Gold nanoparticles: effective as both entry inhibitors and virus neutralizing agents against HIV

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ABSTRACT

Spherical gold nanoparticles of 2 – 10 nm diameter as well as gold nanoparticles coated with Polyethylene Glycol (PEG-AuNP) were tested for their cytotoxicity and anti-viral activity. Gold nanoparticles were synthesized by Turkevich’s chemical reduction method of gold ions; cytotoxicity of the gold nanoparticles and PEG coated gold nanoparticles was assessed by tissue culture based colorimetric MTT assay and anti-viral potential of HIV was assessed by P24 antigen inhibition assay. Both Au NP and AuNP+ PEG were allowed to interact respectively with viral particles before infecting the cells as well as interacted with HIV- infected CD4⁺ T cells. An increase in cytotoxicity was observed with increase in gold nanoparticle concentration. However, it was below 10% in both Au NP and PEG-AuNP at 2 ppm and 4ppm. Nanoparticles showed greater activity in the test where the viral particles were allowed to interact with the nanoparticles before infecting the cells as compared to the test where the cells, previously infected with the virus, were allowed to interact with the nanoparticles. AuNP at 4ppm concentration were found to be effective as virus neutralizing candidate when allowed to interact with virus infected cells. Whereas, Au-PEG at 2ppm and 4ppm were more effective in inhibiting viral entry when interacted with viral particles directly. The results suggest that the gold nanoparticles are effective as both virus entry inhibitors as well as virus neutralizing agent.

Key words: Gold nanoparticles, HIV, PEG, P24 ELISA.

INTRODUCTION

HIV/AIDS is the fourth leading cause of death worldwide, due to its magnitude of infection. AIDS is caused by Human immunodeficiency virus, popularly called HIV [1]. Human immunodeficiency virus (HIV) is the aetiological agent of Acquired Immunodeficiency Disease Syndrome (AIDS). Its primary target is the activated CD4⁺ T helper lymphocytes (T Lymphocytes) but can also infect several other cell types including macrophages. HIV is a lentivirus, a class of retrovirus. Most lentiviruses target cells of the immune system and thus diseases is often characterized as immunodeficiency.

Currently Antiretroviral drugs in "combination therapy" (typically involving 3 or 4 drug combination of nucleoside analogues, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors) are used to treat the human immunodeficiency virus (HIV). However, antiretroviral do not cure the disease and patient remain infected for life;

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neither have they prevented HIV transmission and the problem of developing resistant to a drug remains because HIV reproduces very quickly in the human body and is prone to developing genetic mutations (changes in its genetic makeup). The HIV-1 genetic variability is a result of the highly error prone mechanism of reverse transcriptase due to its lack of a proof-reading mechanism. Due to its high rate of replication ($10^9$ – $10^{10}$ virions/person/day) and error prone reverse transcriptase, HIV can easily develop mutations that alter susceptibility to anti-retroviral drugs.

Scientists are now aiming to an amalgamation of physical and medical sciences for HIV therapy. With nanotechnology, it is possible to move ahead from where traditional medical science puts its hands up [2,3]. A combination of nanotechnology with medical science in context of dreaded diseases such as cancer or HIV/AIDS may lead to possibilities that were never imagined.

In the present study gold nanoparticles were synthesized and assessed as potential antiviral agents against HIV. Gold nanoparticles (AuNPs) are promising candidates in HIV therapeutics due to their facile synthesis, ease of functionalization, biocompatibility and inherent non-toxicity. The unique chemical and physical properties of AuNPs also make them potential alternative to be looked upon for treatment against HIV.

MATERIALS AND METHODS

**Synthesis of Gold Nanoparticles**
Colloidal gold nanoparticles were synthesized by the optimization of classical Citrate Reduction [4] method, involving reduction of gold ion using citrate. 1 ml of 5 mM tetrachloroauric (III) acid solution (H AuCl4·H2O) was diluted by adding 18 ml water and heated until it began to boil. Then 1 ml of a 0.5 % (17 mM) tri-sodium citrate solution was added while stirring rapidly. Heating was continued till the color changed to pale purple or wine red.

**Surface modification of Gold Nanoparticles**
Surface of thus synthesized gold nanoparticles was functionalized by adding poly-ethylene glycol (PEG) of low Mol. Wt (4000) and keeping at room temperature for 10 hrs. Surface modification is necessary to increase its colloidal stability in physiological fluids. Moreover, PEG is biocompatible; it increases the circulation in blood and hides gold nano particles from reticulo endothelial system [5].

Thus two sets of gold nanoparticles i.e. plain gold nano particle (AuNP) and PEG coated gold nano particles (PEG-AuNP), were prepared.

