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Intracellular uptake and fluorescence imaging potential in tumor cell of zinc phthalocyanine

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ABSTRACT

A near IR absorbing phthalocyanine bearing four binaphtyl group has been synthesized in order to investigate its cytotoxicity and intracellular uptake of sensitizer on MCF-7 (human breast cancer), MDAH (ovarian cancer), HeLa (human epitheloid cervix carcinoma), EMT-6 (mouse breast cancer) and WI-38 (human fibroblast lung) cell lines. ZnPc showed four time higher intracellular uptake in carcinoma cells (MCF-7) than normal (WI-38) cell lines. With the aim of studying in detail the biodistribution feature and tumor nuclear imaging capacity, ZnPc was also labeled with I-131. The efficiency of radiolabeled compound was $95 \pm 4.6\%$. In addition, ZnPc reveals to be very efficient singlet oxygen generators ($\Phi\Delta = 0.612$ in DMSO) and promising PS for PDT application. *In vitro* fluorescence imaging study with MCF-7 cells showed that ZnPc localized in cytoplasm of the cells. This results showed that synthesized ZnPc is promising candidate for dual fluorescence/nuclear imaging breast cancer and shows potential PS for PDT application.

1. Introduction

Photodynamic therapy (PDT) has attracted great attention as a medical treatment, in which light energy absorbed by chromophores is transferred to oxygen molecules in the cell to produce toxic singlet oxygen, which causes cell death (Dolmans et al., 2003). PDT has a number of considerable advantages in terms of tumor selectivity, low toxicity and good repeatability over the conventional anti-cancer treatments such as radiation, surgery and chemotherapy that exhibit deleterious side effects (Wilson and Patterson, 2008). In the process of PDT, a non-toxic photosensitizer (PS), which locates in the tumour tissue, absorbs a specific wavelength of light, and then leads the formation of cytotoxic reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) through energy transfer processes to the surrounding oxygen, which induces damage and death of cancer cells (Juarranz et al., 2008). From PDT treatment point of view the photosensitizer plays a crucial role in improving the efficacy of PDT. Ideal photosensor should have high absorption coefficient in the near–infrared (NIR) region in order to penetrate deeper into tissues, thus causes less damage than shorter wavelength light. Moreover, photosensitizers should be selectively accumulated in the target tissue (Nyman and Hynninen, 2004). Phthalocyanines (Pcs), thus, fulfil many essential requirements of efficient sensitizers for PDT (Bonnett, 1995). Up to now, several Pc derivatives have been extensively studied as notable second-generation photosensitizers in photodynamic therapy applications due to their suitable physical and chemical properties. They exhibit intense absorption in the red visible region with high extinction coefficients ($\varepsilon > 10^5\,M^{-M}\,cm^{-c}$) which is required to penetrate deeply into tissues (Allen et al., 2001).

Zinc phthalocyanines present intense fluorescence in the near infrared region which render them an excellent photosensitizer for NIR fluorescence imaging method that has been used the diagnosis of cancer (Moan et al., 1998; Witjes et al., 1996; Nesterova et al., 2009). It is known that radiolabeled phthalocyanines can be used

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as nuclear imaging agent (Ranyuk et al., 2013a,b; Ma et al., 2003; Scasnar and Vanlier, 1993). Lipophilic photosensitizers are preferentially transported by lipoproteins, which are uptaken directly by tumor cells (Jori, 1989).

In this context, ZnPc bearing four binaphtyl group has been synthesized in order to investigate its cytotoxicity and intracellular uptake of sensitizer on MCF-7 (human breast cancer), MDAH (ovarian cancer), HeLa (human epitheloid cervix carcinoma), EMT-6 (mouse breast cancer) and WI-38 (human fibroblast lung) cell lines. ZnPc was also labeled with I-131. The singlet oxygen generation potential was tested. The ZnPc was labeled with ^{131}I using iodogen method in order to investigate intracellular uptake in different cells for as a tumor fluorescence/nuclear imaging agent potential.

