Use of Upconverting Nanoparticles in Photodynamic Therapy

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\section*{ABSTRACT}

Photodynamic therapy (PDT) is an emerging and promising modality for the treatment of cancer. PDT is based on the concept that photosensitizers can be preferentially localized in tumour tissues upon systemic administration. Irradiation with an appropriate wavelength of light excites these molecules to transfer their energy to molecular oxygen in their surroundings, resulting in the formation of free radicals and the irreversible destruction of the treated tissues. PDT is currently clinically limited to treating topical lesions as approved photosensitizers absorb light in the spectral region where depth of penetration into the skin is small. To overcome this problem, the possible use of near infra-red (NIR) light which is in the tissue transparent window is being explored here. Photosensitizer–upconverting nanoparticle conjugates with the ability to play the role of ‘nanotransducers’ have been synthesized, whereby the encapsulated upconverting donor ‘harnesses’ energy from the NIR laser irradiated and emits visible light that activates the attached photosensitizer, the acceptor. The photodynamic production of singlet oxygen was detected by using a molecular probe while a colorimetric metabolic activity assay demonstrated induced cell mortality upon NIR laser irradiation of these photosensitizer-upconverting nanoparticle conjugates incubated with MCF-7 human breast cancer cells.

\section*{INTRODUCTION}

Photodynamic therapy is an emerging and promising modality for the treatment of a variety of oncological, cardiovascular, dermatological and ophthalmic diseases (Roy, Ohulchanskyy et al. 2003); one of its main therapeutic applications is in cancer therapy. Currently, PDT is used against bladder, esophageal, gastric, brain, breast, skin, colorectal, oral, head and neck cancers, in addition to gynaecological and thoracic malignancies (Chen and Zhang 2006).

PDT is based on the concept that light-sensitive species or photosensitizers can be preferentially localized in tumour tissues upon systemic administration (Roy, Ohulchanskyy et al. 2003). Irradiation of such photosensitizers with an appropriate wavelength of visible or near-infrared light causes the excited molecules to transfer their energy to molecular oxygen in the surroundings, which is normally in its triplet ground state (Roy, Ohulchanskyy et al. 2003). This results in the formation of cytotoxic reactive oxygen species, such as singlet oxygen or free radicals, which are

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responsible for oxidizing various cellular compartments including plasma, mitochondria, lysosomal and nuclear membranes, etc., causing irreversible destruction of the treated tissues (Konan, Gurny et al. 2002; Roy, Ohulchansky et al. 2003). Besides tumour cells, the targets of PDT also include the microvasculature of the tumour bed as well as normal vasculature, and the inflammatory and immune host system (Dougherty, Gomer et al. 1998).

Compared to current treatments such as surgery, radiation therapy and chemotherapy, PDT offers the advantages of an effective and selective method (since photosensitizers are inactive without light activation, PDT can ideally be considered to be selective to the illuminated area (Vargas, Pegaz et al. 2004)) of destroying diseased tissues without damaging surrounding healthy tissues, a type of treatment where repeated doses can be given without the total-dose limitations associated with radiotherapy and where the healing process results in little or no scarring (Konan, Gurny et al. 2002; Brown, Brown et al. 2004) In addition, in contrast to most other cancer therapies, PDT can induce immunity, even against less immunogenic tumours, and thus contribute to long-term tumour control (Dougherty, Gomer et al. 1998).

Despite PDT’s advantages over current treatments, PDT has yet to gain general clinical acceptance (Kim, Ohulchanskyy et al. 2007); currently approved PDT photosensitizers absorb in the visible spectral regions below 700 nm, where light penetration into the skin is only a few millimetres, thus clinically limiting PDT to treating topical lesions (Kim, Ohulchanskyy et al. 2007). Most tissue chromophores, including oxyhemoglobin, deoxyhemoglobin, melanin and fat, absorb weakly in the NIR spectral range (700 – 1100 nm) - the wavelengths where the deepest penetration of light can be achieved, but most photosensitizers have absorption bands at wavelengths shorter than 800 nm (Chen and Zhang 2006).
To overcome this limitation, the possible use of light in the tissue transparent window is being explored. This involves the encapsulation of photosensitizers on upconverting nanoparticles. Briefly, upconverting nanoparticles are modified nanometer-sized composites which generate higher energy light from lower energy radiation, usually NIR or infrared, through the use of transition metal, lanthanide or actinide ions doped into a solid-state host. In this novel strategy, these nanoparticles play the role of ‘nanotransducers’, whereby the encapsulated upconverting donor ‘harnesses’ energy from the NIR light irradiated and emits visible light that activates the attached photosensitizing PDT drug, the acceptor. Besides allowing increased depth of penetration, the use of such nanoparticles also enables selective targeting to cells, focused laser activation of sharply delineated areas and absence of incident skin photosensitivity.

