5-aminolevulinic Acid Conjugated Gold Nanoparticles for Cancer Treatment

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ABSTRACT

In photodynamic therapy (PDT) for cancer treatment, effective delivery of photosensitizer to the target tumor and minimal damage to the healthy cell is of paramount importance. In this study novel biocompatible positively charged (ξ = +33 mV) gold nanoparticles (30 nm average diameter) are conjugated with a photosensitizing precursor, 5-aminolevulinic acid (ALA) at physiological pH (pH 7.2 to 7.6). As verified by fluorescence spectroscopy of protoporphyrin IX (PpIX), they are preferentially accumulated on the tumor cells. They are found to promote the formation of reactive oxygen species (ROS) and significantly increase the killing efficiency of WT cells. Furthermore, the gold nanoparticles exhibit surface-enhanced Raman scattering (SERS) activity, making them a powerful imaging vehicle for the monitoring of cellular molecular events during cancer treatment using PDT.

Keywords: photodynamic therapy, photosensitizer, nanoparticles, surface-enhanced Raman scattering,

1 INTRODUCTION

Cancer is a life-threatening disease of global proportion. Photodynamic therapy is one of the minimal invasive cancer treatment techniques. It is based on the administration of a photosensitizing compound (also called photosensitizer) and subsequent irradiation with an appropriate wavelength light to produce reactive oxygen species (ROS) for selective damage of the neoplastic tissue [1]. A few photosentizers have been developed. The Photofrin (PF) and 5-aminolevulinic acid (5-ALA) are the most active photosentizers in clinical application. Photofrin® (PF) has some inherent disadvantages, including prolonged skin sensitivity necessitating avoidance of sunlight for many weeks [2], sub-optimal tumor selectivity [3], poor light penetration into the tumor due to the relatively short wavelength used (630 nm) [4], and the fact that it was a complex mixture of uncertain structure [5]. Kennedy and Pottier [6] were the first to apply ALA-based PDT clinically, where 5-ALA was applied topically. The use of 5-ALA as a protoporphyrin IX (PpIX) precursor for PDT is popular for several reasons: (1) ALA is the only PDT agent that is a biochemical precursor of a photosensitizer, which is naturally produced by the body, and alone shows low dark toxicity to cells. (2) Topical delivery of 5-ALA does not give any prolonged photosensitivity reactions, because the drug can be selectively applied in the areas to be treated. (3) Endogenously produced PpIX is rapidly cleared from the body (24–48 h) because it has a natural clearance mechanism. (4) The short time interval (1–8 h, depending on the mode of administration) needed between the administration of 5-ALA and the maximal accumulation of PpIX in target tissues makes 5-ALA attractive for patients. However, the 5-ALA is hydrophilic and does not easily penetrate through intact skin, nodular skin lesions, the stomach and the intestinal walls and in general through cell membranes [7, 8]. The cellular uptake of 5-ALA is limited by its hydrophilic nature and improved means of delivery are being sought. Therefore, the nanotechnology-based effective drug delivery is certainly an attractive option. This study was intended to develop biocompatible nanoparticles for effective drug delivery and treatment.

2 MATERIALS AND METHODS

2.1 Synthesis of gold nanoparticles

0.2 mg/ml of branched polyethyleneimine (BPEI) (molecular weight = 10,000) 40 ml and 0.01% of $HAuCl_4$ 40 ml were mixed and stirred for 5 min in ice bath. Then, it was placed under 400 W metal halide UV lamp (Cure Zone 2) for 1 hr until the color change from yellow to dark red with the completion of the reduction reaction.

2.2 ALA conjugation

5-aminolevulinic acid (5-ALA) was purchased from Sigma-Aldrich, USA. It was mixed with serum free Dulbecco's Modified Eagle Medium (DMEM) to get 1 mM concentration of 5-ALA followed by filtering with 0.2 μm filter. Gold nanoparticles were filtered with 0.2 μm filter as well. 100 ml 5-ALA containing DMEM media was subsequently mixed with 200 μl gold colloidal solutions. The pH of the final solution was adjusted between pH 7.2 and 7.4 with 1N NaOH solution. Fresh solution was used for every experiment due to its sensitivity to temperature and ambient [9].