2. Materials and methods

2.1. Materials

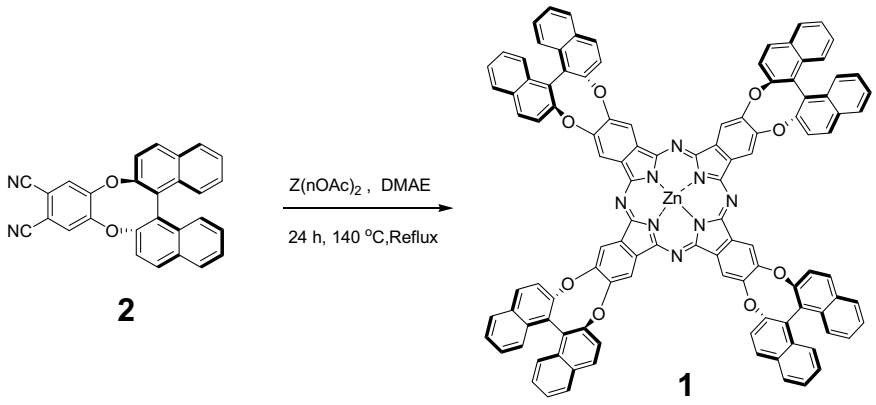
Thin-layer chromatography-cellulose gel (ITLC-F plastic sheets 20 × 20) was supplied from Merck. Iodogen was purchased from Sigma-Aldrich. Radiolabeling experiments were analyzed using a Bioscan AR2000 TLC Scanner. All chemicals used for the *in vitro* studies were supplied from Biological Industries; all other chemicals were provided from Merck. Cell culture studies were performed in a Thermo MSC Advantage 1.2 laminar air flow cabinet. An Olympus Japan inverted light microscope was used for counting cells. A Thermo Multimode microplate reader was used to determine the IC_{50} values of cell cultures. All chemicals and 1,3-diphenylisobenzofuran (DPBF) were purchased from Aldrich Chemical Co. and used without further purification. The monitoring of the reactions has been carried out by thin layer chromatography (TLC), was carried out on aluminum sheets coated with silica gel type 60 F254 (E. Merck). Purification and separation of the synthesized products were performed by column chromatography, using silica gel Merck-60 (230–400 mesh, 60 Å). Infrared spectra (IR) were performed with Perkin-Elmer, FT-IR/MIR-FIR (ATR, Attenuated total reflectance) spectrophotometer. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS spectra were determined on a BRUKER Microflex LT. Nuclear magnetic resonance spectra (NMR) (^{1}H NMR) spectra were recorded with a Bruker AC-400 instrument. UV/Vis spectra were recorded with an Analytic JENA S 600 UV–vis spectrophotometer. 4,5-Dichlorophthalonitrile (Wöhrle et al., 1993) and (*R*)/(*S*)-benzo[b]dinaphtho[2,1-e:1′,2′-g][1,4]dioxocine-5,6-dicarbonitrile (Wang et al., 2012) were prepared according to literature procedures. (*R*)- and (*S*)-2,2′-dihydroxy-1,1′-binaphthyl was commercially available. The ZnPc was prepared following a modification of the synthesis of such compounds by Wang et al., (2012).

2.2. Synthesis of (R)/(S)-tetrakis(dinaphtho)[1,2-e:1′,2′-g]-1,4-(dioxocine)[2,3-b;2′,3′-k;2″,3″-t;2‴,3‴-c′] phthalocyaninato zinc complex

A mixture of (*R*)/(*S*)-benzo[b]dinaphtho[2,1-e:1′,2′-g][1,4]dioxocine-5,6-dicarbonitrile (100 mg, 0.243 mmol) and $(ZnOAc)_2$ (12 mg, 0.061 mmol) in DMAE (3 mL) was stirred at 140 °C under argon atmosphere for 24 h. After cooling to room temperature, the solvent was removed and the residue was washed with a MeOH/H_2O (5:1) mixture. The crude product was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 10:0.1) to give **ZnPc** (70 mg, 0.04 mmol) as a green solid. Yield: 17%.^{1}HNMR (d_8-DMSO, 400 MHz): δ (ppm) = 9.49 (s, 8H), 8.23 (d, J = 8, 8H), 8.14 (d, J = 8, 8H), 8.1–8.0 (m, 8H), 7.62–7.51 (m, 24H). IR (ATR): ν (cm^{-1}) = 2920, 2850, 1588, 1442, 1395, 1262, 1215, 1088, 950, 830, 740. UV/Vis (DMSO): λ_{max} (log ε) = 680 (5.3), 613 (4.6), 359 (4.9). MS (MALDI-TOF, ATRA): m/z calcd for $C_{112}H_{56}N_8O_8Zn$: 1704.35; found: 1704.3.

