Photothermal Therapy of Acute Leukemia cells in the Near-Infrared Region Using Gold Nanorods CD-33 Conjugates

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ABSTRACT

In the present work, we demonstrate a potential use of gold nanorods as a contrast agent for selective photothermal therapy of human acute leukemia cells (HL-60) using a near-infrared laser. Gold Nanorods (GNR) are synthesized and conjugated to CD33, a 67 kDa glycoprotein found on the surface of myeloid cells that belongs to the sialoadhesin family of proteins. After pegylation, or conjugation with CD33 antibody, GNR were non-toxic for acute and chronic leukemia cells. We used a Quanta System q-switched titanium sapphire laser emitting at a center wavelength of 755 nm. Each sample was illuminated with 1 laser shot at either high or low fluence. Both laser modes were used in 3 independent cell probes. HL-60 cells were treated for 45 min with GNR conjugated with mAb CD33, or with GNR-Pegylated particles. After laser application, the cells were resuspended and analyzed to cell viability with Trypan blue exclusion assay. GNR-CD33 conjugates significantly increase the percentage of cell death as compared with a control group after laser illumination: a 3 fold increase is observed.

Key words: contrast agents, optoacoustic, bubble formation, laser nanothermolysis, gold nanorods conjugates, CD33 antibody, human leukemia, cell selective targeting.

INTRODUCTION

The work presented in this paper focuses on the experimental observation of cell destruction through laser-induced microbubble formation. The study is motivated by a well-documented complication arising from leukemia treatment: the contamination of transplants by residual tumor cells. This complication drastically limits the efficacy of auto-transplantation. Laser ablation of leukemia cells may be applied for elimination leukemic cells from the graft. The principles of optoacoustic processes rely on the occurrence of thermal confinement which occurs when the laser pulse duration is small compared to the transit time of sound through the penetration depth of the light[1]. Since the density of the medium cannot change substantially during laser pulse action, an important stress field is created as a result of the inhomogeneous temperature field, and an acoustic signal is generated. In the case of photothermal therapy, this effect is furthered by increasing the light fluence to a point where not only thermal confinement occurs, but also an actual phase transition of the medium surrounding the region of interest[2]. Specific contrast agents for use in optoacoustic spectroscopy were developed to enhance the optoacoustic contrast (i.e. improve signal to noise ratio), and to improve the specificity of target detection. Metallic nanoparticles have high optoacoustic contrast, and can be conjugated with specific ligands, such as antibodies, to produce the targeted contrast agents. Recently, we demonstrated that gold nanorods constituted a new nanoparticulate optoacoustic contrast agent[3]. Gold nanorods feature plasmon resonances: collective charge oscillations excited by light, known to be strong absorbers[4]. These nanoparticles were designed and fabricated to possess exceptionally strong plasmon resonance absorption peaks which can be tuned to any desirable wavelength in the near-infrared spectral range by changing their aspect ratio[5-7]. Tailoring the dimensions and shape of a nanoparticle permits optimization of the plasmon-derived optical resonance for maximum optical absorption[4]. For example, cylindrical or elliptical

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nanoparticles with an aspect ratio of 3.5 will strongly absorb the wavelength of 755-760 nm, which can be obtained from various lasers, such as the Titanium Sapphire (Ti:Sapph) laser used in this work, as well as widely tunable systems like optical parametric oscillators (OPO). Published results have indicated that gold nanorods, being the size of a cluster of a few large protein molecules (roughly 15x50 nm), can absorb light about one thousand times more strongly than an equivalent volume of an organic dye[4,8]. The optoacoustic efficiency of these contrast agents is also due to the localized superheating of the particles. As they are unable to release energy as fluorescence, superheating results in evaporation of a nanolayer of surrounding water[1], which produces acoustic waves up to an order of magnitude stronger than that from a homogeneously absorbing solution with equal average absorption coefficient[9]. Through accurate control of the illumination parameters, it is therefore possible to carefully tune the quantity of radiation energy deposited in the sample to readily destroy cancer cells while excluding normal cells. The number of CD33 receptors is higher on the surface of acute leukemia cells[10] and therefore these cells were used for thermotherapy experiments.

