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Citation	Kannadorai, R. K., Chiew, G. G. Y., Luo, K. Q., & Liu, Q. (2015). Gold nanorods as photothermal agents and autofluorescence enhancer to track cell death during plasmonic photothermal therapy. Proc. SPIE 9537, Clinical and Biomedical Spectroscopy and Imaging IV, 9537, 95371B-.
Date	2015
URL	http://hdl.handle.net/10220/39192
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Gold nanorods as photothermal agents and autofluorescence enhancer to track cell death during plasmonic photothermal therapy

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ABSTRACT

The transverse and longitudinal plasmon resonance in gold nanorods can be exploited to localize the photothermal therapy and influence the fluorescence to monitor the treatment outcome at the same time. While the longitudinal plasmon peak contributes to the photothermal effect, the transverse peak can enhance fluorescence. After cells take in PEGylated nanorods through endocytosis, autofluorescence from endogenous fluorophores such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) in the mitochondria is enhanced two times, which is a good indicator of the respiratory status of the cell. When cells are illuminated continuously with near infrared laser, the temperature reaches the hyperthermic region within the first four minutes, which demonstrates the efficiency of gold nanorods in photothermal therapy. The cell viability test and autofluorescence intensity show good correlation indicating the progress of cell death over time.

Keywords: hyperthermia, gold nanorods, autofluorescence, photothermal therapy, fluorescence enhancement, cell viability

1. INTRODUCTION

Plasmonic photothermal therapy (PPTT) [1-3] involves exploiting the light to heat conversion property of gold nanoparticles to localize heating to the desired region. Nanoparticles such as nanorods [1] and nanoshells [1] absorb NIR laser and produce heat and have been successfully used to treat cancers [4] *in vivo*. Gold nanorods (GNRs) have two absorption peaks, in which the longitudinal plasmon peak contributes to the photothermal effect and while the shorter transverse peak can influence fluorescence [5]. When PEGylated nanorods are internalised inside the cells through endocytosis, the transverse plasmonic peak combined with the enhanced absorption and scattering properties of the nanorods can enhance autofluorescence emission intensity from cells, which could be explored to monitor the progress of cell death. During PPTT, cellular death can be tracked continuously by measuring fluorescence emitted by endogenous chromophores including nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) known to reflect cellular metabolic rate [6]. This non-destructive optical method is an excellent research tool and potentially a clinical method to ensure that a sufficient dose has been deposited to cause adequate cell damage during treatment. In the present work the dual peaks of GNRs are exploited for conducting plasmonic photothermal treatment and enhancing weak cellular autofluorescence simultaneously to facilitate the monitoring of treatment outcome in vitro.

2. MATERIALS AND METHODS

Preparation of gold nanorods and cell line

Bare GNRs with an aspect ratio of 3.8 (10 nm in diameter and 38 nm in length) which had two plasmonic peaks at 765 nm and 520 nm, were purchased from Strem chemicals Inc. The nanorods were PEGylated by incubating it with mPEG (Sigma-Aldrich, Missouri, United States) at a ratio of 1:4 at varying times under constant rotation. Human renal cell carcinoma cells (Caki-2 cell line) were purchased from ATCC. Caki-2 cells were cultured in IMDM supplemented with 10% FBS, and were incubated in the incubator at 37°C and with 5% CO_2. A total of 1×10^6 cells/ml suspension were mixed with PEGylated GNRs and incubated for 24 h in the incubator. The PEGylated GNRs were washed once with culture medium prior to mixing with the caki-2 cell medium. To evaluate cell death due to necrosis, 10 µl of 0.4% (w/v) trypan blue solution was added to 10 µl of the cell sample and mixed well. The mixture was left for 10-15 mins in room temperature before the observation under optical microscope. Dead cells were stained blue while live cells remained unstained as their cytoplasm remained intact.