2.3. Singlet oxygen measurements

The singlet oxygen quantum yield (ΦΔ) is a measurement of the conversion of molecular oxygen into reactive oxygen when a photosensitizer irradiated by the light. In this study, the singlet oxygen quantum yield (ΦΔ) was determined by using 1,3-diphenylbenzofuran (DPBF) as a quencher, according to the literature procedure (Ince et al., 2016). Non-substituted **ZnPc** in DMSO was used as the reference (ΦΔ (DMSO) = 0.67). A halogen lamp (300 W, Optel) was used as a light source. To filter of light under 515 nm and far infrared radiations, a glass (Jenger Glaswerk Schott & Gen., Mainz OG 515) and water filters were used, respectively. DPBF was dissolved in DMSO (2 mL, with an absorbance of ca. 1) in a 1 × 1 cm quartz optical cell and bubbled with oxygen for 60 s. The ZnPc solution in DMSO (with an absorbance of ca. 0,1) was then added to solution of DPBF, to give an absorbance of ca. 0.1. The working solution was placed at 60 cm from the light source and irradiated for defined time intervals by using a halogen lamp (300 W). The experiment was repeated three times. The decrease of DPBF concentration with irradiation time was monitored by UV/vis absorption spectroscopy at 418 nm due to formation of singlet oxygen (Scheme 1).



Scheme 1. Synthetic route to ZnPc.

2.4. Radiolabeling and radiochemical purity analysis

The stock solution of the compound was prepared by dissolving 1 mg of ZnPc in 600 μL of DMSO and 600 μL of bidistileted water. Then 100 μL ZnPc stock solution was diluted with 900 μL bidistilated water and the solution was put into 1 mg iodegen coated tube. Then 11,1MBq ^{131}I was added into the tube and incubated 30 min. After the incubation ^{131}I-ZnPc compound was dropped to cellulose-coated plastic (ITLC-cellulose) sheets (1 × 10 cm; Merck) and to determine the amount of radiolabeling yield three different mobile phases [mobile phase 1: *n*-butanol-water-acetic acid (4-2-1) mobile phase 2: chloroform-ethanol (1-1) and mobile phase 3: chloroform-acetic acid (9-1)] was used. Then the cellulose sheets were scanned on a TLC-scanner (BioScan AR-2000 Washington DC, USA).

2.5. The stability of ^{131}I-Zn(II)Pc

In vitro stability of radiolabeled ZnPc was checked by taking samples at 30 min, 1, 2, 4, and 24 h using TLRC method.

2.6. Lipophilicity test of ^{131}I-Zn(II)Pc

To determine of *n*-octanol/water ratio, radiolabeled ZnPcs; radiolabeled Pc (150 μL) was added into 3 mL *n*-octanol and 3 mL water solution in a tube. The solution was stirred by a magnetic mixer for 1 h and centrifuged (2500 rpm, 5 min.) Then 500 μL sample from each phases were counted using a Cd(Te)-RAD-501 single channel analyzer. The octanol-to-water ratio (Po/Pw) of the radiolabeled Pc was calculated.

2.7. Cell culture

MCF-7 (human breast cancer), MDAH (ovarian cancer), HeLa (human epitheloid cervix carcinoma) EMT-6 (mouse breast cancer) and WI-38 (human fibroblast lung) cell lines were cultured in 75 cm^2 flasks with high glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C in which the CO_2 level was kept constant at 5%.

