 doi:10.20944/preprints202206.0117.v2

Communication

Formation of Stable Cruciform Assembly of Gold Nanoparticles from *Cannabis Indica* Leaves

Anjul Khadria[1,2,*] and Subhankar Paul[1,*]

[1]Structural Biology and Nanomedicine Laboratory, Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela, Odisha, 769008, India
2Current address: Caltech Optical Imaging Laboratory, Andrew and Peggy Cherng Department of Medical Engineering, California Institute of Technology, Pasadena, California, 91125, USA
* Correspondence: anjul.nitrkl@gmail.com; spaul@nitrkl.ac.in,

Abstract: Gold nanoparticles have been increasingly used in several electronic, material fabrication, and biomedical applications. Several methods have been reported to prepare gold nanoparticles of various shapes and sizes with different photophysical properties. Although useful to prepare gold nanoparticles, most of the methods are not stable enough and undergo degradation, if stored at room temperatures (up to 30 °C) for a few days. In this paper, we report a novel environmentally friendly method to synthesize self-assembled gold nanoparticles in cruciform shapes by using leaf extract of *Cannabis indica* as a reducing agent without the aid of any polymers or additional chemicals. The nanoparticles are found to be stable for more than a month when stored at room temperature (30 °C). They were able to form stable conjugates with bovine α-lactalbumin protein that may possess anti-cancerous properties.

Keywords: Gold nanoparticles; nanomaterials; self-assembled nanoparticles; Cannabis indica

1. Introduction

The use of gold nanoparticles has increased exponentially in several biomedical and non-biomedical applications.[1] Gold nanoparticles have been shown to possess anti-cancerous activity by several studies, and recently it has been clinically shown to treat localized prostate tumors.[2] Due to their excellent photophysical properties, they have been explored to be used as theranostic agents.[3] Gold nanoparticles have emerged as one of the most promising agents for the treatment of various types of cancer due to several advantages associated with them such as (a) ease of cellular uptake by endocytosis, (b) ease of large scale synthesis and characterization, (c) ability to form stable complexes with a wide range of biomolecules, (d) biocompatibility, and (e) unique physicochemical properties. Gold nanoparticles of different shapes and sizes have different unique properties with different effects on cancer cells.[4] Conjugates of bovine α-lactalbumin (BLA) protein with oleic acid commonly known as BAMLET (bovine alpha-lactalbumin made lethal to tumors) have been successfully shown to be anti-cancerous; however, oleic acid is toxic to healthy cells as well.[5,6] Biocompatible gold nanoparticles have been shown to replace oleic acid in the BAMLET conjugate to form anticancerous gold nanoparticle-BLA conjugates *in vivo*.[7] One of the significant challenges of synthesizing assembled gold nanoparticles is making them stable at temperatures up to 30 °C, which will give them a long shelf-life and make them biocompatible to form stable conjugates with proteins.[8] Several nanoparticle synthesis methodologies require high temperatures and toxic chemicals, which can be harmful to the proteins. In this study, we report the synthesis of stable cruciformly assembled gold nanoparticles using *Cannabis indica* leaves and the formation of its conjugates with the BLA protein. Normally, assembled gold nanoparticles are prepared with the aid of polymers or other thiol-assisted chemicals; however, in this report, we did not use any such synthetic chemicals to form the cruciform assembly of the gold nanoparticles.[1,9]

2. Materials and Methods

Materials: Cetyl trimethylammonium bromide (CTAB), [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] (MTT) assay kit and Dulbecco's minimum essential medium (DMEM) were obtained from Himedia, India, Oleic acid was obtained from RFCL limited, India and auric chloride hydrate and Bovine α-Lactalbumin (BLA) were obtained from Sigma Aldrich. All the glassware was purchased from Borosil (India). Milli-Q (HPLC grade) water was used in all preparations.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 June 2022 doi:10.20944/preprints202206.0117.v2

Synthesis of cruciform assembly of gold nanoparticles by using *Cannabis indica* leaves extract: To prepare the reducing agent, the *Cannabis indica* leaves were (2.80 g) dissolved in 5 mL of water followed by centrifugation at 5000 rpm for 10 minutes. The resultant supernatant was filtered using a 0.22 μm syringe filter (Whatman), and the pellet was discarded. Solution of auric chloride (5 mL, 2.5 mM) in water was mixed with 18.65 mL of 12.5 mM of cetyl trimethylammonium bromide (CTAB) aqueous solution, and the mixture was stirred at 100 °C. After two minutes the filtered leaf extract (supernatant) was added to the reaction mixture. The reaction was quenched by putting the reaction mixture flask in an ice bath soon after the color of the mixture turned pinkish red, which is the characteristic color of gold nanoparticles.

