Upconverting Nanoparticles as Nano-Transducers for Photodynamic Therapy in Cancer Cells

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ABSTRACT

We demonstrate the application of monodisperse fluorescent upconverting nanoparticles (UCN) with functionalized surfaces for imaging and photodynamic therapy. In contrast to downconversion fluorescent materials, UCN convert near infrared excitation to visible emission and are shown to be advantageous in imaging of cells and tissues because of minimum photo-damage to living organisms, weak background fluorescence, high detection sensitivity and high light penetration depth in tissues. For photodynamic therapy, UCN were functionalized with zinc phthalocyanine (ZnPC) photosensitizer. The nanoparticles act as 'nano-transducers' to convert NIR excitation to emission frequencies suitable to activate the photosensitizer to release reactive oxygen species to kill cancer cells. In this report we present 50nm polymer coated UCN for photodynamic therapy.

Keywords: nanoparticles, photodynamic therapy, cancer, upconversion

1 INTRODUCTION

Photodynamic therapy is a therapeutic option for cancer that relies on the interaction of light and drugs to kill targeted cells. Acceptance of PDT has been limited by fear of high cost of setup, comparative cure rates with conventional therapies, absence of standard protocols established by randomized trials, skin photosensitivity for prolonged periods and inability to easily reach deeper seated tumors. In this report we discuss the development of a 'nano-transducer' which can potentially address some of these problems.

1.1 Hypothesis

The nanodevice comprises of zinc phthalocyanine (ZnPC) photosensitizer bound to up-converting nanoparticles (UCN). UCN are composed of sodium yttrium fluoride ($NaYF_4$) nanocrystals co-doped with the rare earth ions ytterbium (Yb^{3+}) and erbium (Er^{3+}) with a polymeric coat of high molecular weight (25kD) poly(ethylene imine) (PEI). The photosensitizer ZnPC is adsorbed to the nanoparticles' surface.

PEI/NaYF:Yb,Er nanoparticles when excited with 980nm NIR laser emit in the visible range with two relatively sharp peaks at 500-550nm (green light) and 650-675nm. [1] The fluorescence excitation spectrum of ZnPC shows an excitation maximum at about 670nm and considerably overlaps the red emission peak for the upconverting nanoparticles. This overlap ensures that ZnPC in close proximity to the nanoparticles can absorb the emitted luminescent radiation when the nanoparticles are excited with 980nm laser.

To the best of our knowledge there has only been one earlier report of nanotransducer nanoparticles used for PDT [2]. Zhang et al used NaYF particles in size ranges from 60 nm to 120 nm which are then coated with a thin layer of silica incorporating merocyanine as a photosensitizer.

In this work, we aim to use 50nm nanoparticles for photodynamic destruction of cancer cells in vitro.

2 MATERIALS AND METHODS

2.1 Materials

The title should be in boldface letters centered across the top of the first page using 14-point type. FA (approximately 98%), dimethyl sulfoxide (DMSO), N-hydroxy-succinimide (NHS), 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC), 3-mercaptopropionic acid (3-MPA), zinc phthalocyanine boric acid and sodium azide (99.99+%) were purchased from Sigma-Aldrich. ADPA was purchased from Invitrogen. FITC-streptavidin was purchased from Biomeda. Sodium chloride (NaCl) was purchased from LabScan. Polyethylenimine (PEI, branched polymer of molecular weight 25kD, sodium chloride (NaCl, >= 99.0%), yttrium chloride hexahydrate (YCl3•6H2O, 99.99%), ytterbium oxide (Yb2O3, 99.99%), erbium oxide (Er2O3, 99.99+%), ammonium fluoride (NH4F, 98+%), were purchased from Sigma-Aldrich. All of the reagents

were used as received without further purification. PEI stock solution (5 wt%) was prepared by dissolving PEI in DI water. YCl3 and NaCl stock solutions (0.2 M) were prepared by dissolving YCl3•6H2O and NaCl respectively in DI water. YbCl3 and ErCl3 stock solutions (0.2 M) were prepared by dissolving corresponding oxides in hydrochloric acid.

HT29 cells (human colon adenocarcinoma cells) were cultured in a media constituted of DMEM, FBS and antibiotics (streptomycin and penicillin) in a ratio of 100:10:1 in 75 cm2 flasks. The cells were incubated in a 100% humidified incubator with 5% CO2 at 37°C. The cells were maintained using protocol as described before.