Figure 2. Schematic representation of the upconverting nanoparticle with attached photosensitizer

In this paper, the feasibility of the novel concept of using upconverting nanoparticles for photodynamic therapy is being highlighted. The nanoparticles in use are NaYF$_4$ nanocrystals coated with polyethyleneimine (PEI), which encapsulate the absorber ion (Yb$^{3+}$) and emitter ion (Er$^{3+}$), and demonstrate strong emission of visible light upon irradiation with 980 nm NIR laser. We have successfully managed to attach the photosensitizer, zinc (II) phthalocyanine (ZnPc), onto the surface of these upconverting nanoparticles. Based on a standard curve prepared, the entrapment efficiency has been estimated to be approximately 97%. The detection of the photodynamic production of singlet oxygen via the use of a molecular probe indicates that NIR light is indeed able to activate the photosensitizer indirectly via the upconversion process within the nanoparticle. In addition, the substantial cell mortality induced upon the incubation of MCF-7 human breast cancer cells with the folic acid–coated ZnPc-nanoparticle conjugates and subsequent irradiation with NIR confirms the phototoxicity of these nanoparticles in vitro.
Figure 3. Schematic representation of the method of activation of photosensitizer ZnPc via the upconversion process within the nanoparticle

MATERIALS

Ethanol, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin and 3-[4, 5-diethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) solution. Zinc (II) phthalocyanine (ZnPc, Mr 577.92) was purchased from Sigma-Aldrich. The folic acid-coated PEI/NaYF₄ nanoparticles (4.4 mg/ml) were prepared by Wang Feng. Disodium, 9, 10-anthracenedipropionic acid (ADPA) was purchased from Invitrogen.

METHODS

Quantification of ZnPc by the fluorescence emission method

0.0006 g of ZnPc (Mr 577.92) was dissolved in 100 ml of ethanol to obtain 1 µM ZnPc stock solution. 2 ml of 500 nM solution of ZnPc in ethanol was taken in a cuvette and the emission spectrum from 650–700 nm recorded using a SpectroPro 2150i spectrophotometer (Roper Scientific Acton Research, MA). The solution was then diluted progressively and the process repeated. The standard solutions were excited at 610 nm. The standard curve was prepared covering the range of 4-500 nM ZnPc. The area of fluorescence emission spectrum was correlated with the ZnPc concentration (nM) to obtain the standard curve.

ZnPc encapsulation efficiency

The fluorescence emission spectrum recorded between 650-700 nm of 0.5 µM of ZnPc solution was obtained as described above. 1 ml of 1 µM ZnPc solution was added to 1 ml of 4.4 mg/ml folic acid–coated PEI/NaYF₄ nanoparticles in a tube and was shaken for
30 minutes for even mixing. The suspension was next subjected to 10 minutes of centrifugation at 13,000 rpm. The supernatant was carefully extracted, collected in a cuvette and its ZnPc content measured by the area on the fluorescence emission spectrum, recorded between 650-700 nm, using the SpectroPro 2150i spectrophotometer (Roper Scientific Acton Research, MA) equipped with a 1200 g mm-1 grating and a 980 nm VA-II diode pumped solid state (DPSS) laser (current set at 1.50 A).

Synthesis of ZnPc-nanoparticle conjugates  The suspension of 4.4 mg/ml folic acid–coated PEI/NaYF₄ nanoparticles was gently shaken with an equal volume of 1 µM ZnPc solution for 30 minutes at room temperature. The resulting suspension was centrifuged for 10 minutes at 13,000 rpm and the pellet of ZnPc-nanoparticle conjugates was resuspended in phosphate buffered saline (PBS).

Determination of singlet oxygen production using a molecular probe  The detection of singlet oxygen production through the photobleaching of disodium, 9, 10-anthracenedipropionic acid (ADPA) was originally reported by Lindig et al. Since this molecular probe is destroyed by singlet oxygen species, the concentration of ADPA (as determined by absorbance at 400 nm) is inversely proportional to the effectiveness to energy transfer to molecular oxygen. This molecular probe has recently been used to assess the singlet oxygen production from photosensitizers encapsulated in silica and polyacrlamide nanoparticle systems (Wieder, Hone et al. 2006). A number of wells containing equal volumes of nanoparticles (4.4 mg/ml) and ADPA (10 µM) were prepared. Each well was exposed to laser excitation at 980 nm for different time periods. As a control, wells containing similar amounts of ADPA and ethanol without nanoparticles were exposed to similar time periods of equivalent laser exposure. The concentration of ADPA remaining in the wells was read by measuring the absorption at 400 nm in a spectrophotometer. Care was taken to keep initial concentration of ADPA the same in all wells irrespective of laser exposure. Results were expressed as percentage of control sample containing no nanoparticles and not exposed to laser.