Dynamic Light Scattering (DLS) particle size analysis: The DLS particle size analysis was performed to determine the average size and polydispersity index of the nanoparticles using a Malvern Zetasizer Nano-ZS instrument.

Scanning Electron Microscope (SEM): Scanning Electron Microscopic (SEM) image was developed using Jeol JSM-6480 LV SEM. Thin films of the sample were prepared on the grid.

UV-Vis spectra analysis of GNPs: The UV-Vis spectroscopic (PerkinElmer, Lambda35) analysis of the reaction mixture was performed to confirm the formation of gold nanoparticles. The nanoparticles were stored at room temperature (up to 30 °C) in dark between the measurements.

Preparation of BAMLET and GNP-BLA conjugates: The stock solution of bovine α-lactalbumin (BLA) was prepared in a 20 mM phosphate buffer, 7.2 pH. To prepare the conjugates, gold nanoparticles suspension (0.35 mL) was mixed with 0.15 mL BLA solution to get a final concentration of BLA as 1.0mg/mL and GNPs at 1.75 mM, followed by incubation at 60 °C for 10 minutes. To prepare BAMLET, 10 μL of oleic acid was mixed with 0.3 mL of stock solution of BLA (final concentration was 1 mg/mL), 0.69 mL of buffer, and incubated at 60 °C for 10 minutes.[10]

Tryptophan fluorescence emission spectra: Bovine α-lactalbumin (BLA) protein solution was prepared in a 20 mM phosphate buffer (pH= 7.2) at a concentration of 1 mg/mL. Tryptophan fluorescence spectra of the GNP-BLA conjugates and only BLA solution were recorded in a Perkin-Elmer LS-55 Luminescence spectrometer. An excitation wavelength of 280 nm was used, and emission spectra were recorded from 280 to 550 nm. The excitation and emission slit widths were set to 10 nm and 5 nm, respectively.

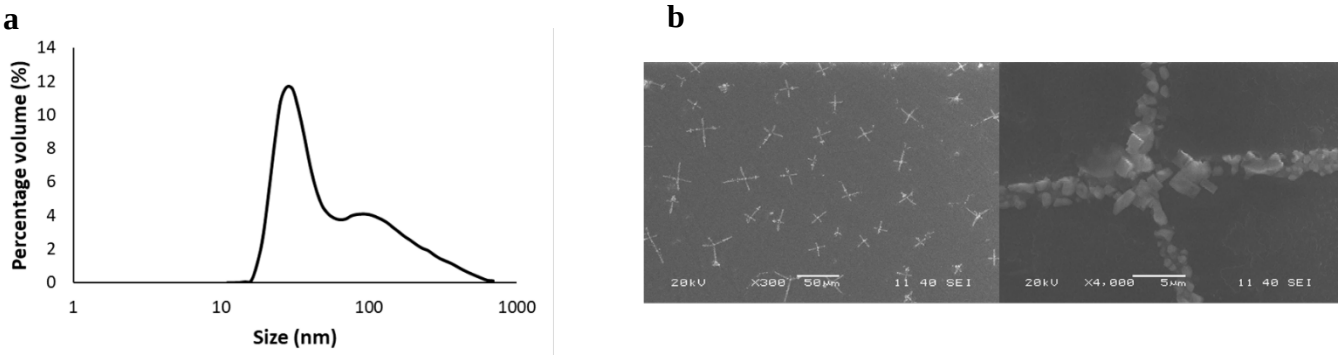

Figure 1: (a) The dynamic light scattering experiment shows that particles are mostly monodisperse (polydispersity index = 0.28). A majority of the number of nanoparticles was found to be less than 100 nm. (b) Cruciform assembly of gold nanoparticles in synthesized from Cannabis indica leaf water extract method. The right image is the magnified version of the cruciform assembly.