EXPERIMENTAL

Laser system

We used a Quanta Systems Nd:YAG second harmonic (532 nm) pumped titanium-sapphire (Ti:Sapph) laser emitting at a center wavelength of 755 nm. Through a motorized system of polarizing optics, the output energy can be varied continuously up to about 100 mJ per 10 ns pulses. The laser is engineered to operate at a repetition rate of 10 Hz. In order to adequately control the experimentation conditions (energy deposited per sample), a custom-made switch box was devised to allow for single-pulse operation. By design, the output radiation exits the laser through an aperture on top of the device, and connects to an articulated arm. For the purpose of this experiment, a simple system shown in the next figure was assembled mostly from off-the-shelf parts available from Thorlabs: it replaces the arm and mounts directly onto the laser system. It features optical tubes and a plano-convex (thus focusing) lens mounted in an X-Y adjustable holder. The curvature of the lens is chosen such that the focal spot is located above the top of the assembly, where a cage is located.

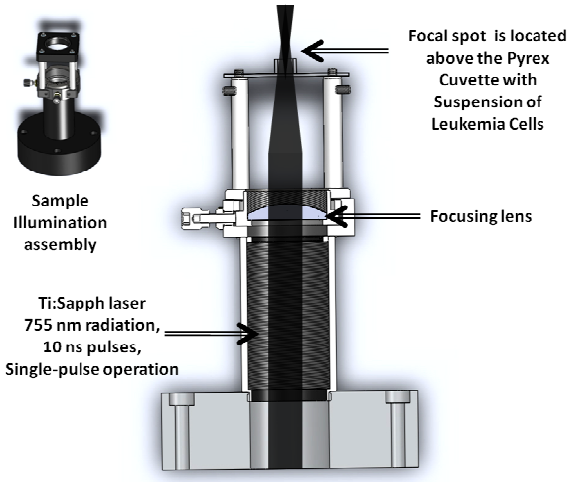

Figure 1: Sample holder for pulsed-laser nanothermolysis experiment

Therefore, it is possible to carefully locate the focus high enough above the sample such that the beam diameter at the bottom of the cuvette corresponds exactly to the diameter needed to achieve the required fluence. In the present case, we have used both a low and a high fluence setting, with values respectively of 0.6 and 1.1 J/cm^2. A diameter of 1.7 mm at 100 mJ output energy allows achieving the high fluence requirement. Furthermore, it ensures the bottom of the cuvette is fully illuminated by the laser beam. Doing so implies the volume of the laser beam traveling

through the cuvette does match that of the cuvette itself and maximizes illumination of the whole sample. Unfortunately, because of the Gaussian nature of the illumination beam profile, a fraction of the sample is experiencing weaker illumination, and our nanothermolysis yields are thus lower than optimal. Our sample holders, or cuvettes, were assembled in-house. They are made of 4mm sections of Pyrex tube with 3 mm inside diameter affixed onto onto a microscope slide with cyanoacrylate adhesive.

Fabrication and covalent conjugation of gold nanorods with PEG and monoclonal antibody CD33

Presented below are the details of our GNR fabrication protocol adapted from previously reported methodology[11,12]. The base procedure is tailored to the needs of the specific experiments presented in this paper. It allows high-yield fabrication of a narrow size distribution of rods with a 755 nm Plasmon Resonance. In a typical procedure, 0.250 mL of an aqueous 0.01 M solution of $HAuCl_4 \cdot 3H_2O$ was added to 7.5 mL of a 0.1 M CTAB solution in a test tube (15 ml glass tube). Then, 0.600 mL of an aqueous 0.01 M ice-cold $NaBH_4$ solution was added all at once. This seed solution was used 2-4 hours after its preparation. In the next step of the fabrication, exact proportions of 4.75 mL of 0.10 M CTAB, 0.200 mL of 0.01 M $HAuCl_4 \cdot 3H_2O$, and 0.030 mL of 0.01 M $AgNO_3$ solutions were added one at a time in the preceding order, then gently mixed by inversion. The solution at this stage appeared bright brown-yellow in color. Then 0.032 mL of 0.10 M Ascorbic Acid was added. The solution became colorless upon addition and mixing of Ascorbic Acid. Ten minutes were allowed for the reaction to fully proceed before adding the required quantity of seed solution. The reaction mixture was gently mixed for 10 seconds and left undisturbed for 1-3 hours. After that the solution was left under thermostatic conditions for 24 hours at the temperature of 30^o C. Before covalent binding with Polyethylene Glycol (PEG), or conjugation with monoclonal Antibody CD33, the GNR were centrifuged at low speed (4000 rpm, 10 min) for separation of other aggregates (platelets, stars). The pellet was removed and for the next steps, only the supernatant fraction was used. For Pegylation[13,14] the GNR solution was centrifuged at 14000 g for 10 minutes, the supernatant was removed, and the pellet was resuspended in DI water to reduce CTAB concentration to 0.01 M. Then, 0.1 ml of 2 mM potassium carbonate (K_2CO_3) was added to 1 ml of aqueous GNR solution and 0.1 ml of 0.1 mM mPEG-Thiol-5000 (Laysan Bio Inc., Arab, AL). The resulting mixture was kept on a rocking platform at room temperature overnight. Excess mPEG thiol was removed from solution by two rounds of centrifugation and final resuspension in PBS (pH 7.4).