**Characterization of Gold Nanoparticles**
Synthesis of both gold nano particles and PEGylated gold nanoparticles were studied by characterized using UV-Vis Spectrophotometer, High Resolution Transmission Electron Microscopy, FTIR and understanding its SEAD pattern.

**Cytotoxicity of Gold Nanoparticles by MTT assay**
Prior to using the synthesized gold nanoparticles for anti-viral assay, cytotoxicity of nanoparticles was determined using Vero cell lines by MTT (a tetrazole) assay [6]. It involves reduction of tetrazole by mitochondrial enzymes to formazan crystals. These crystals were dissolved in acidified alcohol and OD was recorded at 530nm. Vero cell lines were trypsinized and suspended in minimal essential medium and counted using trypan blue exclusion dye.

Cells were then seeded in a 96 tissue culture well plate and cell concentration was adjusted to 5 x $10^5$ cells/ml. After overnight incubation in a humidified incubator having 5% $CO_2$ at 37°C; different concentrations of gold NP were added to the wells and again incubated for 8 to 10 hr at 37°C in $CO_2$ incubator.

A control (only cells) and a positive control (cells and dimethyl sulphoxide (DMSO) were also added. After incubation 10μl (5mg/ml) MTT reagent was added in each well and incubated at 37°C for 4 hrs in CO2 incubator. Then 100 μl of 0.1 N acidified iso-propanol was added to each well and kept in dark for 30 min at RT. The well plates were then kept on a shaker for 1 min and OD was taken at 530 nm wavelength.
Preparation of viral stock by PBMC macro co-culture technique [7, 8]

For anti-viral assay HIV viral stock showing high levels of P24 antigen levels is needed. Viral stock was prepared by co-culturing HIV seropositive patient peripheral blood mononuclear cells (PBMC) or standard viral culture and uninfected PHA-stimulated PBMCs was maintained under ideal conditions to allow viral replication in vitro. Most PBMC cultures from HIV–1 seropositive patients yield detectable HIV–1 antigen (P24) by this method. The method entitles following methods:

Isolation of PBMC’s:
PBMC were separated from whole blood by density gradient centrifugation. 5 ml blood sample was collected in EDTA vacutainer and blood was then carefully layered on 2 ml of ficoll in centrifuge tube and centrifuged at 3000 rpm for 25 min [9]. The PBMC layer formed at the junction of plasma and ficoll was collected and added to another centrifuge tube. The isolated cells were serially re-suspended in PBS and later in plain RPMI medium and centrifuged for 10 min at 1500 rpm to remove dead cells or artifacts in any.

The supernatant was discarded and to the cell pellet 2 ml of complete RPMI medium (with FBS) was added. 2 ml of this re-suspended cell suspension was added to T25 flask containing 8 ml RPMIC and incubated at 37°C in CO₂ incubator for 24 hrs.

PHA stimulation of Donor PBMC’s:
For selective growth of CD4⁺ T cells and over expression of surface marker proteins of T cells to facilitate viral infection, donor PBMC’s were stimulated with mitogen phytohemagglutinin (PHA) in presence of human interleukin (IL–2) for 24 to 72 hrs. This promotes blast formation, T-cell proliferation and over expression of T-cell surface markers (CD4).

Previously incubated donor PBMCs were centrifuged at 1500 rpm for 10 min and resuspended in growth medium containing IL–2 and 50 μl of (1mg/ml) PHA and incubated for 72 hrs in CO₂ incubator.

Infection of Donor PBMC’s with standard HIV stock
After 72 hrs incubation PHA stimulated cells were counted by Trypan blue method. Cells were centrifuged and the cell pellet was resuspended in 5ml RPMI medium. HIV infected PBMC’s were also centrifuged after counting and cell pellet was re-suspended in RPMI medium. 5 to 10 million PHA stimulated donor PBMC’s were then infected with 3 to 4 million HIV sero-positive PBMC’s in T25 flask containing growth medium. The flasks were then incubated for 4 days in CO₂ incubator.

Maintenance and storage of viral stock
The cultures were maintained for four weeks and were provided with feeder cells and aliquots for HIV p24 antigen testing were obtained according to the following schedule:

On 3rd or 4th day, 5 ml of supernatant was carefully removed from the flask. An aliquot for HIV p24 antigen testing was frozen at −70°C. The culture was fed with 5 ml of warmed growth medium. On 7th day, 5ml of supernatant was removed from the flask without disturbing the cells. An aliquot for HIV p24 antigen testing was preserved at −70°C. Culture was replenished with 5 ml of warmed growth medium containing 10 million PHA-stimulated PBMCs. Sampling and feeding in this manner was continued until the end of the culture i.e. twice per week; adding fresh medium once per week and adding fresh medium plus donor PBMCs weekly.