3. Results

Gold nanoparticles are commonly prepared by the chemical reduction method.[11] Different plant extracts like *Hibiscus rosasinesis* and *Ocimum tenuiflorum* have been used as reducing agents for the synthesis of gold nanoparticles from auric chloride.[12,13] Here, we synthesized a stable cruciform assembly of gold nanoparticles by using water extract from the leaves of *Cannabis indica* plants. We confirmed through dynamic light scattering that the particles prepared by our method are monodispersed (poly-dispersity index of 0.28) and nanosized (Figure 1a). The sizes and shapes of the nanoparticles were confirmed by scanning electron microscopy (SEM) experiments (Figure 1b). The SEM experiments revealed a rare cruciform assembly of the nanoparticles.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 June 2022 doi:10.20944/preprints202206.0117.v2

We did not observe any significant change in its absorbance spectra in the visible light region (400 nm – 700 nm) over the range of 45 days when stored at 30 °C, proving the stability of the nanoparticles (Figure 2a). The light absorbance maxima at around 580 nm due to surface plasmon resonance further demonstrates the formation of gold nanoparticles. After synthesizing stable gold nanoparticles, we prepared its conjugate with the BLA protein by heat-shock method and monitored the tryptophan fluorescence spectrum (Figure 2b). The tryptophan fluorescence intensity of the BLA protein changed significantly after binding to gold nanoparticles (to form nanoparticle-BLA conjugate) and oleic acid (to form BAMLET), thus confirming the formation of the conjugates. BLA exhibited its strong intrinsic tryptophan fluorescence emission spectra with maxima at 334.5 nm, whereas the gold nanoparticles did not show any fluorescence. The gold nanoparticles partly quenched the tryptophan fluorescence of BLA apart from causing a minor (4.5 nm) red-shift in the emission band (Figure 2b).

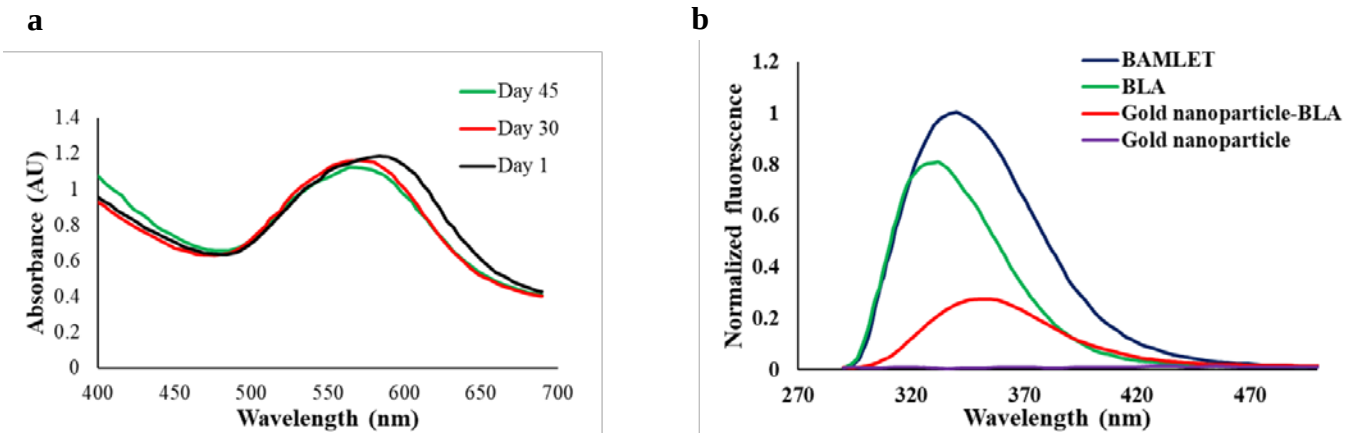

Figure 2: (a) Visible light spectra of gold nanoparticles measured over a period of 45 days proving their stability. (b) Tryptophan fluorescence emission spectra of BLA and the conjugates with tryptophan excitation maxima of 280 nm. The fluorescence intensity of the tryptophan changed from the native structure after formation of conjugates.

4. Discussion

This work reports only the the synthesis and characterization of gold nanoparticles using *Cannabis indica* leaves extract and its conjugate with BLA, which was found to be anticancerous against breast cancer cells in preliminary studies *in vitro* as previously reported as part of an undergraduate thesis.[14] We have synthesized a stable cruciform assembly of gold nanoparticles using water extract of *Cannabis indica* leaves as a reducing agent without usage of any additional synthetic chemicals such as polymers. To the best of our knowledge, such cruciform assembly of individual gold nanoparticles has not been reported before. Self-assembly of nanoparticles is a result of several types of interfacial interactions such as van der Walls attractions, magnetic and Coulombic forces, steric repulsions, hydrophobic and hydrophilic interactions, etc. dependent on the surface chemistry of the nanoparticles.[15,16] The water extract of the *Cannabis indica* leaves contains several chemicals that may have assisted in forming the cruciform assembly of the gold nanoparticles in our experimental conditions. In the future, it may be interesting to isolate the chemicals and test the effects of each of them individually or in the presence of one another in forming self-assembled gold nanoparticles. Given the presence of hundreds of chemicals in the leaves, carrying out such work is beyond the scope of the current study. Self-assembled gold nanoparticles have huge applications in the field of sensors, optics, and electronics. Self-assembled nanoparticles have also found usage in enhanced brain tumor targeting, which remains one of the toughest challenges to solve in medical science.[17]