Clinical and Biomedical Spectroscopy and Imaging IV, edited by J. Quincy Brown, Volker Deckert, Proc. of SPIE-OSA Vol. 9537, 95371B · © 2015 SPIE-OSA
CCC code: 1605-7422/15/$18 · doi: 10.1117/12.2183581

Experimental setup for plasmonic photothermal therapy and autofluorescence measurements

A table top 500 mW, 785 nm laser was used for photothermal therapy. The FC fiber coupled laser was collimated using a fiber collimator with an NA of 0.5 and a focal length of 8 mm. The collimated beam diameter was about 6 mm that yielded a optical power density of about 5.14 W/cm^2. To measure temperature during photothermal therapy, a hypodermic T-type thermocouple with a diameter of 200 µm purchased from Omega was connected to a controller, which was programmed using LabVIEW to record the temperature at desired time points. The schematic of the experimental set up is as shown in Fig. 1.

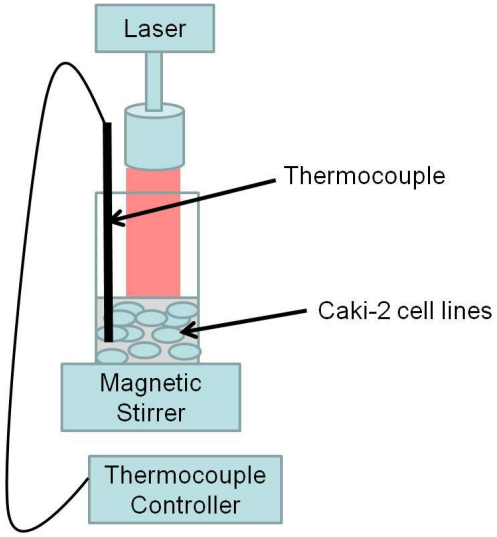

Figure 1: Photothermal therapy experimental setup.

Cells incubated with GNRs were prepared as described earlier before the start of the photothermal experiment. A cell sample of 0.5 ml was placed in a cuvette and exposed to the laser beam from the top. To prevent the cells from settling down during the experiment, which may result in non-uniform illumination of cells, the mixture was gently stirred using a magnetic stirrer throughout the experiment. At every scheduled time point, an amount of 10-µl cell sample was taken out of the cuvette to be tested for viability. During each test, the 10-µl cell sample was mixed with 10 µl of 0.4% (w/v) trypan blue solution. Dead cells were stained blue while live cells remained unstained as their cytoplasm remained intact. Meanwhile, another 120-µl cell sample was taken out and added to a 120-µl cuvette which was placed inside a commercial fluorescence spectrometer to obtain autofluorescence spectrum.

Experimental design

The experiment was categorized into four groups in which the sample of cells incubated with GNRs was abbreviated as cells+GNRs for convenience. Out of the four groups, one was the experimental group consisting of cells+GNRs which was exposed to laser illumination (group 4) and the other three were control groups which were classified as cells+GNRs with no laser illumination (group 3), cells only exposed to laser (group 2) and cells only with no laser illumination as group 1. Experiments in each of these groups were carried out for 0, 2, 4, 6, 8 and 10 mins with the continuous monitoring of temperature. For every time point, a new batch of fresh cells were used in the experiment. At the end of every experiment, autofluorescence was measured from all the four groups immediately and also cell viability test was carried out to determine the percentage of live cells. The entire set of experiments was repeated three times for every group.

The thermal damage due to varying laser exposure time was determined by measuring the autofluorescence and cell viability test at different stages of heating and was compared to the other groups which involved less or no heating. Cell counting was done once for every one of three repititions for each cell sample in the study and was averaged whereas the autofluorescence intensity was averaged between ±10 nm from the peak emission wavelength of FAD (520 nm) and plotted against time for all the groups.

3. RESULTS AND DISCUSSION

The PEGylation layer plays a significant role in the enhancement factor, as the thickness reduces the enhancement reduces which may lead to quenching of autofluorescence. To find the optimal parameter of the PEGylation layer in cells in this study, we have varied the thickness of the PEGylation layer by incubating the mPEG (50 μM) and GNR solution (6.68×10^{11} nanoparticles/ ml) mixture (1:4 ratio) for 6, 12 and 24 h. The PEG layer thickness was later measured using transmission electronic microscope (TEM). Fig. 2 shows the TEM images of the GNRs without and with the PEGylation layer, as the PEGylation duration was increased from 6 to 12 and then to 24 h. With the right PEGylation thickness and by target specific binding to the autofluorescing agent in the mitochondria within the cell may result in higher enhancement. Continuous monitoring of autofluorescence can predict the progress of cell death during plasmonic photothermal therapy thus eliminating the need for alternative time consuming and off-line cell staining techniques to determine cell death.