2.8. Cell viability assay

IC_{50} value of ZnPc was determined by using MTT method in EMT-6, HeLa, MCF-7 MDAH and WI-38 cell lines. Working group consisting of these cells was formed in a 96-well culture plate (1×10^5 cells in each well) and after 1 day incubation, 10–90 μM concentration compound which was prepared with MEM solution (without FBS) and added in each well. After 24 h, the medium on the cells was taken and MTT solution was added in each well. After 4 h incubation, DMSO solution was accrued for solving MTT and absorbance of wells were measured in a micro plate reader (Varioskan Flash Multimode Reader-Thermo, Finland) at 560 nm. Then the percentage of cytotoxicity was calculated.

2.9. Intracellular uptakes of ^{131}I-ZnPc

The *in vitro* cellular uptake of ^{131}I labeled ZnPc was performed by using the EMT-6, HeLa, MCF-7, MDAH cancer cell lines and WI-38 cell line as control group. The cells were placed in 24-well culture plates (1×10^5 cells in each well) and they were incubated throughout two days at 37 °C incubator. Two days later, the medium on the cells was removed and the wells were washed two times with 0.9% NaCl solution and the radiolabeled ZnPc (20 μM, activity: 0.5 MBq) which was diluted with MEM (without FBS) was added on the cells. The intracellular uptake of Na^{131}I as the control group was assayed the given conditions. At this stage it was checked whether the uptake was caused by free iodine or radioiodinated compound. After determined time period incubation (1, 2, 4, 6 and 24 h), the wells were counted by Cd (Te) RAD 501 signal channel analyzer. Then, the radioactive medium on the cells was removed. The cells were washed once and 0.9% NaCl solution was added in each well. After the wells was been counted again, the data were analyzed and the percentage of intracellular uptake was calculated.

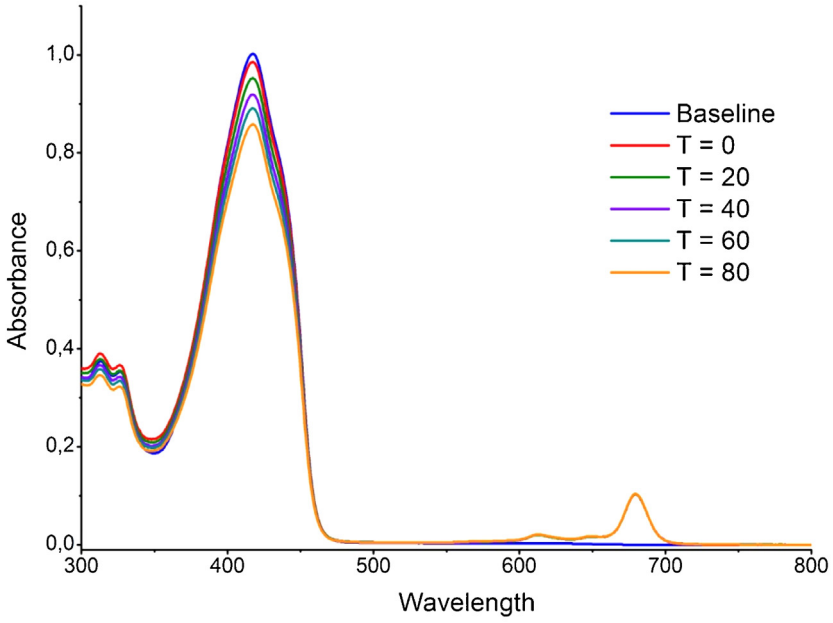


Fig. 1. Time-dependent photobleaching of DPBF absorption in the presence of ZnPc in DMSO.

