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Laser immunotherapy with gold nanorods causes selective killing of tumour cells

Rejiya C.S.[a], Jatish Kumar[b], Raji V.[a], Vibin M.[a], Annie Abraham[a,*]

[a] Department of Biochemistry, University of Kerala, Kariavattom Campus, Thiruvananthapuram 695 581, Kerala, India
[b] Photochemistry Research Unit, National Institute for Interdisciplinary Sciences and Technology (NIIST), CSIR, Thiruvananthapuram 695 019, Kerala, India

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ABSTRACT

Therapeutic approaches that exploit nanoparticles to deliver drugs selectively to cancer cells are currently considered one of the most promising avenues in the area of cancer therapeutics. Recently, gold nanorods (AuNRs) have shown promising biological applications due to their unique electronic and optical properties. In this paper, we have demonstrated the anti-cancer potential of gold nanorods with low power laser light. Gold nanorods (AuNRs), surface modified with poly (styrene sulfonate) PSS and functionalized with epidermal growth factor receptor antibody conjugated with gold nanorods (anti-EGFR–AuNRs) were successfully synthesised and characterized by UV–Visible–NIR spectrophotometry and High Resolution Transmission Electron Microscopy (HR-TEM). Inductively Coupled Plasmon Atomic Emission Spectrometry (ICP-AES) and Immunofluorescence studies confirmed the efficient uptake of these functionalized gold nanorods by human squamous carcinoma cells, A431. The *in vitro* photothermal therapy was conducted in four groups – control, laser alone, unconjugated AuNRs with laser and anti-EGFR conjugated AuNRs with laser. Phase contrast images have revealed cell morphology changes and cell death after the laser irradiation. In order to determine whether the cell death occur due to apoptosis or necrosis, we have evaluated the biochemical parameters such as lactate dehydrogenase release, reactive oxygen species level, mitochondrial membrane potential and caspase-3 activity. Flow cytometry analysis have shown the cell cycle changes after laser irradiation with antibody conjugated gold nanorods. Thus the results of our experiments confirmed that immunolabeled gold nanorods can selectively destruct the cancer cells and induce its apoptosis through ROS mediated mitochondrial pathway under low power laser exposure.

1. Introduction

Past decade has witnessed a significant advancement in utilizing metal nanoparticles for biomedical applications. Gold, which possess several unique shape/size-dependent properties, strong absorption/scattering of light, stability and non-toxic nature [1] and [2], is one among them. Currently, gold nanoshells, nanocages, and nanorods (AuNRs) are the primary candidates being investigated as potential phototherapeutic agents, each demonstrating strong absorption properties in the NIR that results from their surface plasmon resonance (SPR) oscillations [3,4]. Colloidal AuNRs have become increasingly popular due to their NIR tunability [5], ease of surface functionalization for tumour targeting [2] and capacity to efficiently convert pulsed-NIR laser energy to heat when exposed at the longitudinal peak absorbance [5]. These properties collectively make them exceptional candidates that can selectively destroy cancerous and diseased cells through photothermal therapy. Applications of AuNRs have been documented in gene delivery [6], chemical sensing [7], medical diagnostics [8] and photothermal destruction of pathogenic bacteria [9].

Though several techniques have been developed to destroy cancerous cells, photothermal therapy using gold nanorods functionalized with a targeting agent to skin cancer cells has not been demonstrated extensively, especially on AuNRs which are wavelength-tunable photothermal nanoconvertors [10]. Optical excitation of AuNRs with NIR light results in its penetration into tissues by localized hyperthermia [2]. The most common method for synthesising AuNRs involves the seed-mediated approach using cetyltrimethylammonium bromide (CTAB) surfactant as the shape-directing agent. Polyelectrolyte encapsulation of CTAB-stabilized AuNRs mitigates its cytotoxic nature [11]. *In vitro* studies on the toxicity of gold nanoparticles of different size, shape, and surfactant showed

Abbreviations: AuNRs, gold nanorods; anti-EGFR, epidermal growth factor receptor antibody; anti-EGFR–AuNRs, epidermal growth factor receptor antibody conjugated with gold nanorods; A431, human squamous carcinoma cells; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; PTT, photothermal therapy; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; PI, propidium iodide; DCFDA, dichlorodihydrofluorescein diacetate; ICP-AES, Inductively Coupled Plasmon Atomic Emission Spectrophotometer.

* Corresponding author. Tel.: +91 471 2308078; fax: +91 471 2307158; mobile: +91 9447246692.
E-mail address: annieab2001@gmail.com (A. Abraham).

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that free CTAB molecules are detrimental to human cells while bound CTAB molecules are not toxic [12]. The cytotoxicity of CTAB-passivated AuNRs can alternatively be reduced by ligand exchange with phosphatidylcholine [13] and thiolated polyethylene glycol [14]. In the present study, we have aimed at testing the therapeutic efficacy of anti-EGFR–AuNRs. We have successfully synthesised uniform-sized gold nano rods, stabilized with CTAB, surface modified with PSS and functionalized with anti-EGFR [15,16]. These were then administered to squamous cancer cells followed by NIR irradiation and their cancer combating potential was studied. To the best of our knowledge, this is the first report on targeting surface modified and functionalized gold nanorods to skin cancer cells.

2. Materials and methods

2.1. Materials

A431 cells were procured from National Centre for Cell Sciences (NCCS), Pune, India. Monoclonal antibody of epidermal growth factor receptor was purchased from Santa Cruz Biotechnology, Inc., USA. Polystyrene sulfonate (PSS) was procured from Prolab, India. DMEM, FBS, Trypsin, PI, RNase, DCFDA, Secondary goat anti-rat FITC antibody, Caspase 3 colorimetric kit and Mitochondrial membrane staining kit were purchased from Sigma Aldrich, USA. Laser equipment with a power density of 10 mW (650 nm) purchased from Holmarc Opto-mechatronics Pvt. Ltd. All chemicals and biochemicals used were of analytical grade.

