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# Green synthesis of gold nanoparticles for staining human cervical cancer cells and DNA binding assay

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ABSTRACT

Gold nanoparticles have been functionalized by non-ionic surfactants (polysorbates) used in pharmaceutical formulations. This results in the formation of more well-dispersed gold nanoparticles (GNPs) than the GNPs formed in neat water. The synthesized GNPs show good temporal stability. The synthesis conditions are mild and environmentally benign. The GNPs can bind to ct-DNA and displace bound dye molecules. The DNA-binding assay is significant as it preliminarily indicated that DNA–GNP conjugates can be formed. Such conjugates are extremely promising for applications in nanobiotechnology. The GNPs can also stain the human cervical cancer (HeLa) cells over a wide concentration range while remaining non-cytotoxic, thus providing a non invasive cell staining method. This result is very promising as we observe staining of HeLa cells at very low GNP concentrations (1 μM) while the cell viability is retained even at 10-fold higher GNP concentrations.



## 1. Introduction

The potential applications of nanomaterials in biology and medicine [1–3] have been a hot topic of research during the last two decades. Among these, gold nanoparticles are important, as they exhibit the best compatibility with bio molecules [1,2]. Gold nanoparticles can be recognized by their intense color and their distinct surface plasmon resonance (SPR) bands [3,4]. Thus contemporary research has been strongly focussed on the development of synthetic protocols for gold nanoparticle preparation. Parallel to this, the chemistry related to their surface modification has also received considerable attention [1–8]. Often amine [9] and thiol bearing [10] groups have been used for surface modification of GNPs. Protein conjugated GNPs have been used in drug/DNA delivery [11], cell imaging [12] and bio staining.

In the past, some groups have used surfactants for surface modification of GNPs [1–8,13,14]. However, this kind of surfactant-based modification or capping has some adverse consequences. Among surfactants, the cationic surfactant cetyltrimethylammonium bromide (CTAB) is popular for capping GNPs as it is known to be a good structure – directing agent for GNPs [1]. However, there are quite a few reports of the cytotoxicity of CTAB [13], especially CTAB that remains unreacted after GNP functionalization [14]. In view of the reported toxicity of some cationic and anionic surfactants, we were interested to see if GNPs functionalized by non-ionic surfactants may be a viable non-toxic alternative for biological studies employing GNPs.

Among non-ionic surfactants, one popular surfactant is polyoxyethylene isooctyl phenyl ether (Triton X-100) [15]. Another category of non-ionic surfactants are the polyoxyethylene sorbitan esters of fatty acids i.e. the polysorbates or the Tween surfactants [16]. Polysorbates are an important class of non-ionic surfactants that are widely used in pharmaceuticals due to their low toxicity [17,18]. It has been previously reported that Tween-80 and Tween 20 [19] can be used to prepare stable GNPs [19,20].

To the best of our knowledge, there is no report of the use of GNPs for biological studies using polysorbate functionalization. We have used two polysorbates Tween 20 and Tween 40 for functionalization. The primary focus of the present work is to set up a mild and environmentally benign protocol for synthesis of GNPs with the specific aim of using them for biological applications. As in all biological applications, the aim is to use the GNPs for the specific biological purpose while maintaining the viability of the method and simultaneously not destroying the biological system.

## 2. Material and methods

### 2.1. Preparation of the GNPs and their characterization

Chloroauric acid ($HAuCl_4 \cdot xH_2O$), Ascorbic acid (AA), Tween-20 and Tween-40 were purchased from E-Merck. Calf thymus DNA

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(ct-DNA) and Acridine Orange (AO) were purchased from SRL, India. The gold nanoparticles were prepared in different surfactant media. The final surfactant concentrations for synthesis of gold nanoparticles were fixed at (i) $1 \times 10^{-3}$ M i.e. above the respective critical micellar concentration (CMC) of the surfactants and (ii) $1 \times 10^{-6}$ M i.e. below CMC. CMCs of Tween-20 and Tween-40 are $5 \times 10^{-5}$ M and $3 \times 10^{-5}$ M respectively. For all the studies, the precursor ($HAuCl_4$) concentration used was $5 \times 10^{-5}$ M, Ascorbic acid (Vitamin C) was used as the reducing agent and its concentration was $5 \times 10^{-4}$ M. AA was used as it is a mild reducing agent and is bio – compatible [21]. Apart from the chemical synthesis using AA, the GNPs were also prepared by uv-irradiation and ultrasonic method in absence of AA.

The pH of the solutions was measured with a systronics pH-meter. The pH was maintained at ~6 for the GNP synthesis. Photoirradiation was carried out using a Philips uv-lamp (15 W) and sonication was carried out in a mini bath sonicator (Piezo-U-Sonic model). The absorption spectra were recorded with a Shimadzu UV 2401-PC spectrophotometer (Kyoto, Japan). Dynamic light scattering (DLS) studies were performed with a Nano-ZS (Malvern) instrument, which is equipped with a 4 mW He–Ne laser ($\lambda$ = 632 nm). The solutions for this study were prepared by filtering them through a 0.2 μm filter. The resolution of the experimental setup is 0.6 nm. Transmission Electron Microscopic (TEM) studies of the nanoparticles were carried out at a resolution of 1.9 Å unit with a JEOL, JEM-2100 Electron Microscope from Japan. TEM specimens were prepared by placing micro-drops of solutions on a carbon film supported by a copper grid (300 mesh).

