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Lifetime-resolved Photoacoustic (LPA) Spectroscopy for monitoring Oxygen change and Photodynamic Therapy (PDT)

Janggun Jo^{#1}, Chang Heon Lee^{#1}, Raoul Kopelman^{2,*}, and Xueding Wang^{1,*}

¹Department of Radiology, University of Michigan Medical School, Ann Arbor, Michigan 48109

²Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109

These authors contributed equally to this work.

Abstract

The Methylene Blue loaded Polyacrylamide Nanoparticles (MB-PAA NPs) are used for oxygen sensing and Photodynamic therapy (PDT), a promising therapeutic modality employed for various tumors, with distinct advantages of delivery of biomedical agents and protection from other bio-molecules overcoming inherent limitations of molecular dyes. Lifetime-resolved photoacoustic spectroscopy using quenched-phosphorescence method is applied with MB-PAA NPs so as to sense oxygen, while the same light source is used for PDT. The dye is excited by absorbing 650 nm wavelength light from a pump laser to reach triplet state. The probe laser at 810 nm wavelength is used to excite the first triplet state at certain delayed time to measure the dye lifetime which indicates oxygen concentration. The 9L cells (106 cells/ml) incubated with MB-PAA NP solution are used for monitoring oxygen level change during PDT in situ test. The oxygen level and PDT efficacy are confirmed with a commercial oximeter, and fluorescence microscope imaging and flow cytometry results. This technique with the MB-PAA NPs allowed us to demonstrate a potential non-invasive theragnostic operation, by monitoring oxygen depletion during PDT in situ, without the addition of secondary probes. Here, we demonstrate this theragnostic operation, in vitro, performing PDT while monitoring oxygen depletion. We also show the correlation between O₂ depletion and cell death.

Keywords

photoacoustic spectroscopy; oxygen sensing; nanoparticle; methylene blue; photodynamic therapy

1. INTRODUCTION

Photodynamic therapy (PDT) is a promising treatment for various tumors [1-5]. This therapy is a highly localized treatment, as the illumination is exposed to the target tissue, with a certain wavelength of the illumination used to excite a photosensitizer so as to convert the local ground state oxygen (³O₂) molecules into cytotoxic reactive oxygen species (ROS). The ROS cause oxidative stress within the cells, inducing cell death by apoptosis, necrosis or a combination thereof [1-5]. Upon light excitation, absorbed photons of a photosensitizer go

*Corresponding author: kopelman@umich.edu; xdwang@umich.edu.

through internal conversion and intersystem crossing, then quickly relax to the excited triplet state. The emission from the triplet state (phosphorescence) is typically slow due to its being a quantum mechanically forbidden process which requires a change of spin. As a result, the excited photosensitizer state lifetime is long enough to be quenched by collisional interaction with nearby $^3\text{O}_2$ creating ROS. Its advantages over conventional therapies are: 1) have minimal long-term toxicity effects; 2) enable highly selective localized treatments; 3) enable repeated treatments at the same site; and 4) present a significantly lower risk than surgery. In addition, it can be employed to virtually any types of cancer. Numerous worldwide clinical trials have shown PDT as an effective and safe modality for various cancers [1-3]. Due to the increase attention in PDT, researchers have been interested in optimizing nanophotosensitizers owing PDT agents' potentials to increase efficiency, targetability, and biocompatibility for personalized theragnostic (therapy + diagnostics) operations [5, 6].