The use of water extract of *Cannabis indica* leaves as a reducing agent ensures that no toxic waste is generated during the synthesis procedure instead of using harmful chemicals. This finding gives insight into a new class of compounds, which can be used to synthesize stable (at temperatures up to 30 °C) self-assembled gold nanoparticles. Upon studying the fluorescence emission spectra, we found a reduction in the tryptophan fluorescence intensity of BLA protein, which indicates a conformational change of the protein. This is of significance because different types of self-assembled gold nanoparticles with unique photophysical and physicochemical properties can be first synthesized, and then their conjugates with the BLA protein can be formed. The clinical trials that utilized gold nanoparticles to treat tumors have used photodynamic therapy, which relies on using light of specific wavelengths to excite the nanoparticles and generate heat or reactive oxygenated species.[2,18,19] Although photodynamic therapy has been beneficial to treat localized tumors; it cannot be practically used to kill metastatic tumors that have spread to several body parts because it is not always possible to shine light on different deeper parts of the human body. For such purposes, only drugs that can kill cancer cells without an external stimulus could be helpful. Chemotherapy-based techniques that utilize drugs such as doxorubicin and paclitaxel are gold-standard clinical methods to kill metastatic cancer cells, but they are also harmful to healthy cells; hence, alternatives are required, such as gold nanoparticle-BLA conjugates, which are not toxic to healthy cells.[7,20] Gold nanoparticles can be combined with other natural chemicals such as pyropheophorbide-a and related molecules that have been shown to possess anticancerous and photophysical properties, to form conjugates with enhanced efficacies.[21,22] Apart from cancer biology, gold nanoparticles have been used for nonlinear optical imaging such as second harmonic generation.[23] It may be interesting to study the nonlinear optical properties of these nanoparticles in live cells and lipid monolayer droplets by conjugating them with porphyrins, which have been shown to possess extremely high nonlinear optical properties.[24] Gold nanoparticles have also found usage in neuronal modulation as an alternative to optogenetics; and it may be exciting to make its conjugates with different optical dyes to make a single agent, which can modulate neurons and sense action potentials simultaneously.[25,26] Overall, the application of gold nanoparticles is rising exponentially, and new greener methods that can synthesize stable self-assembled nanoparticles are highly required, which we attempt to target through this work.

Author Contributions: A.K. and S.P. designed and conceived the project. A.K. designed the gold nanoparticle synthesis protocol, synthesized all the samples, and characterized them. S.P. supervised the project and arranged the funding. A.K. wrote the paper. All authors contributed to writing the manuscript.

Funding: This research received funding from the Department of Biotechnology, Government of India, and the National Institute of Technology, Rourkela, India.

Acknowledgments: We thank Prof. Sunil Kumar Sarangi, Dr Deependra K. Ban, Prof. Kunal Pal, Prof. S.S. Ray, and Dr. Sailendra Mahanta at the National Institute of Technology- Rourkela for useful discussions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Daniel, M.-C. & Astruc, D. Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology. *Chem. Rev.* **104**, 293–346 (2004).

2. Rastinehad, A. R. *et al.* Gold nanoshell-localized photothermal ablation of prostate tumors in a clinical pilot device study. *Proc. Natl. Acad. Sci.* **116**, 18590 LP – 18596 (2019).

3. Gao, Q. *et al.* Gold Nanoparticles in Cancer Theranostics. *Front. Bioeng. Biotechnol.* **9**, 221 (2021).

4. Sztandera, K., Gorzkiewicz, M. & Klajnert-Maculewicz, B. Gold Nanoparticles in Cancer Treatment. *Mol. Pharm.* **16**, 1–23 (2019).

5. Svensson, M. *et al.* Molecular Characterization of α–Lactalbumin Folding Variants That Induce Apoptosis in Tumor Cells*. *J. Biol. Chem.* **274**, 6388–6396 (1999).