### 2.2. Study of the interaction of the GNPs with calf thymus DNA

We also studied the interaction of the synthesized GNPs with calf thymus DNA (ct-DNA) by performing DNA binding assay using the common DNA intercalating dye Acridine Orange [22,23]. DNA solution was prepared in Tris–HCl buffer at pH 8. The DNA concentration was determined using an extinction coefficient of 6600 $M^{-1}$ $cm^{-1}$ at 260 nm. Purity of the DNA was checked by monitoring the ratio of the absorbance at 260 nm to that at 280 nm. The solution gave an A260/A280 ratio of 1.8 indicating that the DNA was sufficiently free from protein. The fluorescence spectra were recorded with a Perkin Elmer LS 55 spectrofluorimeter. Fluorescence lifetimes were determined from time-resolved fluorescence intensity decays by the method of time correlated single photon counting (TCSPC) using a diode (IBH, UK) nanoLED-07 as the light source at 438 nm. The full width at half maxima (FWHM) of this excitation source is 70 ps. The decay curves were analyzed using IBH-6 decay analysis software. The circular dichroism measurements were done in the region 200–320 nm with a JASCO J – 815 CD spectrometer (model No. J-815-150 S) using a quartz cuvette of 1 mm path length.

### 2.3. Biological studies with the GNPs

The synthesized nanoparticles were used for biological applications such as cell staining and cytotoxicity studies.

#### 2.3.1. Cell culture

Hoechst dye and Trypan Blue were purchased from Sigma Chemicals (USA). Culture media MEM and serum were purchased from HiMedia, India. Other molecular biology grade fine chemicals were procured locally. Human cervical cancer cell line HeLa was obtained from National Centre for Cell Sciences, Pune, India. HeLa cells were grown in MEM supplemented with 10% bovine serum (complete medium) at 37 °C in humidified atmosphere containing 5% $CO_2$.

#### 2.3.2. Cell staining by the GNPs in phosphate buffered saline (PBS)

HeLa cells were inoculated on a cover slip in complete MEM medium. After overnight incubation in $CO_2$ incubator, the cover slip was taken out, washed with PBS twice and then incubated in presence of 1 μM, 5 μM and 10 μM of the GNPs in PBS for 15 min in the dark and at room temperature. Then the cover slip was taken out and placed upside down over a glass slide (cells were in contact with glass slide) under a fluorescence microscope (Carl Zeiss) with UV excitation and photographed using Axio Vision 3.1 software. The entire cell staining experiments were repeated five times independently.

#### 2.3.3. Cell viability and morphology study

After inoculation in a fresh medium, HeLa cells were incubated overnight and treated with different concentrations of GNPs (0–10 μM) for 24 h. After treatment, cells were harvested by trypsinization and suspended in phosphate buffered saline (PBS). After washing twice, the cells were incubated with 0.2% Trypan Blue for 5 min at room temperature and counted using hemocytometer under light microscope. The dead cells appeared to be blue, whereas the viable cells were colorless. Each experiment was repeated 3 times and the mean of percentage viable cells at each dose was compared with the mean of untreated control using one way ANOVA with a post hoc test such as the Dunnett's test. The significance values ($p$ values) are denoted in the respective figure.

For cell morphology study, cells were grown on cover slips overnight and treated with different concentrations of GNPs (0–10 μM) for 24 h in culture medium. Then cells were washed with phosphate buffered saline (PBS) twice and placed upside down over clean microscopic slides. The photographs were taken under normal light microscope.

## 3. Results and discussion

### 3.1. Nanoparticle synthesis and characterization

#### 3.1.1. In aqueous Tween micelles

When chemical synthesis was performed in aqueous Tween-20 medium using AA, GNPs were formed at Tween-20 concentrations above the CMC only. GNP formation was realized from the observation of deep pink coloration and the occurrence of the surface plasmon resonance (SPR) spectra with peak at ~530 nm. The GNP containing solution was stable for several hours. Table S1 in the supplementary section gives the DLS results.

In Tween-20 micelles, GNPs with hydrodynamic radii 25 nm are formed. Fig. 1a shows the TEM image of the GNPs in Tween-20 micelles. We note that well-dispersed spherical particles of diameter 20–25 nm are produced in high density.

Tween-40 micelles too promote GNP formation. Here too deep pink colored GNP containing solutions are observed with SPR peak at ~530 nm. The DLS study indicates that $r_h$ of the GNPs is ~50 nm i.e. higher than in Tween-20 where $r_h$ was 25 nm (Table S1 in supplementary data). This is probably because DLS gives the hydrodynamic radius ($r_h$) of the particles. Tween-40 is a higher homologue of Tween-20 and thus an increase in $r_h$ can be well understood from the increase in chain length of the surfactant. Fig. 1b shows the TEM image of GNPs synthesized in Tween-40 micelles. Here the particle density is lesser than for Tween-20 while GNP size is larger. Also a few non-spherical and rod-like GNPs are formed. This arises due to preferential adsorption of $[AuCl_4^-]$ along certain crystal facets of already existing Au (0) particles [24].

GNPs were also prepared by other methods i.e. uv-irradiation in absence of reducing agent. Immediately after passing UV light, violet–pink color was seen accompanied by a slightly broad peak in