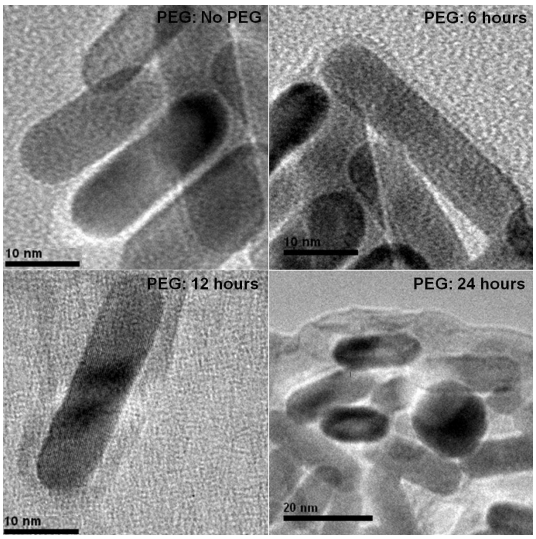

Figure 2: TEM images of the gold nanorods (GNR) without and with the PEGylation layer as the PEGylation duration was increased from, 6 to 12 and then to 24 h. The PEG: 24 h image is zoomed out (scale bar 20 nm) to show the aggregation of GNRs.

The cells incubated with GNRs were illuminated for 10 mins by laser with a power density of 5.14 W/cm^2, the local temperature increased to about 47.5^{o}C resulting in cellular death. The subsequent trypan blue based cell viability test (Fig. 3(a)) showed cell death which can be primarily attributed to necrosis. Due to the sudden increase in temperature during first 4 mins, the cell membrane is damaged [7] allowing the trypan blue to enter the necrotic cell. However, cell death due to apoptosis may be negligible since cell apoptosis becomes significant only when the cells are exposed for longer durations at enhanced temperature between 42°C and 44°C Harmon, Corder [7]. The autofluorescence measurement immediately after the treatment showed a drastic decrease in the emission intensity (NADH and FAD) as compared to the control group as shown in fig. 3(b). The decrease in the measured intensity of the treated cells was due to the mitochondrial damage (cell damage) resulting in the alteration in the redox status of cells [8]. The fluorescence microscope images of the cells can illustrate the autofluorescence and cell viability for monitoring the treatment outcome. The other advantage of using GNRs for autofluorescence to monitor the treatment outcome in PPTT is that the relatively weak cell autofluorescence is enhanced by GNRs, which could increase the sensitivity of this technique to the variation in cell viability. Two-time enhancement in autofluorescence intensity from cells with GNRs as compared to cells without GNRs is achieved.

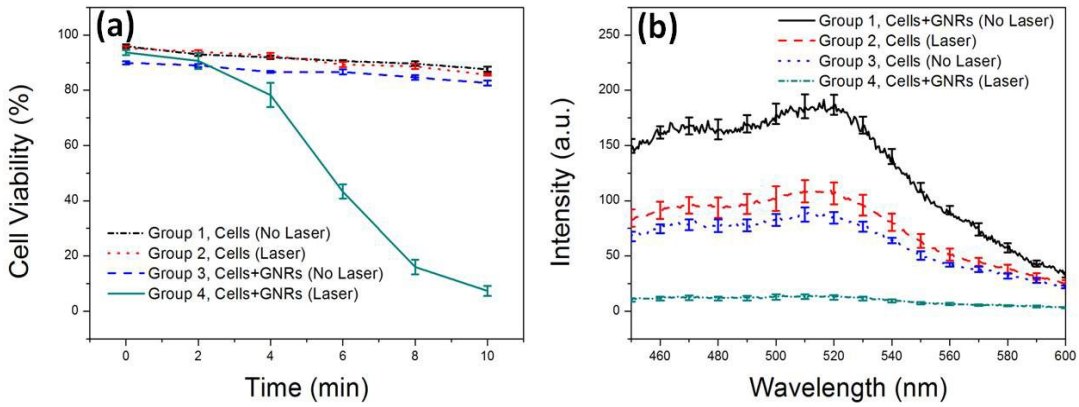

Figure 3: Cell viability and autofluorescence measured in the treatment and control groups. (a) Cell viability as a function of time. (b) Autofluorescence emission spectra of the treatment and control group measured 10 min after the start of the experiments.