A protocol for conjugation of GNR has been developed at TomoWave Laboratories. It replaces CTAB as was described before[14,15] on the surface of gold nanorods with Nanothink-16 and polyethyleneglycol, and monoclonal antibody CD33. One mL of synthesized GNR in CTAB was centrifuged twice in a 1.5 mL Eppendorf tube at 14000 RPM for 10 minutes and resuspended in one mL of Milli-Q water (MQW) to a concentration of 1 nM. Then, 10 µL of 5 mM Nanothinks acid16 (Sigma) in ethanol was added to the GNR solution, and the solution was sonicated for 30 minutes at 50°C to prevent aggregation. The solution was centrifuged at 12000 RPM for 10 minutes, the supernatant was removed and the pellet was resuspended in MQW. 10 µL EDC (1-ethyl-3-[3-imethylaminopropyl] carbodiimide hydrochloride (Pierce) and sulfo-NHS (Pierce) was added from stock solution in MES (2-(4-Morpholino)ethane Sulfonic Acid) buffer in 10 mM and 0.4 mM, respectively. The mixture was sonicated for 30 minutes at room temperature to produce activated GNR (GNR that are capable of binding to the amine side chain of proteins). Commercial, purified, mAb CD33 (BD Pharmingen) was added to a final concentration of 25 μg/mL to 1 mL of 1 nM activated GNR. The mixture was sonicated at room temperature for 1 hour and then left on a rocking platform overnight. Following the removal of excess CD33, 10 μL of PEG-Thiol (1 mM) was added to 1 mL of GNR-CD33 conjugates and the mixture was incubated at room temperature for 12 h. The solution of GNR CD33 PEG conjugate was centrifuged at 12000 g for 10 minutes, the supernatant was removed and the pellet was resuspended in PBS pH 7.4 to a concentration of 1 nM (or optical density around 4.0 as measured by Beckman 530 and Thermo Scientific Evolution 201 Spectrophotometer).

Zeta-potential and Protein determination

The zeta-potential of GNR before and after conjugation was measured with a high performance particle sizer (Malvern Instruments Ltd., Southborough, MA, USA) at 25^{o}C, and ten 20-second runs were performed for each sample. Zeta-potential is a measure of both particle stability and adhesion. More negative or positive values of zeta-potential are associated with more stable particle solution, because repulsion between the particles reduces the

particle aggregation[16,17] . A measure of total and bound protein (mAb CD33) was performed with the Pierce Micro BCA™ Protein Assay Reagent Kit (Pierce). Concentration of CD33 was measured before, and after adition of GNR-activated solution: it is dependent upon either level of Antibody, or incubation time. The determination was performed through measurement of absorbance at or near 562 nm. It is important to note that the ratio of absorbances at 562 nm (proteins relative to BSA) has a coefficient of variation of only around 10%.

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Cell culture and laser application

Two human cells lines were used. These are K-562 (chronic leukemia, CML) and HL-60 (acute leukemia), both grew at 37.0°C, 95% air and 5% carbon dioxide, with renewal of the medium every 2-3 days. All cell lines were obtained from ATCC (Manassas, VA) and were grown in 10 ml flasks in RPMI-1640 medium with 10% fetal bovine serum (Hyclone). For toxicological experiments cell were slowly centrifuged (500 RPM) and gently resuspended in medium. The toxic effects of GNR were quantified through use of Trypan blue staining. GNR-CD33 conjugates and GNR different modification and control cells were added to each small non-hermetically closed Eppendorf tube at the required final concentration. For laser thermotherapy acute leukemia cells were pretreated for 45 min with GNR conjugates in concentration of 250 pM (OD 1.0). After centrifugation and removal of supernatant, the cells were resuspended in a small volume of PBS (pH 7.4) and put in a cuvette (25 µl), ready for irradiation. The laser focal spot is located above the sample cuvette for maximum coverage of the number of cells being irradiated. After this, cells were resuspended, and stained with Trypan blue in order to count the number of dead and living cells. All experimental data presented is analyzed as mean ± SD

