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Biogenic gold nano-triangles: Cargos for anticancer drug delivery

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ABSTRACT

We present synthesis of biogenic gold nano triangles (GNTs) using *Azadirachta indica* leaf extract at inherent pH (5.89) and its application in efficient drug delivery of doxorubicin (DOX) (anticancer drug). The main idea was to take advantage of large surface area of GNTs which has 3 dimensions and use the plant peptides coated on these triangles as natural linkers for the attachment of DOX. Sucrose density gradient centrifugation (SDGC) and dialysis methods were used for separation of the GNT from mixture of GNPs. Flocculation parameter (FP) was used to check stability of GNT which was found to be exceptionally high (0–0.75) due to the biological capping agents. DOX attachment to GNT was verified using Fourier transformed infra-red (FTIR) spectroscopy. The complex thus formed was found to be less toxic to normal cells (MDCK cells) and significantly toxic for the cancerous cells (HeLa cells). Drug loading efficiency was found to be 99.81% and DOX release followed first order release kinetics. Percentage drug release was found to be more than 4.5% in both acidic (5.8) as well as physiological pH (7.2) which is suitable for tumor targeting.

1. Introduction

Nanotechnology is getting flourished as a consequence of developing nanomaterials and their enormous applications in various fields e.g. drug delivery [1], improvement of cancer diagnostics [2] and other diseases as well as in the field of catalysis [3], fuel cell [4], heavy metal detection [5] and therapeutics [6]. Prevailing among nanoparticles, gold nanoparticles (GNPs) have gathered more attention not only due to their higher efficiency of light absorption at their longitudinal plasmon resonance but also greater efficacy of conjugated drug delivery [7] and less toxicity [8].

The shape, morphology and size of nanoparticles govern the physical, chemical and optical properties of nanoparticles [9,10]. Among these, shape plays a significant role in tuning the properties and is the exigent task to manipulate methodically [10]. In the last few decades, several chemical and physical methods for synthesis of gold nano rods [11], discs [12], multipods [13], triangular prisms [14], cubes [15] and nano-shells [16] have been reported.

However, biological methods are more simplistic and eco-friendly and result in the formation of thermodynamically stable nanoparticles which extrudes the disadvantages of chemical methods requiring expensive instruments and result in the release of toxic chemicals [17,18]. Biological methods also produce nanoparticles having natural linkers onto which drugs can be loaded [7] directly. To this date, metal nanoparticles are synthesized with the aid of algae [19,20], bacteria [21,22], fungi [23,24] and plant [25–27] systems.

The application of GNPs as drug delivery vehicles resulted in a more efficient drug delivery system because of their controlled release of chemotherapeutic agents to diseased site and minimum use of drug [28,29]. This property of GNPs is explored to anchor drugs and transport them to specific site evading immune mechanism and avoiding damage to healthy tissues. Additionally, gold nano triangles (GNTs) also get internalized inside the cell cytoplasm [30]. Biologically synthesized GNTs are biocompatible [31] and have a large surface area which provides covalent binding of various chemical compounds like drugs [32], proteins [33], genes [30] and other molecules. The unique optical properties of GNTs make them a promising candidate for photothermal treatment and hyperthermia of tumors [34]. Extremely flat morphology is the key feature of GNTs which provides high thermal contact with tumor cells, thereby reducing exposure time. This is not possible with gold nanospheres and rods [35]. Hence, GNTs are considered to be the best option in comparison of gold nanorods and gold nanospheres for cancer treatment [34].

In the present paper we report use of *Azadirachta indica* leaf extract for synthesis of GNPs in which uniquely shaped triangles were found to be dominant among all other shapes such as cubes, hexagons and spherical structures. For drug delivery, the need of GNTs is envisaged; hence, efforts were directed toward standardizing suitable separation technique. Sucrose density gradient centrifugation (SDGC) technique takes the advantage of the difference in the densities of anisotropic GNPs thus forming different layers of GNPs from which the required nanoparticles can be easily separated. Therefore, GNTs were separated using SDGC based on their different sedimentation rates. Moreover,

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stability of biogenic GNT was assessed by flocculation study to figure out the efficiency of the surface proteins to resist flocculation under physiological pH (7.2) with addition of increasing concentration of NaCl and calculating integrated absorbance between 600 nm and 800 nm. An anticancer drug doxorubicin (DOX) was attached for treatment of tumor cells and drug release kinetics was studied using zero order, first order, Higuchi and Hixson–Crowell model [27,36].

2. Materials and methods

2.1. Materials

Gold aurochlorate ($HAuCl_4$), doxorubicin (DOX) and triethylamine (TEA) were purchased from Sigma–Aldrich, USA. All the experiments were carried out in nanopure water. In order to remove the traces of metal contaminants glasswares were washed with Aqua regia.

2.2. Preparation of plant extract

Leaves of *A. indica* were washed with nanopure water to remove dust particles. 5 g of leaves was crushed in 20 mL nanopure water and then filtered through 0.22 μ filter to remove cellular debris. Extract was diluted 100 times using double distilled water for experimental use. This extract was stored at 4 °C until further use.