Fig. 4 shows the experimental cell viability and autofluorescence data for Group 4. All the values in each curve have been normalized to facilitate comparison in the trend across curves. The data for other groups are not shown because the calculated cell viability shows no change in the cell viability and remained 100%. At 6 mins the calculated cell viability is around 57% as compared to 43% experimentally. After 10 mins, the temperature eventually reaches 47.5°C, and the cell viability drops to less than 10% in the experimental study. The curves show a good agreement between the experimental cell viability and measured autofluorescence especially when the heating time is shorter than 4 minute.

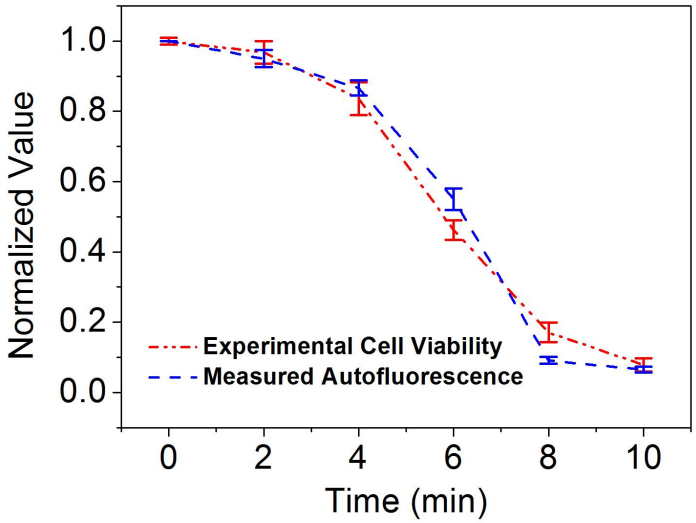

Figure 4: Comparison of the experimental cell viability and the measured autofluorescence as a function of time. The autofluorescence curve was obtained by taking the average cell autofluorescence intensity at the FAD peak (520 ±10 nm).

In the present study, the cell death can be primarily attributed to necrosis due to the sudden increase in temperature in the first 4 mins which reaches the hyperthermic region which in turn results in cell membrane disruption [7, 9] allowing the trypan blue to enter the necrotic cell. At longer durations of laser exposure when temperature steadily increases, the cell viability decreases to as much as 40 % at 6 mins as observed in fig. 3(b) as a result of necrosis. However, cell death due to apoptosis may be negligible since cell apoptosis becomes significant only when the cells are exposed for at least 30 mins at enhanced temperature between 42°C and 44°C [9]. Hence the change in autofluorescence was primarily a result of necrosis. This phenomenon was confirmed by cell imaging results in Fig. 5(a) and 5(b). Autofluorescence image of cells were captured at 5× magnification, in which the excitation wavelength was from a white light source with a bandpass filter at 436±10 nm and the emission band was ranged from 535 ± 15 nm. It can be

observed that the laser exposed section, i.e. the top portion of the figure, of gold nanorods internalized cells showed nearly no autofluorescence in Fig. 5(a) and the cells appear blue when stained with trypan blue in Fig. 5(b). The latter observation suggests that cells exposed to the laser underwent necrosis. In contrast, the cells that were not treated by laser illumination, i.e. the bottom portion of the figure, showed strong autofluorescence in Fig. 5(a) and no blue color in Fig. 5(b).