2.3 Cell seeding and culture

Human neonatal dermal fibroblasts (NHDF, passage 1) were purchased from Cambrex BioScience Walkersville, Inc. (Walkersville, MD). Cells were subcultured in FGM-2 medium (Cambrex) till passage 4-6 for use in the experiments. Cell splitting was performed when the monolayer cells reached 70-80% confluence. WT cells (HT 1080) were purchased form ATCC. The same medium was used for both cell lines.

To seed the cells into 96 wells, cells were trypsinized, centrifuged and resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, ATCC, Manassas, VA) and 1% penicillin and streptomycin at a final concentration of 1.5×10^4 cells/mL. 200 µL cell suspensions were seeded in each well. After seeding, cells in the wells were cultivated in DMEM with 10% FBS and 1% penicillin and streptomycin in a humidified incubator at 37 ºC with 5% CO_2 for two days.

2.4 PpIX formation

The 96 seeded well plates were replaced with serum free medium (for control), gold nanoparticles-containing medium, 1 mM 5-ALA medium and a medium containing 5-ALA conjugated Au nanoparticles. Kinetics of PpIX formation can be observed using multi-detection microplate reader (Synergy^{TM} HT, BIO-TEK) for fluorescence intensity at 630/20 nm with excitation wavelength 470/50 nm for different samples at different incubation time. Before using the microplate reader, the medium in the well was aspirated and washed with Phosphate Buffer Solution (PBS). Each well was replaced with 100 µL Hank's Balanced Salt Solution (HBSS).

2.5 Photodynamic therapy

The sample preparation procedures were the same as above. The incubation time was 4 hr for PDT. The well plates were washed with PBS under dark condition and solution-exchanged with 100 µL HBSS. Lasers as well as non-coherent light sources have been generally used for PDT [10]. In our investigation, the samples were irradiated with non-coherent light sources (broadband halogen lamp, 150 W, Dolan-Jenner Fiber-Lite MI-150) for 1 min. The wells were then exchanged with the serum-containing DMEM medium and incubated for one day before MTT test.

2.6 MTT assay

Thiszolyl Blue Tetrazolium Bromide (MTT) was dissolved in HBSS (5 mg/ml). After PDT treatment, 100 µl MTT working solution (0.5 mg/ml) was added into each of the 96 wells. After one hour incubation, the MTT was replaced with 100 µl Dimethyl sulfoxide (DMSO) to dissolve the formazan crystal and the absorbance was measured using Synergy HT microplate reader (BIO-TEK) at 570 nm wavelength.

2.7 Co-culture experiment

WT cells were stained with CellTracker^{TM} Green CMFDA (Invitrogen) for 30 minutes. After one day culture, cells were trypsinized, centrifuged and resuspended in DMEM. The same amount of NHDF and WT cell were co-cultured using the same medium, followed by the PDT procedure as above. After PDT treatment, treated culture was further cultured for 24 hrs. Then the fluorescence images were taken for the co-culture using Nikon Eclipse TE 300 fluorescence microscope.

3 RESULTS AND DISCUSSION

Positively charged gold nanoparticles (Au NPs) were synthesized by UV assisted reduction method. Absorption maximum of gold colloid was found at 520 nm wavelength (Fig.1). They had zeta potential (ξ) +33 mV and average diameter of 30 nm. 5-ALA, a zwitterionic compound, [11] can form end groups of COO^- and NH_3^+, the extent of which depends on the pH of the solution. It has net negative charge at the physiological pH (pH7.2 ~ 7.6), which creates favorable condition for conjugation with positively charged nanoparticles. According to absorption spectroscopy, addition of 1 mM 5-ALA in the gold colloids did not result in no change in the absorption band. However, changes occurred as 5-ALA concentration increases to 10 mM and 100 mM where a new absorption peak emerged. The appearance of new absorption band can be attributed to nanoparticles conjugation promoted by a high concentration of 5-ALA. The SEM micrograph insets in Fig.1 show the gold colloid without (left) and with 100 mM 5-ALA (right).

