of Fe^{2+} ions (5 mol.%) into the particles increased fluorescence intensity in the red region. Covalent binding of mTHPC to the surface of UCNP@PMVEMA particles led to the ability to generate ROS demonstrated by DPBF assay after NIR excitation. *In vitro* study on pancreatic cancer cells incubated with the particles demonstrated significant cell destruction after excitation with 980 nm laser. This proved efficient energy transfer from the particles to mTHPC molecules. The pilot *in vivo* PDT study showed promising therapeutic efficiency against human pancreatic adenocarcinoma in mice.

ACKNOWLEDGEMENTS

This research has been financially supported by the project National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) - Funded by the European Union – Next Generation EU, and research grant from The League Against Cancer Prague (recipient - David Větvička).

REFERENCES

[1] GUNAYDIN, G.; GEDIK, M.E.; AYAN, S. Photodynamic Therapy for the Treatment and Diagnosis of Cancer - A Review of the Current Clinical Status. *Front. Chem.* 2021, vol. 9, pp. 686303.

[2] YANOVSKY, R.L.; BARTENSTEIN, D.W.; ROGERS, G.S.; ISAKOFF, S.J.; CHEN, S.T. Photodynamic Therapy for Solid Tumors: A Review of the Literature. *Photodermatol. Photoimmunol. Photomed.* 2019, vol. 35, pp. 295-303.

[3] VAN STRATEN, D.; MASHAYEKHI, V.; DE BRUIJN, H.; OLIVEIRA, S.; ROBINSON, D. Oncologic Photodynamic Therapy: Basic Principles, Current Clinical Status and Future Directions. *Cancers.* 2017, vol. 9, pp. 19.

[4] SENGE, M.O.; BRANDT, J.C. Temoporfin (Foscan®, 5, 10, 15, 20-tetra(m-hydroxyphenyl)chlorin) - A Second-Generation Photosensitizer. *Photochem. Photobiol.* 2011, vol. 87, pp. 1240-1296.

[5] JIANG, Y., HUANG, J., ZHEN, X., ZENG, Z., LI, J., XIE, C., MIAO, Q., CHEN, J., CHEN, P., PU, K. A Generic Approach Towards Afterglow Luminescent Nanoparticles for Ultrasensitive *In Vivo* Imaging. *Nat. Commun.* 2019, vol. 10, pp. 2064.

[6] HAMBLIN, M.R. Upconversion in Photodynamic Therapy: Plumbing the Depths. *Dalton Trans.* 2018, vol. 47, pp. 8571.

[7] TSAI, Y.C.; VIJAYARAGHAVAN, P.; CHIANG, W.H.; CHEN, H.H.; LIU, T.I.; SHEN, M.Y.; OMOTO, A.; KAMIMURA, M.; SOGA, K.; CHIU, H.C. Targeted Delivery of Functionalized Upconversion Nanoparticles for Externally Triggered Photothermal/Photodynamic Therapies of Brain Glioblastoma. *Theranostics.* 2018, vol. 8, pp. 1435-1448.

[8] YAKAVETS, I.; MILLARD, M.; ZORIN, V.; LASSALLE, H.-P.; BEZDETNAYA, L. Current State of the Nanoscale Delivery Systems for Temoporfin-based Photodynamic Therapy: Advanced Delivery Strategies. *J. Control. Release.* 2019, vol. 304, pp. 268-287.

[9] SHAPOVAL, O.; BRANDMEIER, J.C.; NAHORNIAK, M.; OLEKSA, V.; MAKHNEVA, E.; GORRIS, H.H.; FARKA Z.; HORÁK D. PMVEMA-Coated Upconverting Nanoparticles for Upconversion-linked Immunoassay of Cardiac Troponin. Talanta, 2022, vol. 244, 123400.

[10] LUO, X.; CHEN, Q.; GUO, H.; ZHANG, H.; HE, X.; ZHAO, W. One-step Hydrothermal Synthesis of Cit-$NaYbF_4$:Er^{3+} Nanocrystals with Enhanced Red Upconversion Emission for *In Vivo* Fluorescence Molecular Tomography. *J. Rare Earths.* 2022. Available from: https://doi.org/10.1016/j.jre.2022.09.027.

[11] KAMIMURA, M.; OMOTO, A.; CHIU, H.S.; SOGA, K. Enhanced Red Upconversion Emission of $NaYF_4$:Yb^{3+}, Er^{3+}, Mn^{2+} Nanoparticles for Near-infrared-induced Photodynamic Therapy and Fluorescence Imaging. *Chem. Lett.* 2017, vol. 46, pp. 1076-1078.