RESULTS AND DISCUSSION

The following figure presents the UV-VIS spectra of Gold Nanorods before (CTAB) and after Pegylation (PEG), and Conjugation with Antibody (CD33). Normalization was performed to even out the intensity of the absorption maximum at the transverse plasmon resonance. We demonstrate constant absorption line width as well as insignificant red shift of the Plasmon resonance maximum, thereby indicating the success of the conjugation.

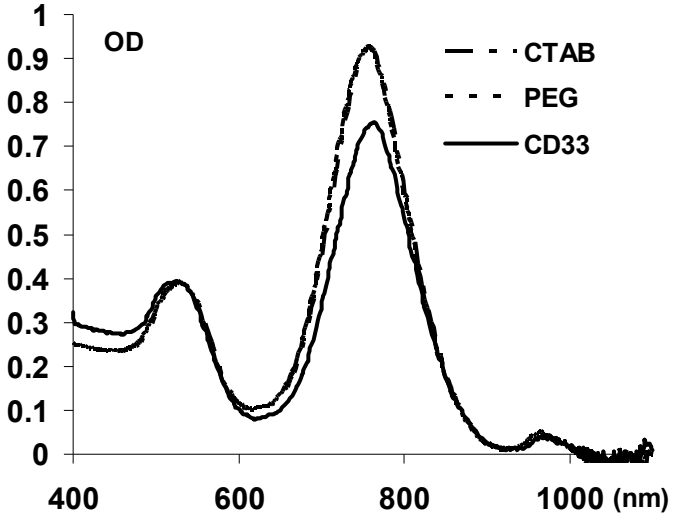

Figure 2: Normalized UV-VIS spectra of Gold Nanorods before (CTAB) and after Pegylation (PEG), and Conjugation with CD33 Antibody (CD33)

The intensity of the spectral features in the UV VIS at a level of OD 1 corresponds to a concentration of GNR in solution around 250 nM, or 1.55×10^{11}GNR/ml. In this project we then proceeded to determine the extinction coefficient of our GNR. Gold has a face-centered cubic (fcc) unit cell with 4 atoms per unit. The density of the solid

is 19.32 g/cm^3. From this each nm^3 contains 59 atoms of gold. GNR size from the protocol of our synthesis is around 15 x 50 nm[11]. The GNR is cylindrical with hemispheric caps, yielding a volume of around 7850 nm^3 and surface area of 2700 nm^2. From the volume, we determine that GNR have a molar weight of 9.1×10^7. Therefore, one GNR weighs 1.51×10^{-13} mg. We have measured the yield of GNR formation after synthesis for a solution with maximum plasmon resonance of 760 nm and OD 2.0. The stock solution was first concentrated by centrifugation and then by evaporation in dry tubes. The total mass of GNR was found to be 47 ± 7.37 mg/L. It is important to note the theoretical maximum mass yield of GNR from our protocol is 158.6 mg per liter of solution. Our yield is near 30% after removal of the pellet containing mostly platelets and stars, and other non-rod-like particles (around 12 mg/l). From this we conclude that our solution with OD 2.0 has 3.11×10^{14} GNR/l, or a concentration of 0.52×10^{-9} M and an extinction coefficient of 3.85×10^9 $M^{-1}cm^{-1}$. These data corroborate results from several groups[18 15,19]

The zeta-potential of the GNR-CTAB complex was highly positive due to the presence of the positively charged CTAB molecules on the surface of the rods. The GNR-mAb CD33-PEG complex nanoparticle solution showed a zeta-potential which is slightly negative, but significantly different from zero. These results (UV VIS Spectra, and zeta-potential measures) suggested that this composition is non-precipitated complex. Zeta potential changes do confirm surface chemistry modifications, and correspond to previously published data[20,21].

GNR-CTAB	GNR-PEG	GNR-CD33-PEG
52 ± 18.9	- 17 ± 13.3	- 9 ± 3.3

Table 1: Zeta-potential of the GNR-CTAB, after GNR Pegylation and conjugation with CD 33 antibody in table.