Methylene Blue (MB) is a promising candidate for working as a dual PS/O₂ activator/sensor, due to its high triplet quantum yield (~0.5), enabling efficient energy transfer to oxygen and thus leading to higher efficacy PDT. Moreover, MB has Near Infrared absorption wavelength ($\lambda_{\text{max}} = 664 \text{ nm}$ in aqueous media), allowing deeper tissue penetration [7]. The feasibility of MB for quantitative imaging of oxygen via the method of lifetime-resolved photoacoustic (LPA) measurement has already been explored previously [8, 9]. However, MB in free molecule form has only limited use *in vivo*, because the MB molecules, without being protected, get reduced into PDT inactive, colorless isomeric molecules (*leuko*-MB) by blood enzymes [7, 10, 11]. Also, at higher concentrations, MB molecules may form aggregates that reduce their luminescence quantum yield, due to "self-quenching", leading to a decrease in PDT efficiency [7, 11, 12]. Furthermore, MB molecules cannot specifically target tumors when intravenously injected. In some of our previous works, MB loaded polyacrylamide nanoparticles (MB-PAA NP) have been developed, using several different methods to maximize their PDT efficacy [10-14]. Notably, the PAA matrix protects the MB molecules from the blood enzymes, thus avoiding isomerization of MB into *leuko*-MB. According to our previous measurements, in the presence of reducing enzymes, more than 80% of the free floating MB molecules' fluorescence disappears after 1 hour; however, only less than 10% of the MB-PAA NP fluorescence disappears under the same condition [10]. Also, the amine functionalized PAA NPs enable surface functionalization, for attachment of tumor targeting moieties, such as the F3 tumor homing peptide, via conjugation with bi-functional polyethylene glycol, thus navigating the MB PS specifically to and into the tumor cells [13, 15-17].

Oxygen depletion during PDT is well known and has been studied previously and its importance in PDT efficacy and its effects in tumor survival have been monitored very closely [18-20]. However, most experiments were performed using secondary probes such as hypoxia markers or invasively sticking oxygen sensing optodes. We still do not have a good to monitor oxygen depletion during PDT non-invasive and real time monitoring. We have recently developed photoacoustic chemical imaging [21-23], and, in particular photoacoustic imaging of tissue oxygen using LPA measurements [22, 23]. LPA spectroscopy uses two laser beams for pump and probe beams. Upon the pump beam excitation, absorbed photons of an oxygen sensitive dye go through internal conversion and intersystem crossing, then

quickly relax to the excited triplet state. The probe beam response measures its transient absorption in the excited triplet state. By changing the time delay between pump and probe beams, the exponential decay curves of the photoacoustic amplitudes can be obtained, from the probe beam. The rates of the exponential decay correlate directly to the oxygen concentration in the medium.

In this study, we demonstrate a method for the personalized theragnostics operation. MB-PAA NP, itself, has a unique capability of both PDT and O₂ monitoring which may, not only perform PDT and O₂ monitoring separately, but also be applicable to the study of PDT efficacy by *in situ* oxygen depletion monitoring through LPA measurements. Monitoring oxygen depletion during PDT allows direct quantification of therapeutic efficacy to monitor tumor suppression (cell death) without the aid of secondary probes and also allows us to determine the correct drug/light dosage (Therapeutic window) for different types of cancers *in vivo* non-invasively.

2. MATERIALS AND METHODS

2.1 MB-PAA NPs

Acrylamide (AA), poly(ethylene glycol) dimethacrylate, Mn 550, (PEGDMA), ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dioctylsulfosuccinate (AOT), Brij 30, dimethyl sulfoxide (DMSO), Ethanol, phosphate buffered saline (BioReagent, pH 7.4, for molecular biology) and hexane were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(3-aminopropyl)-methacrylamide hydrochloride (APMA) was purchased from Polysciences (Warrington, PA, USA). Dicarboxymethylene blue NHS ester (DCMB-SE) was purchased from European Molecular Precision Biotech (Berlin, Germany). Anthracene-9, 10-dipropionic acid disodium salt (ADPA), RPMI 1640 Medium, 100X Antibiotic-Antimycotic, Heat Inactivated Fetal Bovine Serum (FBS), Calcein AM, and Propidium Iodide (PI) from Life Technologies (Carlsbad, CA, USA). Annexin V:FITC Apoptosis Detection Kit I was purchased from BD Biosciences (San Jose, CA, USA). 9L rat gliosarcoma cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The water was purified with a Milli-Q system from Millipore Corporation (Billerica, MA, USA). All chemicals were used without further purification.