Tissue Culture Infectivity Dose 50 (TCID₅₀)

The TCID₅₀ assay measures the level of replication competent infectious virus from culture supernatants. This assay is performed prior to viral input-sensitive experimental procedures such as phenotypic drug susceptibility testing.

The TCID₅₀ assay estimates viable virus using a streamlined endpoint dilution assay that is analyzed by the Spearman-Karber statistical method. The TCID50 infectivity titration determination uses PHA-stimulated PBMC from normal donors. TCID₅₀ determinations are performed after high titer (assessed by p24 determination) culture supernatants are harvested, aliquoted and stored at −70°C or colder (preferably at liquid nitrogen temperatures).
To do this assay seven serial four-fold dilutions of virus stock, ranging from 1:16 to 1:65536 were titrated in triplicates in 96 well flat bottomed tissue culture plates.

PHA stimulated healthy donor PBMCs were centrifuged at 1200 rpm for 15 minutes at room temperature. The supernatant was discarded and the cells were resuspended in growth medium.

The cell viability was determined using 0.4% Trypan Blue exclusion dye. The cell density was then adjusted to 4 x 10⁶ cells/ml. 150 µl of the growth medium was added to previously labeled tissue culture wells. The virus stock was rapidly thawed at 37°C using a water bath. The virus sample was then diluted 1:12 using growth medium and transferred to the wells labeled 4⁻ flame and mixed properly. 50 µl from these wells were then transferred to the next wells labeled 4⁻² continuing such transfer till the wells labeled 4⁻⁸. 50 µl of the enumerated donor PBMCs were then added to each well and covered plate was then incubated at 37°C, 5% CO₂ with humidity. After seven days of incubation, the supernatants were tested for HIV p24 antigen by performing p24 capture ELISA.

The TCID₅₀ was calculated by the Spearman – Karber formula:

\[ M = xk + d \left[ 0.5 - \left( \frac{1}{n} \right) ( r ) \right] \]

Where; xk is dose of highest dilution; d is spacing between the dilutions; n is wells per dilution and r is sum of number of negative responses.

Interaction of viral particles with Gold Nanoparticles:

As the Nanoparticles synthesized were in the range of 2 – 10 nm; well below the size of the virus, it was expected that the AuNP will interact with the surface proteins of the virus and denature them and thus it will hinder virus from infecting T cells. To study the mode of action of Nanoparticles with HIV, they were interacted with the viral particles in two different ways:

Method I - In the first method the viral particles were interacted with the Nanoparticles for 1 hr and then allowed to interact with the PHA stimulated PBMC's.

50µl of viral suspension was added with 50µl of the different concentrations of the synthesized Nanoparticles in eppendorf tube and incubated for 1hr at 37°C. The concentrations of Nanoparticles used (2ppm and 4ppm) were based on the results of the Cytotoxicity assay done for the Nanoparticles showing less than 50 % cytotoxicity.

Method II - In second attempt the viral particles were first allowed to infect the PBMC’s and the Nanoparticles were added to the suspension afterwards.

A volume of 50 µl of viral suspension was added to different wells of tissue culture well plate containing 500 µl of PHA stimulated PBMC’s and incubated for 1 hr in CO₂ incubator. After 1 hr of incubation the wells were added with 2ppm and 4ppm concentration of different Nanoparticles and incubated for 72 hr at 37°C in CO₂ incubator. Following three controls were also kept parallel for incubation.
1. One control containing only cells and viral suspension.
2. Other control containing cells and viral suspension along with the standard anti-retroviral AZT.
3. One control containing only cells.

Anti-viral susceptibility test by P24 ELISA [10, 11]

The in vitro HIV drug susceptibility assays are performed with a cell line infected with HIV being exposed to various concentrations of the drug in question. An end-point determination of p24 antigen using an antigen capture ELISA method was done to quantify the levels of p24 antigen. The decrease of p24 antigen from control sample (without drug) to test sample (drug) is indicative of the drug being effective. A percent inhibition of p24 antigen can then be directly correlated to the efficacy of that drug.

Microwells are coated with a monoclonal antibody specific for the p24 gag gene product of HIV–1. Viral antigen in the specimen is specifically captured onto the immobilized antibody during specimen incubation. The captured antigen is then reacted with a high-titered human anti-HIV–1 antibody conjugated with biotin. Following a subsequent incubation with Streptavidin-Peroxidase, color develops as the bound enzyme reacts with the substrate.
Resultant optical density is proportional to the amount of HIV–1 p24 antigen present in the specimen. The procedure was followed according to the manual of the Zeptometrix RETRO-TEK HIV-1 p24 Antigen ELISA kit.