2.2 Characterization of the UCN

2 ml of a 500nM solution of ZnPC in alcohol was taken in a cuvette and the emission spectrum from 550nm – 700nm for excitation at 400nm was recorded using a SpectroPro 2150i spectrophotometer (Roper Scientific Acton Research, MA). The solution was then diluted progressively and the process repeated. The area under the curve was used to plot the standard curve of fluorescent peak and concentration of ZnPC. Absorbance spectrophotmetry was performed on the 2ml sample of ZnPC to determine best overlap with upconversion fluorescence.

A one-pot synthetic procedure was used to synthesize the PEI/NaYF:Yb,Er nanoparticles, as described previously. FA was attached to the surface as a targeting ligand using the EDAC-NHS reaction described earlier. 1 ml of ZnPC in alcohol (500nm) was added to 1 ml of 4.4 mg/ml FA-PEI/NaYF in alcohol and the mixture mildly shaken in an automated shaker for half an hour. The mixture was then centrifuged at 10,000rpm for 10 minutes to spin down the nanoparticles. The supernatant was carefully withdrawn and the particles re-suspended in deionized water. The washing process was repeated twice to remove unbound ZnPC.

Unlike FA, ZnPC is adsorbed to the surface of PEI/NaYF:Yb,Er nanoparticles rather than being covalently bound. This is because ZnPC, being highly non-polar, lacks the charged side-groups necessary for formation of salt bridges; furthermore, non-polar regions of the polymer PEI bind strongly to the non-polar ZnPC in polar solvents, preventing free dissociation.

FTIR was used to confirm the presence of ZnPC on the nanoparticles. Samples containing pure ZnPC, PEI/NaYF and ZnPC-PEI/ZnPC were dried in vacuum and finely ground with KBr and then pressed into pellets. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Bio-Rad FTS156 spectrometer.

Production of singlet oxygen was determined through the photobleaching of disodium, 9, 10-anthracenedipropionic acid (ADPA). Since this molecular probe is destroyed by singlet oxygen species, the concentration of ADPA - as determined by absorbance at 400nm - is inversely proportional to the effectiveness to energy transfer to molecular oxygen and has been used for different nanoparticle systems.[3] A number of wells containing equal volumes of nanoparticles (4.4mg/ml) and ADPA (10μM) were prepared. Each well was exposed to laser excitation at 980nm for different time periods. The concentration of ADPA remaining in the wells was read by measuring absorption at 400nm 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.

2.3 PDT using UCN

HT29 cells were cultured on in wells of a 24 well plate for 24 hours at about 10000 cells/well. The cells were cultured in with ZnPC-PEI/NaYF nanoparticles (440 μg/ml). The cells were incubated for 1 hour at 37°C and 4% CO_2, then washed thoroughly. The wells were washed free of unattached ZnPC-nanoparticles, then exposed to 980nm laser for 5 minutes. The wells were then incubated at 37°C for another 2 days.

To assess the effect of exposure to NIR laser on the cells with and without the nanoparticles, the cell viability was measured by MTT assay. The media was removed and the wells rinsed with 300ul of PBS twice. 50ul of MTT solution was added to each well and topped up with 250ul media then incubated for 1 hr. Then all media was removed. After removing the medium, the wells are washed in PBS and intracellular formazan crystals were extracted into 300 μl of DMSO and quantified by measuring the absorbance of the cell lysate at 595 nm in a microplate reader. Cell viability was expressed as a percentage of the control. All results are averages ± SD of four samples.

3 RESULTS AND DISCUSSION

3.1 Nanoparticle characterization

The standard curve for ZnPC fluorescence was plotted as the area of the fluorescence emission spectrum (650nm - 700nm) versus known concentration of ZnPC in alcohol and a high encapsulation efficiency (> 95%) demonstrated. It is likely that ZnPC binds to the to the relatively non-polar C-C-N backbone of PEI. High encapsulation efficiencies of ZnPC (80%) have also been demonstrated by others using PLGA nanoparticles.[4]

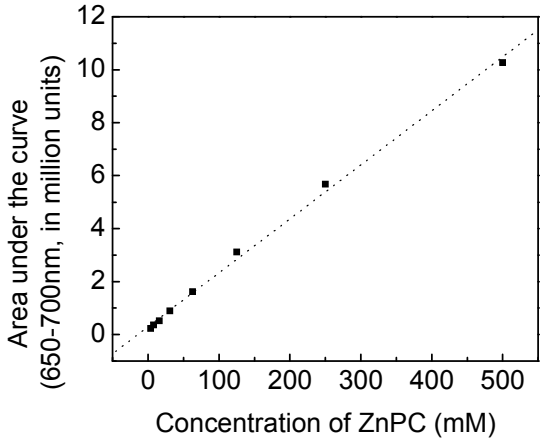

Figure 1: Standard curve for ZnPC.