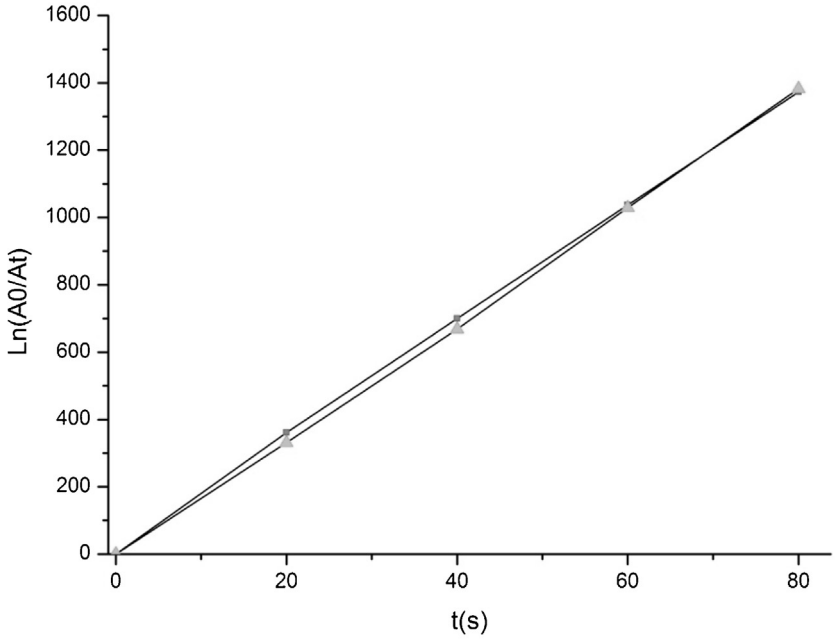


Fig. 2. Plots of the decrease of DBPF absorption induced by reference ZnPc (▲) and compound ZnPc (■).

2.10. Cells imaging

Fluorescence imaging study was performed to investigate the interactions of ZnPc with MCF-7 cells. The cells were fixed on a slide 2 days before experiment. The medium was removed and ZnPc solution was added on cells for 2 h at 37 °C incubator. The cells were washed two times with PBS and DAPI solution added for staining the cell nucleus. The cells were washed again with PBS after 15 min. Photographs were taken under green and DAPI filter with Fluorescence microscope (Olympus BX53F) equipped with a CCD camera (Olympus DP72).

3. Result and discussion

3.1. Singlet oxygen result

The singlet oxygen quantum yield measurement of ZnPc was carried out using chemical trapping method which is described in detail elsewhere (Makhseed et al., 2012; Kuznetsova et al., 2000). Singlet oxygen generation capacities of ZnPc were studied in dimethylsulfoxide (DMSO) and using 1,3-diphenylisobenzofuran (DPBF) as the scavenger. The formation of singlet oxygen was monitored by UV/vis absorption spectroscopy (Fig. 1) which shows

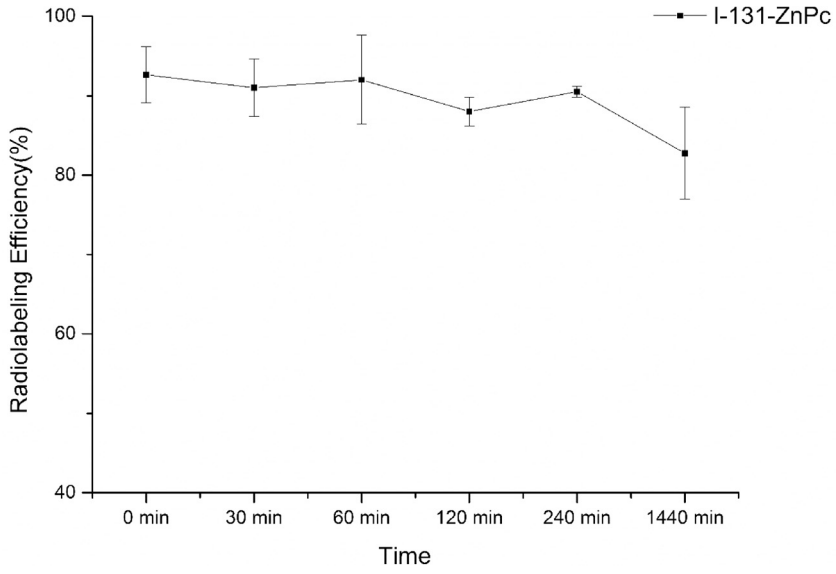


Fig. 3. *In vitro* stability of ^{131}I labeled ZnPc at different times after labeling.