RESULTS AND DISCUSSION

Synthesis of Gold Nanoparticle
Gold nanoparticles were synthesized using Turkevich method using citrate ion as a reducing and nucleating agent. This citrate ion first gets converted to acetone dicarboxylate and then due to its collision with gold ions, gold nuclei are synthesized. Once gold nuclei are formed, they are capable enough to further reduce gold ions to grow on them. This process continues till the potential barrier is augmented so much that there is no further reduction and addition of Au+3 on the surface of the nanoparticle. Such nanoparticles have negative charge on their surface due to the strong adsorption of citrate ions forming stern layer on the colloidal particle. In the quest to decipher the characteristics of Gold nanoparticle, UV-Visible spectrophotometric analysis was performed and its Surface Plasmon Resonance was found to be at 530nm (Fig. 1A). Since there is only one transverse peak, it indicates that the nanoparticles are isotropic in nature i.e. they are spherical nanoparticles. This was further attested with the help of High resolution Transmission Electron Microscope which exhibited nanoparticles of size 2-10nm (Fig. 1B). It is postulated that small atomic clusters aggregated from the precursor nuclei, forms a single crystal of multiple twins, often with flat sides. Selective Area Electron Diffraction (SAED) pattern (Fig. 1C) further indicates that the particles are face-centred cubic which has (1,1,1) twin planes.

Figure – 1 (A) UV Spectrophotometric analysis of AuNP showing peak at 530 nm (B) HRTEM micrograph showing spherical nano particles of gold having 2 – 10 nm diameter (C) SAED pattern shows particles that are face-centred cubic which has (1,1,1) twin planes.
Pegylation of Gold nanoparticles

Gold nanoparticles were then pegylated using Polyethylene glycol (PEG) which showed a redshift in SPR peak as seen in UV VIS spectra (Fig 2) indicating interaction of PEG with Gold nanoparticles. An α-acetal PEG layers forms on GNP that imparts the stability to nanoparticle in aqueous solution (SERUM) and increases the solubility, it increases the size and prevents the clearance by RES, surface modification with PEG hides GNP from immune system Moreover it is a biocompatible compound and is known to prevent the cleavage of metallic bonds and it increase circulation in blood.

![UV-vis spectroscopy of AuNP and Pegylated AuNP (PEG-AuNP) showing a red shift](image)

Figure 2- UV-Visible spectrophotometer of Gold nanoparticles attached with PEG

![Cytotoxicity of Gold Nanoparticles by MTT assay](image)

Figure 3, a histogram showing % cytotoxicity, with respect to cell control of AuNP as assessed using the MTT.

Cytotoxicity assessment

Cytotoxicity assay of five concentrations of AuNP (2, 4, 6, 8 and 10ppm), of the two types of gold nanoparticles (AuNPs, Pegylated AuNPs) (Fig. 3) by MTT-assay shows that with increase in gold nanoparticle concentration there was an increase in cytotoxicity. It was below 10% in both the types of Au NP at 2 ppm. Hence, Anti-HIV activity was tested at 2 and 4 ppm concentrations only.
Anti-HIV activity of selected nanoparticles was studied using the in vitro drug susceptibility assay with an endpoint determination of p24 antigen. Percent inhibition of p24 antigen is co-related to the anti-HIV activity of the nanoparticles. Figure 3a and b shows that anti HIV activity of gold nanoparticles was more for post infection virus at both the tested concentrations; but after PEGylation the activity was more profound in virus prior to infection, but there was an inhibition in antiviral activity in post infection samples.

![Figure - 4: Anti HIV activity of (a) gold nanoparticles and (b) PEG-conjugated gold nanoparticles](image)

The nanoparticles showed greater activity in the test where the virus was allowed to interact with the nanoparticles before infecting the cells as compared to the test where the cells, previously infected with the virus, were allowed to interact with the nanoparticles. This may be due to the fact that the nanoparticles coated with charge stabilizers may be of a larger size and hence cannot enter the cells. This was also demonstrated by the uncoated Gold particles at 4 ppm that were directly added to previously infected cells showed a higher percent inhibition of p24 (77.78%) than the test in which the virus was first interacted with the nanoparticles (55.56%).

**CONCLUSION**

Gold nanoparticles were synthesized using Turkevich method and analyzed using UV-spectrophotometer, which exhibited Surface Plasmon Resonance at 535nm. The size of nanoparticles were determined by Transmission electron microscope and found to be 2-10nm. Such nanoparticles were then functionalized by polyethylene glycol so that RES clearance is delayed in the body.

This whole study demonstrates anti-HIV activity of Gold as well as Pegylated Gold nanoparticles. But before this study, it was imperative to analyze cytotoxicity of both the particles and then minimum inhibitory concentrations were determined. It was then found that Pegylated Gold nanoparticles were more effective as an entry inhibitor before infection of the cell rather than as a virus neutralizer after infection.

**REFERENCES**

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