Fluorescence lifetime-based intracellular thermometry for photothermal therapy

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Abstract. Photo-thermal therapy (PTT) is a rapidly developing approach for cancer treatment, that has greatly benefited from the tremendous advances in the synthesis of plasmonic nanoparticles (NPs), which can be used as light sensitive agents. This approach is based on the local induction of hyperthermia via light irradiation of plasmonic NPs in order to thermally kill cancerous cells. Such an approach ensures a high precision of treatment and the mechanism of action makes it as a valid alternative for treatment of malignant neoplasms with a multi-drug resistance. However, in order to minimize the adverse effects of PTT it is necessary to precisely measure and control the achieved temperatures at the tumor foci during light irradiation. Herein we report a novel technique to monitor intracellular temperatures during PTT based on temperature sensitive fluorescent dye, Rhodamine B. These findings may improve the quality of treatment and reduce unwanted adverse effects.

1. Introduction
Photo-thermal therapy (PTT) is a minimally invasive approach for cancer treatment that has gained a lot of attention in recent years as an additional therapy, which can be combined with traditional approaches of cancer treatment to improve therapy [1]. The main operating principle of this therapy is to convert the energy of electromagnetic radiation using photosensitizer to heat. The excessive heat leads to local hyperthermia, which causes cancer cell death. Recent works reported on the use of various photosensitizers, such as nanographene sheets [2], carbon nanodots [3], and a large number of organic molecules [4]. Plasmonic nanoparticles are the most extensively studied due to their superb optical properties, great tunability, and bio-targeting capabilities [5]. Owing to the effect of plasmon resonance, plasmonic NPs possess enhanced near-infrared (NIR) absorption, which is crucial for effective PTT. Depending on the temperatures reached during the PTT, cancerous cells can die via either apoptosis or necrosis pathway [6]. The difference is crucial for the effectiveness of cancer treatment since a large number of necrotic cells can cause a cytokine storm that may lead to various complications during cancer treatment. Therefore, despite all the advantages of PTT, it is required to precisely control the temperatures reached during the light irradiation.

Different studies have used various techniques to determine temperatures reached during PTT, which include infrared thermometers and thermocouples. However, these methods allow detecting of changes in temperature of not only cells but also the surrounding media, which significantly decreases the accuracy of the measurements. Thus, to accurately assess the temperatures reached in the cancerous cell during the irradiation another, more precise thermometry approach is required. For this purpose, certain
fluorescent dyes can be utilized since their optical properties, such as fluorescence intensity and fluorescence lifetime depends on the temperature changes in the surrounding media [7]. Therefore, by detecting these changes it is possible to precisely measure temperatures reached in certain parts of cells, which were previously described in a number of works [8]. However, there are almost no studies that have utilized fluorescent probes for temperature monitoring during PTT.

In the present work we have developed an easy and precise method for intracellular thermometry based on fluorescence lifetime microscopy during PTT using Rhodamine B as a fluorescent temperature sensor.

2. Materials and methods

2.1. Materials
Gold (III) chloride trihydrate (HAuCl4·3H2O, ≥99.9%, Sigma-Aldrich), ascorbic acid (AA, ≥99.0%, Sigma-Aldrich), sodium borohydride (NaBH4, ≥98%, Sigma-Aldrich), cetyltrimethylammonium bromide (CTAB, ≥99%, Sigma-Aldrich), sodium oleate (NaOL, > 97.0%, Sigma-Aldrich), Rhodamine B (RhB, ≥95%, Sigma-Aldrich).