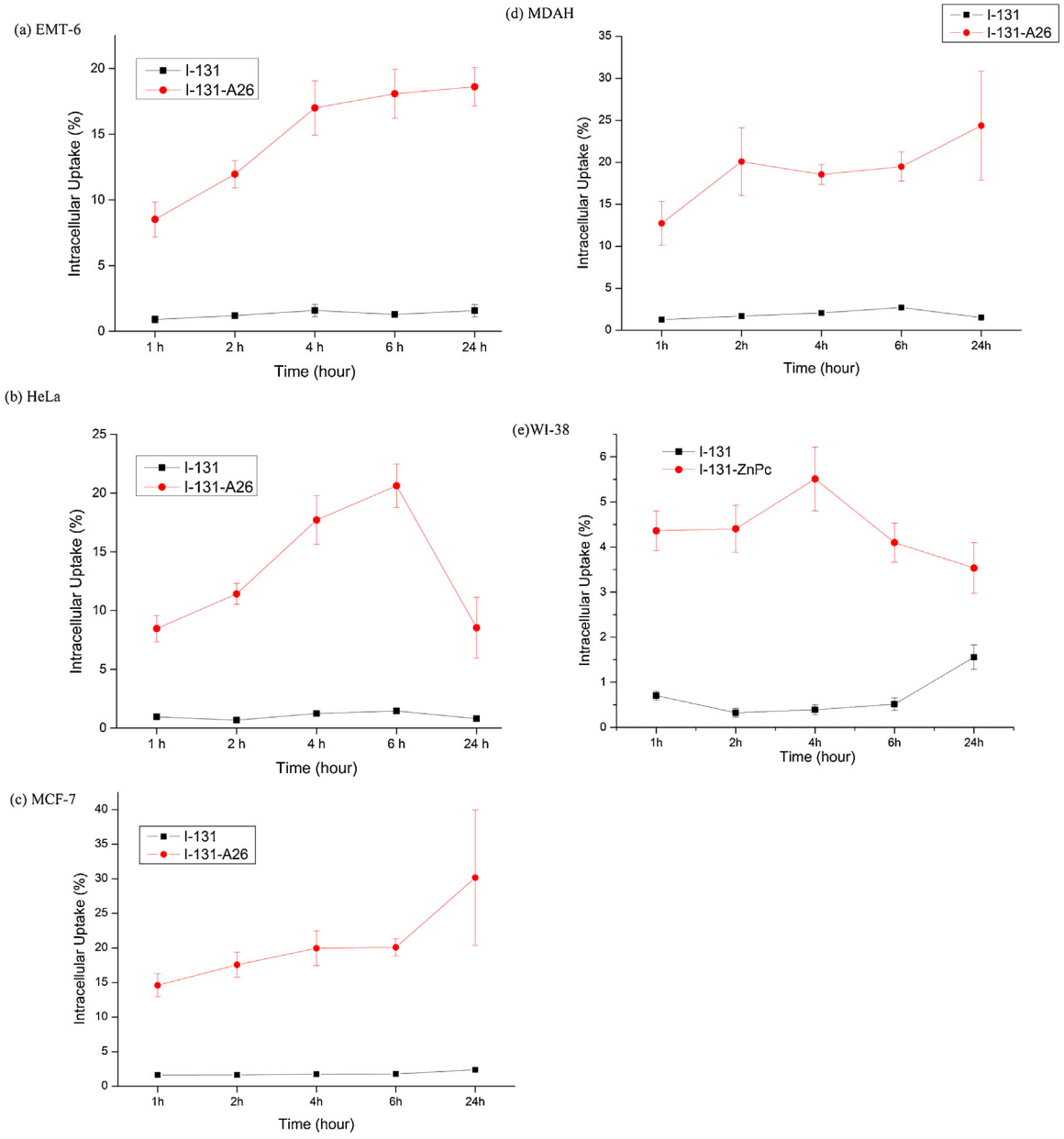


Fig. 4. Uptake Intracellular Uptake (%) of ^{131}I and ^{131}I-ZnPc in cells; (a: EMT-6, b: HeLa, c: MCF-7, d: MDAH, e: WI-38).

the concentration decay of DBPF at 418 nm in the presence of ZnPc photosensitizer. The singlet oxygen generation mechanism does not cause any remarkable change in the absorption intensity of ZnPc which exhibits the stability of ZnPc during this experiment. Fig. 2 shows that the plots of the decrease of DBPF absorption ln (A_0/A_t) over time irradion (t) induced by reference non-substituted ZnPc and ZnPc photosensitizer. ZnPc reveals to be efficient singlet oxygen generators ($\Phi\Delta$= 0.612) in DMSO.