FT-IR was used to determine the presence of ZnPC on PEI/ NaYF:Yb,Er nanoparticles after surface modification. In the figure below, the additional twin absorption peaks around 1450 cm^{-1} (marked with thick black arrows) were observed in the spectra of ZnPC-adsorbed nanoparticles but not in that of uncoated nanoparticles. This suggests the presence of ZnPC molecules on the nanoparticles.

PEI/NaYF:Yb,Er nanoparticles emit at 500-550nm and 650-675nm (red light) when excited at 980 nm [5], while ZnPC is maximally excited at about 670nm. Thus the red emission peak of the upconverting nanoparticles overlaps the excitation peak for ZnPC. Thus, ZnPC can be excited by the emitted luminescent radiation from the nanoparticles.

The molecular probe ADPA (disodium, 9, 10-anthracenedipropionic acid) is destroyed by toxic oxygen species. The concentration of ADPA can be determined by absorbance at 400nm.This is inversely proportional to the amount of toxic effectiveness to energy transfer to molecular oxygen and has been used for different nanoparticle systems.[3].

Production of singlet oxygen on irradiation of the ZnPC-nanoparticle complex with 980nm was determined through the photobleaching of ADPA. The decreasing absorbance intensity at 400 nm demonstrated increased destruction of ADPA with time and hence the effectiveness of singlet oxygen production by the nanodevice on NIR excitation.

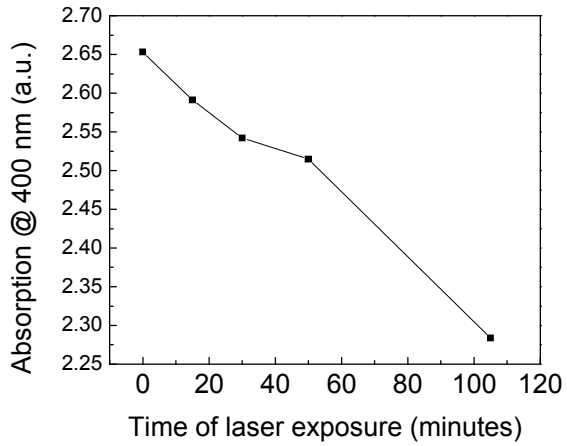

Figure 2: ADPA destruction (shown by reducing absorption at 400 nm) upon laser exposure in the presence of ZnPC-PEI/NaYF nanoparticles.

To target to colon cancer cells, folic acid was covalently attached to the PEI using a standard EDAC/NHS reaction as previously described.[5] The ZnPC-FA-PEI/NaYF:Yb,Er nanoparticles were incubated with HT29 human colonic adenocarcinoma cells and imaged by confocal microscopy at different time points. Although the nanoparticles rapidly clustered on the cell membrane bearing the receptors, uptake into the cells was slow with significant internalization requiring upto 48 hours of incubation at 37^{o}C.

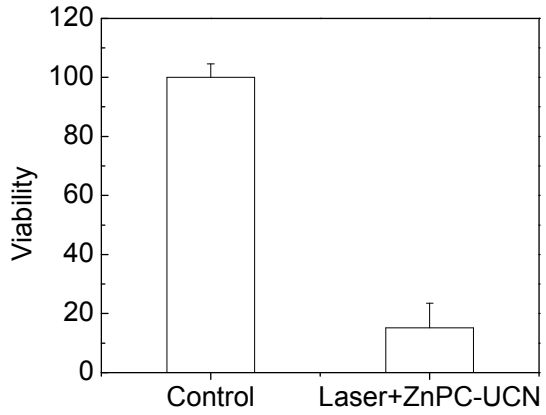

Figure 3: Effectiveness of PDT with UCN. HT29 cells were incubated with 440µg/ml of ZnPC-FA/PEI-NaYF nanoparticles and exposed to 30 minutes of NIR laser.

Effectiveness of this nanodevice in destruction of cancer cells was determined in vitro. HT29 cells were cultured according to standard protocols. About 10^4 cells were then transferred to the wells of a 96-well plate and incubated with 440μg/ml of ZnPC-nanoparticle complex. Each well was irradiated with 980nm laser for 30 minutes and incubated for a further 24 hours. Cell viability was determined using standard MTT protocol. Cell viability was reduced.

4 CONCLUSION

In conclusion, we have described a novel nanodevice for cancer PDT where 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. It can be appreciated that this is essentially a platform technology with the scope to substitute ZnPC with other photosensitizers which absorb in the 540nm or 650nm range. For example, tin etiopurpurin (SnET2) requires 660nm while tetra(m-hydroxyphenyl)chlorine (mTHPC, Foscan) is excited at 652nm. Further studies into effectiveness of this therapy in animals are being explored.

5 ACKNOWLEDGEMENT

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