2.2. Au NRs synthesis
Au NRs with absorbance peak in the NIR region were synthesized using a seeded growth approach and binary surfactant mixture [9]. The initial seed solution was prepared as follows: 5 mL of 0.2 M CTAB solution was added to 5mL of 0.5 mM HAuCl4. Afterward, freshly prepared 0.6 mL of 0.01 M NaBH4 was diluted to 1 ml and then added to Au(III)-surfactant solution, which changed color to brownish. Stirring was stopped after 2 minutes and the seed solution was left undisturbed for 30 minutes prior to further use. Meanwhile, the growth solution was prepared. For this 1.234 g of NaOL and 9 g of CTAB were added to 250 mL of water and then heated up to 50°C until they dissolved. Afterward, the solution was cooled down to 30°C and 36 mL of 4 mM AgNO3 were added. The solution was gently mixed and left undisturbed for 15 minutes. Then 250 mL of 1 mM HAuCl4 solution was added and was left under stirring (700 rpm) for 90 minutes. After this 3 mL of HCl was injected to adjust pH and stirred for another 15 minutes. Finally, 1.25 mL of 0.064 M AA was added to the solution, quickly stirred for 30 seconds, and injected with 0.2 mL of previously prepared seed solution. The obtained solution was left for 12 hours at 30°C for Au NRs to grow. On the following day, the solution was centrifuged at 7,000 rpm for 30 min to remove the supernatant and consequently stabilized using H2N-PEG-SH via ligand-exchange procedure [10].

2.3. Cells
Murine melanoma cell line (B16-F10 cells) was purchased from the American Type Culture Collection. Cells were cultivated in AlphaMEM with 10% of vol. fetal bovine serum and 2mM UltraGlutamine I at 37°C in a sterile humidified atmosphere with 5% CO2.

2.4. Fluorescence lifetime temperature dependence
In order to obtain a calibration curve, describing the relation between fluorescence lifetime of RhB and various temperatures, 100 µM water solution of RhB was used. Values of fluorescence lifetime were obtained using Picoquant Picoharp 300 time-correlated single-photon counting system. The heating of the RhB solution was realized using a custom temperature control system, in which the temperature of the solution was monitored via a thermocouple.

2.5. Intracellular temperature measurements
For the temperature measurements, cells were seeded into confocal cell imaging dishes (d = 35 mm, Eppendorf) at the amount of 1.0 x 10^4 per dish. On the next day, Au NRs were added to the cells, and once again cells were left overnight. On the following day, cells were stained with 15 µL of 1 mM RhB solution. After 30 min, cells were washed twice with PBS and left in PBS supplemented with 4%
glucose. Fluorescence lifetime imaging microscopy (FLIM) was performed with a custom setup, which consisted of Picoquant PDL 800-D picosecond pulsed diode laser driver, Picoquant laser head LDH-FA 530XL, single-photon avalanche diode (MPD PDM PD-100-CTC-FC), and Picoquant Picoharp 300 time-correlated single-photon counting system.

3. Results
Synthesized Au NRs were characterized using Scanning Transmission Electron Microscopy (STEM) (Figure 1A). As determined from STEM pictures, the average width of Au NRs was around 20±1.1 nm and the length was approximately 124±5.4 nm. The obtained Au NRs had an absorbance maximum at 1173 nm (Figure 1B).

![Figure 1. A. STEM images of Au NRs. B. Absorbance spectrum of Au NRs. C. Calibration curve of RhB fluorescence lifetimes depending on the solution temperature.](image)

In order to estimate the relation between temperatures of the RhB solution and fluorescence lifetime, a calibration curve was obtained (Figure 1C). Afterward, FLIM of cells associated with Au NRs under NIR laser irradiation was performed. Depending on the laser intensity, the heating from Au NRs changed, which led to different intracellular temperatures and therefore to different fluorescence lifetimes detected via FLIM (Figure 2). Using the previously obtained calibration curve, we matched values of lifetime, which we detected with their corresponding values of intracellular temperature. Finally, based on the obtained microphotographs and calibration curve, the optimal parameters for achieving an intracellular temperature of 45.2±1.4 °C was power density equal to 0.32 mW/mm² using a 1064 nm fiber laser with 100MHz repetition rate. According to the published works, this temperature may be considered optimal for the effective PTT, as it maximizes the apoptosis of cancer cells [6,11].

![Figure 2. FLIM images of cells associated with Au NRs irradiated with NIR laser of various power densities. Each row corresponds to a certain power density and demonstrates a map of intracellular fluorescence intensities, lifetimes and their representation as a histogram.](image)
4. Conclusion
In this work, we developed a novel technique that allows one to precisely estimate temperatures achieved during laser irradiation using fluorescence lifetime imaging microscopy and fluorescent dye – Rhodamine B. This approach can be used to select optimal parameters of laser irradiation to achieve the most efficient therapy. This method allows to control temperatures in an intracellular environment and, therefore, paves the way for the broader introduction of PTT in clinics.

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