Maintaining the MCF-7 cell line  The MCF-7 (human breast cancer cells) cell line was maintained by routinely culturing the cells in cell culture flasks in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and incubated in a 100% humidified incubator with 5% CO₂ at 37°C. A subculture was carried out whenever confluency reached approximately 80%. Medium was first removed from the culture flasks before the flasks were washed with PBS to dilute any remaining serum. Trypsin was then added to detach the cells, and the flasks were placed back in the incubator for 3-5 minutes. After which, an equal volume of culture medium was added to stop the enzymatic action of trypsin. The contents of the flasks were pipetted into appropriately labeled test tubes which were then subjected to centrifugation at 1200 rpm for 4 minutes. The supernatant from each tube was discarded and the remaining cell pellets were resuspended in culture medium. A fraction of the cell suspension in each tube was pipetted into a new culture flask, topped up with culture medium and incubated again. The remaining volumes of cell suspension left would usually be discarded but for the purpose of carrying out the MTT assay, the cells were retained.
Targetted binding to cancer cells HT29 cells (human colonic adenocarcinoma cells) were cultured on in wells of a 24-well microtiter plate for 24 hours. Folic acid-coated ZnPc-PEI/NaYF₄ nanoparticle conjugates (4.4 mg/ml) were added and the cells were incubated for 1.5 hour at 37°C and 5% CO₂, then washed thoroughly with PBS. Cells were imaged in bright field and under infrared excitation. The sample was excited with a specially fitted continuous wave infrared laser source and images captured in the bright field and confocal fluorescent modes.

Photoexposure and MTT assay Cell viability was assessed using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay. The MTT assay is used to measure cell viability because only living cells contain active mitochondrial reductase enzymes that can reduce yellow MTT to purple formazan crystals. To determine the effects of amount of photosensitizer-nanoparticle conjugates used on cell viability, a cell irradiation protocol-incorporated colorimetric MTT metabolic activity assay was carried out. Using a hemacytometer, approximately 100,000 MCF-7 cells from the cell suspension obtained after subculture (as described above) were added to each well of a 24-well microtiter plate, topped up to 300 µl with culture medium, and incubated at 37°C to allow the cells to attach to the bottom of the wells. The medium from each well was removed and replaced with new medium after 3 days. After another 3 days, the suspension of folic acid–coated ZnPc-nanoparticle conjugates was added to the wells in differing volumes (0 µl, 50 µl, 100 µl, 150 µl of 4.4 mg/ml) then topped up to 300 µl with media. The microtiter plate was returned back to the 37°C incubator after it was placed in an automated shaker for 30 minutes to ensure the even distribution of folic acid-coated ZnPc-nanoparticle conjugates in the wells. After another 2 days of incubation, the contents of the wells were removed, and the wells were rinsed once with PBS to remove any free and unattached folic acid-coated ZnPc-nanoparticle conjugates. 300 µl of new culture medium was added to each well and then each well was irradiated using the 980 nm NIR laser for 5 minutes. The cells were incubated for a further 2 days. Medium was removed from each well, and the wells were rinsed twice with PBS. 50 µl of MTT solution and 250 µl of culture medium were then added to each well and the cells were incubated for a further 1 hour. Any remaining solution was removed from the wells, and the resultant formazan crystals were dissolved in 300 µl of DMSO and quantified by measuring the absorbance of the cell lysate at 595 nm using a FLUOstar Optima microplate reader. Cell viability was expressed as a percentage of the control. All results are averages ± SD of four samples.

RESULTS AND DISCUSSION

Quantification of ZnPc by the fluorescence emission method The standard curve for ZnPc fluorescence emission was plotted as the area of the fluorescence emission spectrum (650-700 nm) versus known concentrations of ZnPc in ethanol. The area of fluorescence emission spectrum of ZnPc correlated approximately linearly with different concentrations of ZnPc in ethanol.
ZnPc was physically adsorbed to the surface of the nanoparticles by mixing equal volumes of 1 µM of ZnPc solution and 4.4 mg/ml of folic acid–coated nanoparticles (both in ethanol) and gently shaking for 30 minutes at room temperature. This resulted in the complete disappearance of the bluish colouration of the ZnPc solution. Based on the standard curve in Figure 4, the encapsulation efficiency was determined to be approximately 97%. This is significantly higher than the 80% encapsulation efficiency of ZnPc by poly-(D,L lactic-co-glycolic acid) (PLGA) nanoparticles, as reported by Ricci-Junior and Marchetti in 2006 (Ricci-Junior and Marchetti 2006). This is surprising since PEI is primarily a cationic polymer while ZnPc is highly non-polar. It seems likely that ZnPc binds strongly either to the surface of highly non-polar nanocrystalline core wherever it is left uncovered by the PEI or else to those stretches of the PEI chain comprising solely of carbon backbone without charged amine groups.
Determination of singlet oxygen production using a molecular probe. Production of singlet oxygen on irradiation of the ZnPc-nanoparticle conjugates with 980 nm NIR laser was determined through the photobleaching of disodium, 9, 10-anthracenedipropionic acid (ADPA). The ZnPc-nanoparticle conjugates suspension in PBS was irradiated with a 980 nm diode laser for different time periods and absorption spectra recorded. The decreasing absorbance intensity at 400 nm demonstrated increased destruction of ADPA with time and hence the effectiveness of singlet oxygen production by the nanotransducer on NIR excitation. A control sample containing only ADPA showed a marginal decline over time, probably due to photobleaching from the laser exposure.
Figure 6. ADPA destruction representing singlet oxygen production (measured by absorbance intensity at 400 nm) as a function of exposure time to NIR laser showing steady fall from original (100%) for the ZnPc-PEI/NaYF₄:Yb³⁺,Er³⁺ nanoparticles (squares) while pure ADPA control undergoes slight bleaching on continuous exposure to laser (circles).