Statistical projections of the number of antibodies that can attach to a nanorod aid in understanding later biochemical and optoacoustic events. Using simple geometry, we can estimate the total area of a nanorod measuring 15x50 nm (aspect ratio of 3.5, plasmon resonance around 760 nm) to about 2700 nm^3. Crystallographic data of common antibodies is widely available; herceptin (HER), the antibody used in the following experiments, has a footprint of about 3-4 nm in radius. Assuming general closest bi-dimensional packing ratio of 0.9, we can calculate we'd observe a maximum of 45-55 antibodies on one nanorod (statistical upper limit). This value has also been determined experimentally, yielding a number of CD33 per one GNR in range 27-40 with maximum conjugation after 12 hours (Fig.3).

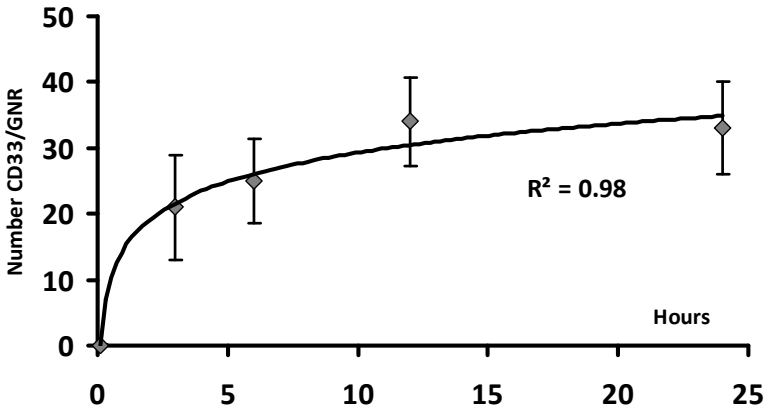

Figure 3: Binding of CD33 on the Surface of GNR: Incubation Time Effects (Hours)

A series of toxicology experiments was needed in order to optimize working concentration of GNR in cell suspension with minimum, or without, toxic damage. Fig.4 presents the results for GNR administration, in different

concentrations, for acute (HL-60), and chronic leukemia (K-562) cells. We cannot observe statistically significant cell damage for concentrations lower than 1 nM, and a level of GNR lower or equal to 0.25 nM has not significantly increased the number of dead cells.

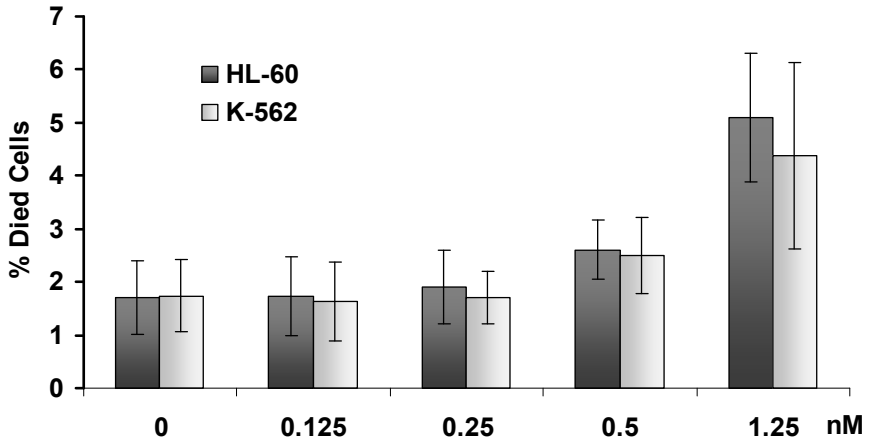

Figure 4: Cell death for Chronic (K562) and Acute (HL-60) Leukemia Cells after GNR-Peg administration at different concentrations (24 hours, n=4)

We know that CTAB alone, as a quaternary ammonium surfactant, can kill living mammalian cells in sufficient doses[21]. Our experiments demonstrated similar results (Fig.5). The number of dead cells after GNR-CTAB administration is 6-9 times higher than with untreated cells, or with cell after pretreatment with pegylated GNR (GNR-PEG). The anti-CD33 (Siglec-3) antibody Gemtuzumab (Mylotarg™) is approved for treatment of acute myeloid leukemia[10]. We can see (Fig.4) that after treatment of leukemia cells with CD33 alone, the number of dead HL-60 cells increases twofold (220 %), with an increase of 150% for K-562 cells. The treatment of leukemia cells with GNR-conjugates with CD33 and PEG demonstrated the same effect: the number of dead acute leukemia cells is significant for both cell lines. It is confirmed that after conjugation, mAb CD33 retains its activity for selective binding with CD33 antigen.