The MB-PAA NPs were prepared as in a previously reported method [10]. All reactions were performed in the dark. The monomer solution was prepared as follows. DCMB-SE (5mg dissolved in 100 μ L of DMSO) was added into 0.93 mL of Phosphate Buffered Saline (PBS, pH 7.4) containing AA (368 mg) and APMA (28mg). The monomer solution was stirred for 2 hr at room temperature. Then, AOT (1.07g) and Brij 30 (2.2 mL) were added into 30 mL of Hexane in a round bottom flask equipped with a stirring bar. After 30 min of argon flushing, the monomer solution was injected and flushed with argon for another 15 min. The radical polymerization was initiated by addition of 100 μ L of TEMED and 100 μ L of APS (15mg / 100 μ L in water), while stirring. After 2 hr, the hexane was evaporated with a rotary evaporator and the resulting MB-PAA NPs were suspended in Ethanol and transferred into an Amicon Stirred Ultrafiltration Cell equipped with a Biomax 300 kDa membrane. The solution was washed with Ethanol and water several times so as to remove

any unreacted monomers and surfactants. Then, the MB-PAA NPs water suspension was freeze-dried and stored at -20°C .

A Dynamic Light Scattering instrument (DLS, Delsa Nano C particle analyzer instrument, Beckman Coulter, Brea, CA, USA) was used to determine the particle size. A UV-1601 Spectrometer (Shimadzu, Kyoto, Japan) was used for the Absorption spectra and a FluoroMax-2 Spectrofluorometer (Jobin Yvon Horiba, Kyoto, Japan) was used for the Fluorescence spectra. Both spectrometers were used to determine the dye loadings and NP's characteristics, comparing to the previously reported MB-PAA NP [10].

2.2 LPA Spectroscopy experimental setup

The schematic of the experiment setup for LPA is shown in Figure 1. An optical parametric oscillator (Surelite OPO plus, Continuum), pumped with the second harmonic of a pulsed neodymium-doped aluminum garnet (Nd: YAG) laser, was used as the pump beam, with a pulse width of 5 ns and a pulse energy of 10 mJ, at 650 nm wavelength. For the probe beam, another optical parametric oscillator laser (Vibrant B, Opotek) generated pulsed beam, with a 7-mJ pulse energy, at 810 nm of wavelength was used. The triggers of the two lasers were controlled by a delay generator (DG535, Stanford Research Systems). The two laser beams were overlapped on the transparent cuvette containing the 9L cells pre-incubated with the MB-PAA NPs. The incubation method and conditions were identical to those in the Flow Cytometry experiments (2 hr). The Photoacoustic (PA) signal was detected by a hydrophone (HNC-1500, ONDA) and amplified with a preamplifier (AH-2010-CDBNS, ONDA) and an amplifier (5072PR, Olympus). The signal, digitalized by an oscilloscope (DTS540, Tektronix), was collected (averaged over 50 pulses).

In our LPA study, the MB-PAA NPs are exposed with a pump laser at 650 nm, re-exposed by a probe laser at 810 nm. The probe beam response measures the MB's transient absorption in the excited triplet state. By varying the time delay between pump and probe beams, the exponential decay curves of the photoacoustic amplitudes can be obtained, by measuring the signal from the probe beam. The oxygen concentration in the medium can be measured with the rate of the exponential decay.

The 9L cells (106 cells/ml) were incubated with a MB-PAA NP solution, containing a 1mg/ml FITC Annexin Binding Buffer, for 2 hr. Then, the cell containing solution was transferred into clean plastic cuvettes and sealed with a rubber septum to minimize air flow into the cuvette. An optical parametric oscillator (Surelite OPO plus, Continuum) pumped with the second harmonic of a pulsed neodymium-doped aluminum garnet (Nd: YAG) laser was used, with a pulse-width of 5 ns, at 650 nm wavelength (10mJ), for different time periods (0 min, 5 min, 10 min, and 20 min). The light source was scattered so as to cover the entire area of the cuvette. Each sample was then transferred into a round bottom tube and then FITC-Annexin V (5 μL /105 cells) and PI (5 μL /105 cells) were added into the test tubes. The tubes were incubated for 10 min, at room temperature, before introduced to a Flow Cytometer (MoFlo Astrios, Beckman Coulter). The oxygen concentration was measured by an oximeter (Microx TX3, Presens), every 5 min. The 9L cells (106 cells/ml) were incubated with MB-PAA NPs for 2 hr and the oxygen concentration was monitored with and without 650 nm laser illumination (i.e., with or without PDT).