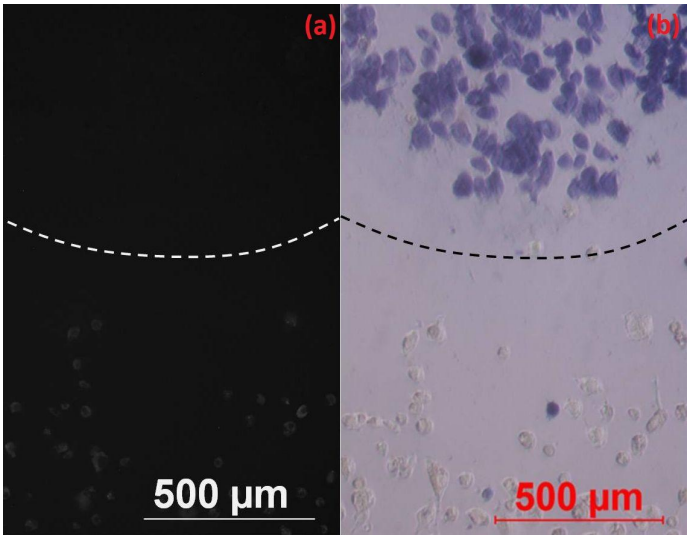

Figure 5: (a) Autofluorescence Image of unstained cells that internalised the GNR for 24 h, in which cells on top of the dashed line were treated by 10-min constant laser illumination and those below the dashed line were not treated. (b) Brightfield image of the same cells stained by trypan blue to highlight necrotic cells.fluorescence emission spectra of the treatment and control group measured 10 min after the start of the experiments.

The results in this study show that apart from enhancing light absorption to facilitate plasmonic photothermal therapy, GNRs can also be used for enhancing the relatively weak cell autofluorescence that can serve as a direct indicator of cell viability. If cell autofluorescence is measured quickly during therapy, one can determine the progress of the treatment online by just monitoring the autofluorescence intensity and thus eliminating the need of performing the time consuming and tedious cell viability test to determine the cell treatment outcome. The continuous monitoring of autofluorescence can help optimize the treatment outcome in real time by providing instant feedback

4. CONCLUSION

Plasmonic photothermal therapy on endocytosed gold nanorods in Caki-2 cell lines was performed. The results show the steady rise in temperature photothermal effect of nanorods when continuously illuminated with near infrared laser. The autofluorescence measurement and cell viability test reveals the progress of cell death when the temperature reached the hyperthermic region. The autofluorescence measurement and cell viability test show a similar trend over the duration of laser exposure. Apart from nanorods assisting the laser therapy of cancer cells it also enhanced the weak autofluorescence by 2 times. The PEGylation layer also plays a significant role in the enhancement factor as the thickness reduces the enhancement reduces. By specific binding of nanorods to the mitochondria within the cell may results in higher enhancement. Continuous monitoring of the autofluorescence can predict the progress of cell death during plasmonic photothermal therapy thus eliminating the need for alternative time consuming and off-line cell staining techniques to determine cell death.

REFERENCES

1. Huang, X., et al., *Plasmonic photothermal therapy (PPTT) using gold nanoparticles.* Lasers in medical science, 2008. **23**(3): p. 217-28.
2. Kannadorai, R.K., et al., *Dual functions of gold nanorods as photothermal agent and autofluorescence enhancer to track cell death during plasmonic photothermal therapy.* Cancer Lett, 2015. **357**(1): p. 152-9.
3. Kannadorai, R.K. and Q. Liu, *Optimization in interstitial plasmonic photothermal therapy for treatment planning.* Med Phys, 2013. **40**(10): p. 103301.
4. Hirsch, L.R., et al., *Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13549-54.
5. Kannadorai, R.K., et al., *Nanosphere Templated Metallic Grating Assisted Enhanced Fluorescence.* Journal of fluorescence, 2011.
6. Wang, H.W., Y.H. Wei, and H.W. Guo, *Reduced Nicotinamide Adenine Dinucleotide (NADH) Fluorescence for the Detection of Cell Death.* Anti-Cancer Agents in Medicinal Chemistry, 2009. **9**(9): p. 1012-1017.
7. Harmon, B.V., et al., *Cell-Death Induced in a Murine Mastocytoma by 42-47-Degrees-C Heating Invitro - Evidence That the Form of Death Changes from Apoptosis to Necrosis above a Critical Heat Load.* International Journal of Radiation Biology, 1990. **58**(5): p. 845-858.
8. Dressler, C., et al., *Fluorescence imaging of heat-stress induced mitochondrial long-term depolarization in breast cancer cells.* Journal of Fluorescence, 2006. **16**(5): p. 689-695.
9. Huff, T.B., et al., *Hyperthermic effects of gold nanorods on tumor cells.* Nanomedicine, 2007. **2**(1): p. 125-132.