2.2. Synthesis and characterization of gold nanorods

AuNRs was prepared in high yield in the presence of cetyltrimethylammonium bromide (CTAB) and silver nitrate using seeded growth condition [17]. Briefly, to 3 mL of an aqueous solution of 0.08 M hexadecyltrimethylammonium bromide (CTAB), 0.42 mg/mL tetradodecylammonium bromide was added as the growth solution. Hydrogen tetrachloroaurate (0.25 mL of 0.024 M, $HAuCl_4{\cdot}3H_2O$) was then added to the solution as the precursor of gold followed by addition of 0.065 mL of acetone and 0.045 mL of cyclohexane to loosen the micellar structure. Different amounts of 0.01 M silver nitrate aqueous solution were added to the solution. The solution was then irradiated with a 254 nm UV light (420 $\mu W/cm^2$) for about 30 h. The resulting solution was spun at 3000 rpm for 10 min and the supernatant was collected, which was again centrifuged at 10,000 rpm for 10 min to yield a precipitate that was collected and redispersed in deionized water. UV–Vis spectra showed one transversal surface plasma peak at 519 nm and longitudinal ones at 645 nm. For HR-TEM studies, samples were prepared by dropcasting nanoparticle solution on a carbon coated copper grid and the solvent was allowed to evaporate. It was then examined by means of a 300 KV (JEOL 3010) transmission electron microscope.

2.3. Surface modification and bioconjugation of AuNRs

The surface modification of AuNRs with PSS was carried out using a previously reported procedure [18]. In brief 0.1 mL of AuNR solution was diluted to 10 mL. To this, 10 mL of 2 g/L PSS prepared in a 6 mM NaCl solution (previously sonicated for 30 min) was added drop-wise and the resulting solution was stirred vigorously for 3 h. The solution was then centrifuged twice at 10,000 rpm to remove excess polyelectrolyte and re-dispersed in 10 mL of PBS buffer (pH 7.6). Coating of PSS on the surface of gold nano rods was confirmed by analysing UV–Vis–NIR spectrum of the resulting solution, which was further used for bio-conjugation.

Anti EGFR–gold conjugates were prepared according to the method described by Huang et al. [2]. The PSS capped AuNR was allowed to react with antibody for 20 min, at room temperature, then centrifuged and redispersed in PBS to form a stock solution. The extinction spectra in a UV–Vis–NIR spectrophotometer (Model V-670 PC, Jasco International Co. Ltd., Japan) confirmed the conjugation of anti-EGFR antibody to the gold nanorods.

2.4. Cellular uptake of AuNRs

A431 cells were used to study the cellular uptake and targeting efficacy of bioconjugated nanorods. 1×10^6 cells was incubated with unconjugated AuNRs and another with anti-EGFR conjugated AuNRs for different time intervals such as 30 min, 1, 3 and 6 h. Approximately 6.023×10^{11} number of gold nanorods suspended in 0.5 mL DMEM medium in 12 well plates was used for the study. After incubation, the cells were washed thrice with PBS to remove unbound AuNRs, then fixed with 4% paraformaldehyde in PBS for 25 min at room temperature. Then they were treated with secondary goat anti-rat FITC antibody for 1 h at 37 °C. Unbound secondary antibodies were removed by PBS wash four times. The cells were then viewed under a fluorescent microscope (Leica microsystems) [19].

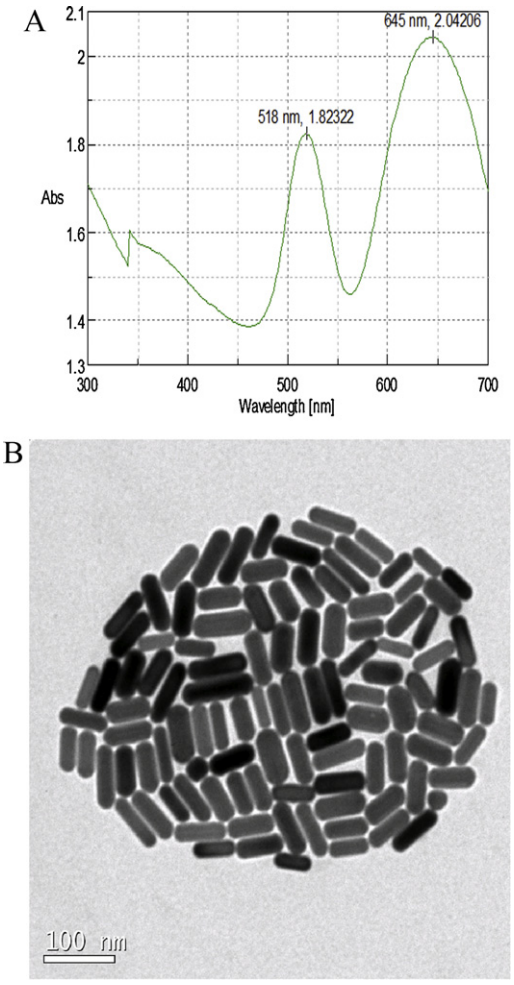

Fig. 1. Characterization data of CTAB capped AuNRs. Optical absorption spectra showed the characteristic peak of AuNRs corresponding to the surface plasmon of gold nanorods. HR-TEM images show that AuNRs are rod shaped and monodispersed with an average length of 50 nm.