3. RESULTS

MB-PAA NPs were synthesized as previously described [10]. Dynamic Light Scattering measurements were taken to determine the hydrodynamic size of the MB-PAA NPs. The average size distribution was 73.7 nm (± 6.8 nm). The optical absorption spectra, at different concentrations of MB-PAA NPs in PBS pH 7.4 buffer. MB-PAA NPs exhibit absorption max at 669 nm and 619 nm as previous study [10]. The peak at 669 nm is responsible for MB monomer peak and the peak at 619 nm is responsible for aggregated MB dimer peak.[7, 10]

The PDT was performed without rinsing off the MB-PAA NP solution in the cell media. Figure 2 shows the comparison with latter pulsed laser PDT experiments and PALT experiments where we used the MB-PAA NP solution without rinsing. The blue box in Figure 2 indicates the illumination area. After 30 min of illumination, all of the cells had strong PI (red) uptakes and reduced Calcein AM (green) signals indicating the cell death. Interestingly, the top half of the cells showed PI uptake while bottom half did not indicating that the PDT was only performed on the area of illumination.

Pulsed laser light was used for PDT and the cell viability was monitored as Figure 3. After 20 minutes of illumination with the pulsed laser, almost the whole population of cells underwent cell death. Also, the oxygen concentration was monitored, with and without PDT. Oxygen concentration dropped more drastically with continuous pump beam exposure compared to without light exposure.

The oxygen concentration during PDT was measured using LPA measurement. An overlay of oxygen concentration decays, measured by oximeter and LPA is depicted in Figure 4. The 650 nm laser was continuously illuminating during the 20 min period while the 810 nm laser was only illuminating during the LLPA measurements (less than 11 min). The PAA signal from the 810 nm laser for the probe beam were exponentially fitted with delay time (1, 2, and 44 μ s) signals, too give the exponential decay rate constants for the MB-PAA NPs at different oxygen concentrations. The oxygen concentration (red squares, Figure 4) was measured by the oximeter.

4. CONCLUSION

In this study, we demonstrate the feasibility of MB-PAA NP to be applied for both diagnostic (measuring oxygen) and therapeutic modality (PDT). The unique dual capability of MB-PAA NP allowed us to demonstrate a potential non-invasive theragnostic operation by monitoring oxygen depletion during PDT in situ without addition of secondary probes in vitro. We demonstrated the correlation between oxygen concentration and cytotoxicity of PDT the 9L glioma cells. Then, oxygen depletion was monitored using LPA spectroscopy based measurements. It can be further applied for in vivo studies to treat tumors with PDT and oxygen monitoring/imaging during PDT. Another potential advantage of MB-PAA NP is that 619 nm light may be applicable for regular PA imaging minimizing cytotoxicity effect from MB-PAA NP. The 619 nm excitation's PDT efficacy was ~70% less than the 669 nm excitation's PDT efficacy while absorptions on 619 nm and 669 nm were similar. This allows longer time for PA structural imaging for better resolution without actually affecting

the cells too much. Also, like Coomassie blue PAA NPs [15, 16], we presume that it could also be applied for tumor delineation with naked eyes being a good contrast agent (blue) against blood (red). PA spectroscopy is non-invasive imaging technique which only uses non-ionizing light and ultrasound detectors. Ultrasound detection allows for deeper tissue penetration compared to optical detection (e.g. fluorescence microscopy). Powerful advantages of optical sensors are no longer limited by the light penetration depth when PA spectroscopy is employed. Combination of nano-technology and PA spectroscopy may open a new branch of interesting studies on tumors and other areas.

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Figure 1.
Schematic diagram of the experimental setup.

Figure 2. Fluorescence microscope images of Live (Calcein AM, green)/Dead (PI, red) cell assays, before and after PDT treatment. The blue box (top half) designates the illumination area.

Figure 3.

The cell survival rate during PDT, using pulsed laser, and the oxygen concentration at each time point during PDT.

Figure 4.

The Green Triangles indicate the LPA decay rate measurements, after illumination (0 min to 20 min), and the Red Squares indicate the oxygen concentrations measured using the oximeter as the gold standard.