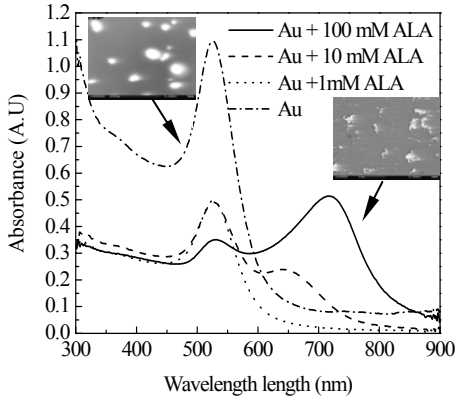

Figure 1: UV-Vis Absorption spectra of with and without 5-ALA conjugated gold nanoparticles

While 5-ALA itself is neither fluorescent nor a photosensitizer, it can induce the biochemical formation of Protoporphyrin IX (PpIX). Two molecules of ALA are converted into porphobilinogen, and then five enzymes, three cytosolic and two mitochondrial, lead to the formation

of PpIX, which is a very efficient photosensitizer. The PpIX formation is dependent on cell type and tissue [12]. The PpIX formation increases (1) with the pH value of 5-ALA, (2) with the 5-ALA incubation time in a moderate sigmoidal manner, and (3) with the 5-ALA concentration up to 700 μg ml^{-1}. Other parameters, such as cell washing procedures, have no influence on the PpIX production [13]. 5-ALA with serum-free medium was used throughout the research because albumin causes efflux of PpIX from cells [14]. The kinetics of PpIX conversion form 5-ALA was studied with above mentioned experimental method for two cell lines, NHDF and WT. Fig. 2 and 3 are the fluorescence intensity results of NHDF and WT respectively. It can be seen clearly that WT cells produce 5-7 times of PpIX than NHDF cells. The kinetics of PpIX formation is highly dependent on cell type. Cancer cells produce more PpIX than normal cells. Moreover, ALA conjugated Au NPs exhibit a similar trend with ALA for up to 20 hrs. Gold nanoparticles did not obstruct the function of PpIX production in both cell lines.

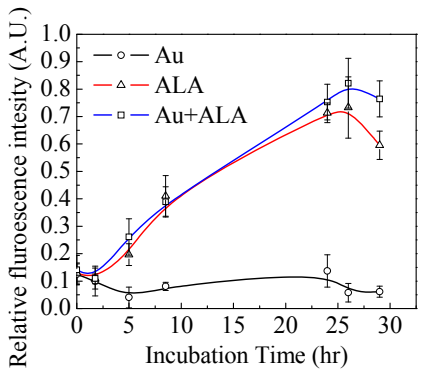

Figure 2: NHDF cell fluorescence intensity for various incubation time with Au NPs, 5-ALA and 5-ALA-Au NPs

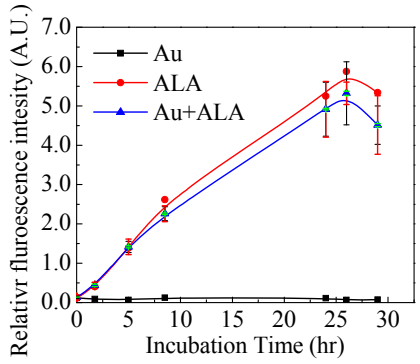

Figure 3: WT cell fluorescence intensity for various incubation time with Au NPs, 5-ALA and 5-ALA-Au NPs

The processes of light absorption and energy transfer are at the heart of PDT. The ground state PS has two electrons with opposite spins (this is known as singlet state) in the low energy molecular orbital. Following the absorption of light (photons), one of these electrons is boosted into a high-energy orbital but keeps its spin (first excited singlet state). This is a short-lived (nanoseconds) species and can lose its energy by emitting light (fluorescence) or by internal conversion into heat [15]. Highly reactive $^1O_2$, formed during PDT, was found to be the main toxic reagent. Evidence for the production of $^1O_2$, its role in cell photoinactivation during PDT and the importance of oxygenation status in tumor tissues were extensively studied [16-18]. The $^1O_2$ can diffuse less than 0.05 μm during its short lifetime from its site of origin before reacting with, or being quenched by, a variety of cellular targets [18]. Therefore, photodynamic damage occurs close to the localization of photosensitizing molecules during light exposure. The subcellular localization of the photosensitizer is of crucial importance, since it determines the localization of the primary damage and its impact.