3.2. Radiolabeling results

The labeling efficiency of ZnPc was examined by using TLRC method. The radiolabeling conditions of ZnPc are 1 mg iodogen, pH = 6, room temperature and 30 min reaction time. The efficiency of ^{131}I-ZnPc was determined as 95 ± 4.6%. TLRC analysis showed that the relative front (R_f) values of Na^{131}I and ^{131}I-ZnPc were 0.04, and 0.8, respectively when mobile phase 1 [*n*-butanol-water-acetic acid (4-2-1)] was used. When mobile phase 2 [chloroform-ethanol (1-1)] was used the R_f values were 0.04 and 0.94 respectively. For mobile phase 3 [chloroform-acetic acid (9-1)], the R_f values were 0.05 and 0.95 respectively.

3.3. The stability of ^{131}I-ZnPc

The shelf-life of radiolabeled ZnPc was obtained by *in vitro* stability study (Fig. 3). The ^{131}I-ZnPc's radiolabeling efficiency was determined as 91 ± 3.6% at 30 min and the radiolabeled yield shown almost same at 240 min (90.5 ± 0.7%). However, ^{131}I-ZnPc was quite stable and labeling efficiency of 82.7 ± 5.7% was maintained a day after.

3.4. Lipophilicity test of ^{131}I-ZnPc

The lipophilicity of ^{131}I-ZnPc was calculated as 2.36 according to the result of lipophilicity experiment. It is indicated that the radiolabeled ZnPc has lipophilic property.

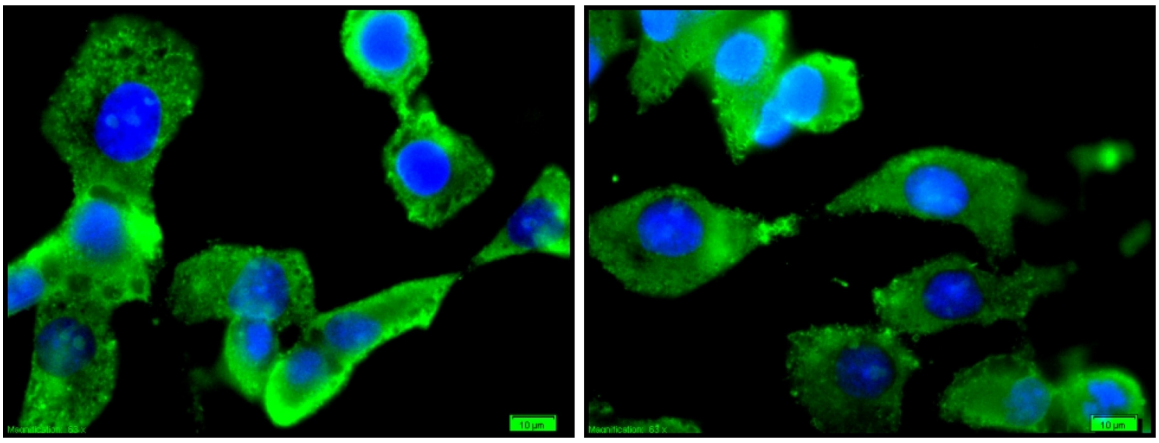


Fig. 5. Fluorescence microscope images of ZnPc in MCF-7 cells.

3.5. Cytotoxicity of ZnPc

MTT cytotoxicity test results showed that the IC_{50} value of ZnPc was tested in the range of 10 μM- 90 μM. There is no toxic effect was observed in MCF-7, MDAH and WI-38 cells (>90 μM). However the IC_{50} values were observed as to be 50 μM in EMT-6 cells and 35 μM in HeLa cells.