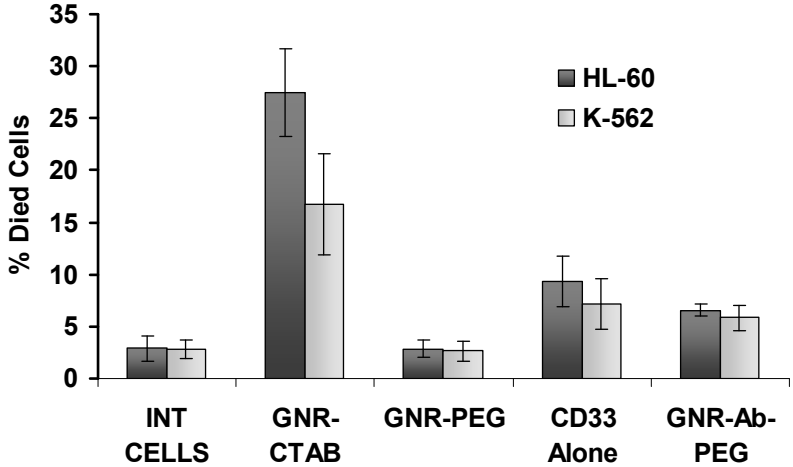

Figure 5: HL-60 and K562 Cells Death (percentage) after Gold Nanorods Conjugate Administration (for 48 hours, 500 pM or 3×10^{11} GNR/ml)

The effect of pulsed-laser nanothermolysis of Acute Leukemia Cells (Fig.6) demonstrated that laser application without prior treatment shows no measurable increase in cell death. The treatment increases the number of dead cells after pretreatment with GNR-PEG (non specific binding condition). However, for cells treated with GNR-CD33 conjugates, the fraction of dead cells was significantly higher for both high fluence laser (more than 4 fold increase) and also low fluence (3 fold increase). This level of cell death correlates with high-specificity and selective binding of GNR-CD33 conjugates with receptors on the surface of acute cancer cells.

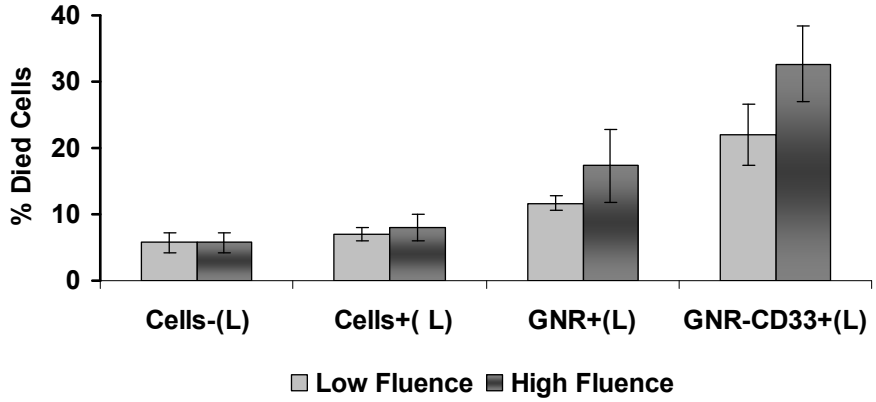

Figure 6: Pulsed-laser Nanothermolysis of Acute Leukemia Cells HL-60 Targeted with Gold Nanorods Conjugated with CD-33 Antibody: Cells Death (percentage)

CONCLUSIONS

Gold nanorods display exceptionally high optical absorption coefficient tunable in the NIR. Specificity is provided by molecular vectors against cancer-specific receptors: improved accumulation of GNR-CD33 complexes on the surface of human leukemia cells. GNR-CD33 conjugates significantly increase the damage of Leukemia cells after laser illumination. We demonstrated the potential use of gold nanorod conjugates as a contrast agent for selective Nanothermolysis of human acute leukemia cells using a near-infrared pulsed-laser. We hypothesize our yields could be closer to optimal through beam shaping the illumination laser from a Gaussian to a top hat in order to ensure irradiation of the entire volume at the specified fluence.

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