6. Shabbirahmed, A. M., Kumaravel, M., Somu, P., Paul, S. & Khadria, A. Recent Advancements in Nanomaterials for Photodynamic Therapy of Cancers BT - Handbook of Oxidative Stress in Cancer: Therapeutic Aspects. in (ed. Chakraborti, S.) 1–24 (Springer Singapore, 2021). doi:10.1007/978-981-16-1247-3_211-1

7. Yang, J. *et al.* Gold/alpha-lactalbumin nanoprobes for the imaging and treatment of breast cancer. *Nat. Biomed. Eng.* **4**, 686–703 (2020).

8. Wang, A. *et al.* Gold Nanoparticles: Synthesis, Stability Test, and Application for the Rice Growth. *J. Nanomater.* **2014**, 451232 (2014).

9. Ofir, Y., Samanta, B. & Rotello, V. M. Polymer and biopolymer mediated self-assembly of gold nanoparticles. *Chem. Soc. Rev.* **37**, 1814–1825 (2008).

10. Kamijima, T. *et al.* Heat-treatment method for producing fatty acid-bound alpha-lactalbumin that induces tumor cell death. *Biochem. Biophys. Res. Commun.* **376**, 211–214 (2008).

11. Turkevich, J., Stevenson, P. C. & Hillier, J. A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discuss. Faraday Soc.* **11**, 55–75 (1951).

12. Philip, D. & Unni, C. Extracellular biosynthesis of gold and silver nanoparticles using Krishna tulsi (Ocimum sanctum) leaf. *Phys. E Low-dimensional Syst. Nanostructures* **43**, 1318–1322 (2011).

13. Philip, D. Green synthesis of gold and silver nanoparticles using Hibiscus rosa sinensis. *Phys. E Low-dimensional Syst. Nanostructures* **42**, 1417–1424 (2010).

14. Khadria, A. Preparation of gold nanoparticles-α lactalbumin binary complex for breast cancer therapy. (National Institute of Technology, Rourkela, 2012). doi:10.13140/RG.2.2.14896.02565

15. Bian, K. *et al.* Formation of self-assembled gold nanoparticle supercrystals with facet-dependent surface plasmonic coupling. *Nat. Commun.* **9**, 2365 (2018).

16. Wang, C., Siu, C., Zhang, J. & Fang, J. Understanding the forces acting in self-assembly and the implications for constructing three-dimensional (3D) supercrystals. *Nano Res.* **8**, 2445–2466 (2015).

17. Feng, Q. *et al.* Self-Assembly of Gold Nanoparticles Shows Microenvironment-Mediated Dynamic Switching and Enhanced Brain Tumor Targeting. *Theranostics* **7**, 1875–1889 (2017).

18. Hu, F., Xu, S. & Liu, B. Photosensitizers with Aggregation-Induced Emission: Materials and Biomedical Applications. *Adv. Mater.* **30**, 1801350 (2018).

19. Moan, J. & Peng, Q. An outline of the hundred-year history of PDT. *Anticancer Res.* **23**, 3591–3600 (2003).

 doi:10.20944/preprints202206.0117.v2

20. Sudha, T. *et al.* Targeted delivery of paclitaxel and doxorubicin to cancer xenografts via the nanoparticle of nano-diamino-tetrac. *Int. J. Nanomedicine* **12**, 1305 (2017).

21. Kang, S. H. *et al.* Hybrid photoactive nanomaterial composed of gold nanoparticles, pheophorbide-A and hyaluronic acid as a targeted bimodal phototherapy. *Macromol. Res.* **23**, 474–484 (2015).

22. Khadria, A. *et al.* Push-pull pyropheophorbides for nonlinear optical imaging. *Org. Biomol. Chem.* **15**, 947–956 (2017).

23. Clark, H. A., Campagnola, P. J., Wuskell, J. P., Lewis, A. & Loew, L. M. Second Harmonic Generation Properties of Fluorescent Polymer-Encapsulated Gold Nanoparticles. *J. Am. Chem. Soc.* **122**, 10234–10235 (2000).

24. Khadria, A. *et al.* Porphyrin Dyes for Nonlinear Optical Imaging of Live Cells. *iScience* **4**, 153–163 (2018).

25. Huang, K., Dou, Q. & Loh, X. J. Nanomaterial mediated optogenetics: opportunities and challenges. *RSC Adv.* **6**, 60896–60906 (2016).

26. Khadria, A. Tools to measure membrane potential of neurons. *Biomed. J.* (2022). doi:https://doi.org/10.1016/j.bj.2022.05.007