In Fig.4 shows the MTT results of NHDF and WT which were incubated for 24 hrs with gold nanoparticles, 1 mM 5-ALA and 5-ALA conjugated gold nanoparticles at dark condition. No significant cytotoxicity was observed. Fig. 5 shows the PDT treatment of NHDF and WT cells which were irradiated with 150 W broadband halogen light source for 1 min. No cytotoxicity was observed for gold nanoparticles- containing medium. However, at the 5-ALA-containing medium, only 50% of cells were alive in both NHDF and WT. Moreover, the efficiency of killing WT cells was significantly increased (by ~ 30-50%) in the 5-ALA conjugated gold nanoparticles treated group compared to 5-ALA treated group. This enhancement most likely results from the plasmon resonance effect of Au nanoparticles.

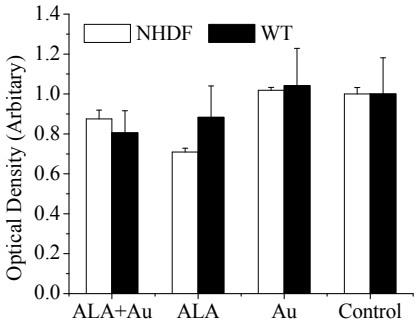

Figure 4: MTT test for without irradiation

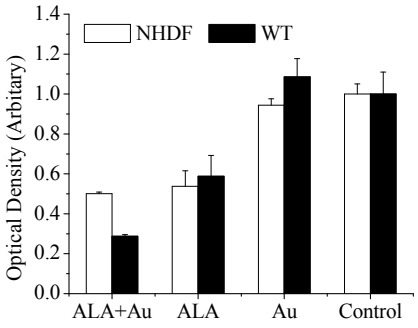

Figure 5: MTT test for 1 min irradiation

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Co-culture experiment was performed to evaluate the selectivity of ALA-AuNPs. As shown in Fig. 6 (a), WT cells were labeled green and remained green during the co-culture. The co-cultured cells were treated with ALA-AuNPs followed by PDT. After cultured for 24 hours, the culture was examined under a fluorescent microscope. The examination showed that the number of WT after PDT treatment was lower than that prior to PDT. As marked in Fig. 6, 39 WT cells before treatment and 24 WT cells after treatment were found. Taking the average of 5 randomly selected regions, it was found that about 50% WT cell were killed after PDT treatment. In contrast, NHDF (non-labeled) became more confluence than before treatment, and cells remained spindle shape. This observation suggests that ALA-AuNPs have more killing effect on WT cells than on NHDF.

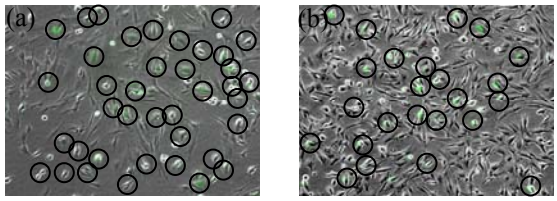

Figure 6: Light microscopy image of the co-culture. (a) before PDT treatment and (b) after 1 min irradiation and further cultured for 24 hours. WT cells labeled green.

4 CONCLUSIONS

The feasibility of biocompatible ALA conjugated gold nanoparticles that offer selective and effective drug delivery has been demonstrated for PDT treatment of cancer. The killing efficiency of WT cells was significantly increased (by ~30-50%) in the ALA-Au treated group compared to ALA treated group by MTT assay. This enhancement most likely results from the plasmon resonance effect of Au nanoparticles that intensified local light field. Given the SERS-active nature of the gold nanoparticles, concurrent Raman imaging of cellular molecular activities during PDT can be carried out.

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