**Targetted binding to cancer cells**  Confocal images of HT29 cells after incubation with folic acid-coated ZnPc-PEI/NaYF₄ nanoparticle conjugates indicated that the nanoparticles mainly clustered on the cell exterior.

Figure 7. Confocal image of HT29 cells incubated with the nanoparticle conjugates
Photoexposure and MTT assay  To determine the in vitro efficiency of the ZnPc-nanoparticle conjugates for photodynamic therapy, the parameter, variation of amount of ZnPc-nanoparticle conjugates, was investigated upon via a cell viability test using the colorimetric MTT reagent. Figure 7 shows that the MCF-7 cell viability, as determined by the MTT assay, generally decreased as the amount of ZnPc-nanoparticle conjugates incubated with the cells increased.

![MTT assay](image)

Figure 8. MTT assay to demonstrate the phototoxic effect of the nanoparticles. Each well was exposed to 5 minutes of 980 nm laser after incubation with different amounts of folic acid-coated ZnPc-PEI/NaYF nanoparticles for 48 hours. (n=4, bars show standard error)

CONCLUSION

The use of photosensitizer-upconverting nanoparticle conjugates as transducers of low energy light to toxic oxygen species has several advantages. Optical absorption coefficients of principal tissue chromophores in the human body show a very sharp ‘valley’ in the NIR spectral region and hence allow deepest tissue penetration. NIR light can penetrate depths an order of magnitude higher than visible light and can potentially be used for deeper structures. NIR light is also less harmful to cells and tissues and reduces the risk of inadvertent tissue destruction. Use of cheaper continuous wave diode lasers for upconversion (as opposed to expensive pulsed lasers for two-photon PDT) reduces cost of set-up and enhances the cost-effectiveness of therapy. The use of laser rather than broad spectrum lamps has both advantages and disadvantages. While laser gives exquisite control
over the specific area of exposure, it also means that large areas cannot be illuminated simultaneously, thereby increasing duration of therapy. Although it remains to be proved, it seems reasonable to suppose that PDT systems that require NIR laser for activation will not be effectively excited by sunlight exposure. Side effects of skin photosensitivity will thus be reduced if not eliminated. This is also supported by rapid clearance of nanoparticles from the body - initial results from animal studies appear to suggest that unmodified nanoparticles are cleared from circulation within 24 hours in the absence of tumours. Finally, nanoparticles have a natural tendency to concentrate in tumours due an enhanced permeation and retention effect contributed by disordered tissue architecture, increased vascularity, larger microvascular fenestrations and absence of lymphatics. This enables the specific delivery of the nanodevice to tumours, which can be further enhanced through the use of other targeting moieties (such as antibodies) attached to the surface.

In conclusion, we have described a novel ‘nanotransducer’ for PDT whereby upconverting nanoparticles convert deeply penetrating NIR light to visible emissions, which are in turn used by the attached photosensitizer to convert molecular oxygen to toxic singlet species. Irradiation of the conjugates incubated with MCF-7 human breast cancer cells led to significant cell mortality, indicating the phototoxicity of these photosensitizer-nanoparticle conjugates through the photodynamic effect. These results suggest that the photosensitizer-nanoparticle conjugates are ideally suited for PDT of cancer indications. In addition, it can be appreciated that this is essentially a platform technology with the scope to substitute ZnPc with other photosensitizers which absorb in the 540 nm or 650 nm range. For example, tin etiopurpurin (SnET2) requires 660 nm while tetra(m-hydroxyphenyl)chlorine (mTHPC, Foscan) is excited at 652 nm. While these results are extremely encouraging, the PDT efficacy of these upconverting conjugates has to be evaluated in vivo via animal studies to truly establish and secure the feasibility of this concept.

REFERENCES