2.3. Synthesis of GNT

A stock solution of 50,000 ppm gold aurochlorate was prepared in nanopure water. In order to use 100 ppm gold aurochlorate, 0.04 mL stock solution was added in 20 mL boiling solution of reaction vessel containing diluted plant extract (inherent pH 5.89). The extract was boiled till the appearance of wine red color.

2.4. Separation of GNTs by SDGC

Separation of GNTs from polydispersed and anisotropic GNPs was achieved with the help of sucrose density gradient centrifugation in which 2 mL solutions of each of 60%, 50%, 40%, 30%, 20% and 10% sucrose (*w*/*v*) were layered one above other in same order in a centrifuge tube. At the end i.e. above 10% sucrose layer, 2 mL of biogenic GNPs solution was carefully poured. Tube was spun at 5000 rpm in centrifuge (Remi Industries, India) for 40 min. Fractions were collected separately using a micropipette (Eppendorf Research Pipettes, Germany) and characterized spectrophotometrically. After SDGC, for further purification dialysis method was employed using pre-activated dialysis bag (12–14 kD) against nanopure water for 3 h under mild stirring.

2.5. Characterization

UV–vis spectroscopy (Lambda 25 PerkinElmer, USA) was carried out using plant extract as reference. Clean quartz cuvette having a path length of 1 cm was used to record the spectra. Morphology of GNTs was studied using field emission scanning electron microscopy (FE-SEM) on a Carl Zeiss Microimaging, GmbH, Germany. 2–3 drops of the colloidal gold solution were dispensed onto a silicon wafer and dried under ambient condition before examination. Involvement of diverse functional groups and molecular interactions as well as molecular orientation of the complexes were verified using Fourier transformed infra red spectroscopy (FTIR) on a MAGNA-550, Nicolet instruments, USA. The sample was prepared by loading 0.1 mL of GNTs in aqueous form onto the source.

2.6. Stability testing of GNTs using flocculation parameter (FP)

The stability of GNTs was checked by analyzing the changes in the optical properties of GNTs in response to the varying concentrations of NaCl. Salt solution was added to cuvette containing GNT solution and UV–vis spectrum was recorded. The procedure was repeated with increasing concentrations (0.017–3.4 M) of NaCl to observe the red shift in the peak as compared to the original peak of GNT. FP is an empirical term used for measurement of integrated absorbance between longer wavelengths (600–800 nm in this case). The equation used to calculate the integrated absorbance is as follows:

$$P = \int_{600}^{800} I_{Abs}(\lambda)dx$$

where, P—flocculation parameter, I_{Abs}—Intensity of absorbance, and λ—wavelength.

2.7. Attachment of DOX to biologically synthesized GNT

0.4 mM stock solution of DOX was prepared by dissolving 0.29 g in 10 mL nanopure water. In order to use 0.25 mM, 6.2 mL of stock solution was added in 3.8 mL of GNTs and allowed to react with 70 μL of TEA. The solution was subjected to purging under argon atmosphere for 4 h and stirred continuously using a magnetic stirrer. After 4 h, both inlet and outlet valves were closed. The reaction was allowed to take place for 12 h. Attachment of DOX to GNT was analyzed spectrophotometrically. The resultant GNT–DOX conjugate was dialyzed overnight against nano-pure water to remove unbound DOX. Unbound drug concentration was calculated using standard calibration curve of DOX (straight line equation $y = 7.428x$).

The drug loading efficiency (DLE) of GNPs was calculated using following equation:

$$DLE = \frac{\text{Theoretical amount of drug loaded} - \text{Free drug}}{\text{Theoretical amount of drug loaded}} \times 100$$

2.8. In vitro release of DOX

Dialyzed GNT–DOX solution was taken in two different pre-activated dialysis bags (2 mL each) and transferred to beakers containing 80 mL of phosphate buffer solution at pH 5.8 and 7.2. The drug release study was conducted at 37 °C with continuous stirring at 100 rpm. To measure the drug release content, samples (3 mL) were periodically removed and replaced with an equivalent volume of the phosphate buffer solution. The amount of released DOX was analyzed with a spectrophotometer at 485 nm and calculated using the standard calibration curve of DOX (straight line equation $y = 7.428x$). The experiments were performed in triplicate for each sample.

With precise control of the GNT–DOX complex, the release of the drug can be tuned to achieve a desired kinetic profile. Four of the most common kinetic profiles are zero order, first order, Higuchi and Hixson–Crowell. These drug release kinetics was calculated using the standard equations as per our previous studies [27,36].

2.9. Cytotoxicity studies

Cytotoxicity effects of Neem extract, GNT, GNT–DOX and free DOX were studied on MDCK and HeLa cells using MTT assay which is based on the conversion of pale yellow MTT to violet colored formazan crystals by mitochondrial enzyme succinate dehydrogenase. Cells were seeded (5×10^5/mL) in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. The culture medium was then replaced with test solutions and incubated further for 48 h. These solutions were later replaced with MTT (200 μg/mL) and cells were incubated for 2.5 h at 28 ± 2 °C to initiate formation of formazan. After completion of the reaction, the medium was replaced with 200 μL of DMSO. The microtitre plate containing complex was agitated slowly to dissolve formazan crystals. Finally, the