3.6. Intracellular uptake of ^{131}I-ZnPcs

The intracellular uptakes of ^{131}I-ZnPcs in EMT-6, HeLa, MCF-7, MDAH and WI-38 cell lines were evaluated. It was observed that the uptake of free ^{131}I was low when compared with the uptake of radiolabeled ZnPc in all cell lines. The highest uptake of labeled compound was determined in MCF-7 cells (30.2 ± 9.8%) at 24 h. Additionally the labeled compound showed the highest uptakes which are 24.4 ± 6.5% and 18.6 ± 1.5% in MDAH and EMT-6 cells respectively at 24 h. However, maximum uptake was determined in HeLa cells at 6 h (20 ± 1.8%) and the uptake was decreased at 24 h (8.5 ± 2.6%). The uptake of ^{131}I labeled ZnPc in WI-38 reached maximum at 4 h (5.5 ± 0.7%). Radiolabeled ZnPc uptake in WI-38 decreased with time (4.1 ± 0.4% at 6 h and 3.5 ± 0.5% at 24 h) (Fig. 4).

When the maximum uptakes between cancer and normal cells were compared, the uptakes in cancer cells were higher about four times than normal cell. As known the uptake of lipophilic photosensitizers by tumour cells is higher than that of normal cells (Allen et al., 2001). Tumor-to nontumor (T/NT) ratio of 2 is adequate for imaging of tumor (Deland and Shih, 1984). Therefore radiolabeled Pc has enough high uptake in MCF-7 cells for nuclear imaging. Due to maximum uptake of radiolabeled Pc in MCF-7 cells, the radiolabeled Pc may be useful in breast cancer nuclear imaging. In another study, new synthesized water-soluble sulfonated zinc phthalocyanine radiolabeled with ^{68}Ga and ^{64}Cu was investigated and the compound showed promising results for dual fluorescence/PET imaging (Ranyuk et al., 2013a,b).

3.7. Cells imaging

The fluorescence imaging experiment was carried out to determine the relations between MCF-7 cells and ZnPc in Fig. 5. For this reason ZnPc compound was added on cells and fluorescence microscope images were taken under green and DAPI filter. The images show that ZnPc have significant uptake in MCF-7 cells.

The lipoproteins play a role on transport of hydrophobic ZnPc structures. In this case lipophilic ZnPc directly is uptaken in tumor cells and localized in subcellular membrane structures (Berard et al., 2006; Fabris et al., 2001). In our cell imaging study, it is observed ZnPc localized in MCF-7 cell cytoplasm. In literature the fluorescence imaging studies show that ZnPcs are localized in Golgi apparatus at shorter incubation periods. In the longer incubation periods, ZnPc presents in Golgi apparatus, at the same time it could be observed in mitochondrial localization also (Fabris et al., 2001).

Ramos et al. synthesized new phthalocyanines that were modified with optically active alcohols. And *in vitro* fluorescence imaging potential of the compound was determined in MCF-7 cells. The results obtained by fluorescence microscopy and cell viability have shown that these phthalocyanines interact with MCF-7 cells (Ramos et al., 2015). In another study Muehlmann et al. investigated the *in vitro* activity of Al-Pc in MCF-7 cells. They observed that the Pc localized in the cytoplasm of MCF-7 cells (Muehlmann et al., 2015). Our result is in agreement with above the studies. As seen in the studies, Pcs and its derivatives could be used for fluorescence imaging.

4. Conclusions

ZnPc was synthesized by cyclotetramerization reaction of corresponding phthalonitrile in the presence of $Zn(OAc)_2$ in DMAE in good yield. ZnPc was labeled with ^{131}I with high yield. The radiolabeled Pc exhibits higher intracellular uptake in MCF-7 cells then other cells. In addition, ZnPc reveals to be very efficient singlet oxygen generators ($\Phi\Delta$= 0.612 in DMSO). *In vitro* fluorescence imaging study with MCF-7 cells showed that ZnPc localized in cytoplasm of the cells. This results showed that synthesized ZnPc is promising candidate for dual fluorescence/nuclear imaging breast cancer and shows potential PSs for PDT application. Although the results are still preliminary, further detailed *in vivo* investigations are also necessary in breast tumour implanted animals to clarify the potential of ZnPc for fluorescence/nuclear imaging agent. The research directed on these lines are undergoing in our group.

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