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Gold nanoparticles conjugation enhances antiacanthamoebic properties of Nystatin, Fluconazole and Amphotericin B

Ayaz Anwar¹, Ruqaiyyah Siddiqui¹, Muhammad Raza Shah² and Naveed Ahmed Khan¹*

¹Department of Biological Sciences, School of Science and Technology, Sunway University, Subang Jaya 47500, Selangor, Malaysia; ²International Center for Chemical and Biological Sciences, H.E.J. Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan.

Running header: Antiacanthamoebic gold nanoparticles.

*Address for correspondence: Naveed Ahmed Khan, Department of Biological Sciences, School of Science and Technology, Sunway University, Selangor, Malaysia; E-mail: naveed5438@gmail.com; Tel: +603-74918622 Ext. 7169; Fax: +603-56358630.
Abstract

Parasitic infections have remained a significant burden on human and animal health. In part, this is due to lack of clinically-approved novel antimicrobials and a lack of interest by the pharmaceutical industry. An alternative approach is to modify existing clinically-approved drugs for efficient drugs delivery formulations to ensure minimum inhibitory concentration is achieved at the target site. Nanotechnology offers the potential to enhance the therapeutic efficacy of drugs through modification of nanoparticles with ligands. Amphotericin B, Nystatin, and Fluconazole are clinically available drugs in the treatment of amoebal and fungal infections. These drugs were conjugated with gold nanoparticles. To characterize these Gold-conjugated drugs atomic force microscopy, ultraviolet-visible spectrophotometry, Fourier transform infrared spectroscopy were performed. These drugs and their gold nanoconjugates were examined for antimicrobial activity against the protist pathogen, *Acanthamoeba castellanii* of the T4 genotype. Moreover, host cell cytotoxicity assays were accomplished. Cytotoxicity of these drugs and drug conjugated gold nanoparticles was also determined by lactate dehydrogenase assay. Gold nanoparticles conjugation resulted in enhanced bioactivity of all three drugs with Amphotericin B producing most significant effects against *Acanthamoeba castellanii* (*P*<0.05). In contrast, bare gold nanoparticles did not exhibit significant antimicrobial potency. Furthermore, amoebae treated with drugs-conjugated gold nanoparticles showed reduced cytotoxicity against HeLa cells. In this report, we demonstrated the use of nanotechnology to modify existing clinically-approved drugs and enhancing their efficacy against pathogenic amoebae. Given the lack of development of novel drugs, this is a viable approach in the treatment of neglected diseases.

Keywords: *Acanthamoeba*; Antimicrobial; Gold nanoparticles; Amphotericin B; Nystatin; Fluconazole.
Introduction

Nanomedicine has recently emerged as a viable alternative to its’ organic drugs counterparts especially in the improvement of new and more efficacious antimicrobials [1,2]. Due to continuous lack in drugs approvals, and ever growing resistance of microbes against common antimicrobial drugs, nanoconjugates are anticipated as the next generation of antimicrobial agents [3]. Nanoparticles are effective drug delivery agents and can increase the bioavailability of drugs at target sites. Their small size can be loaded with high amount of drug molecules, hence reducing the risks of toxicity and side effects. Numerous metallic and metal oxide nanoparticles have been developed and utilized in biomedicines for example copper, silver, gold, platinum, iron oxide, zinc oxide and etc. These nanoparticles have shown tremendous diagnostic as well as therapeutic potential against cancer, central nervous system, and infectious disease. The most common examples of nanoparticles used against infectious diseases are of silver and gold due to their biocompatibility. Gold nanoparticles have been associated with enhancing bioactivity and reducing cytotoxicity of antimicrobial drugs [4,5], as well as successfully used for improvement of bioavailability of drugs by improving routes for drugs delivery [6].

*Acanthamoeba* is an opportunistic free-living amoeba, and a causative agent of a rare but fatal central nervous system infection: granulomatous amoebic encephalitis (GAE), and a blinding eye infection, recognised as *Acanthamoeba* keratitis [7-9]. Currently there are none or very limited options for treatment and management of *Acanthamoeba* infections which include a combination of drugs including biguanides, amidine derivatives, and azoles; nonetheless these drugs suffer from some serious limitations such as long treatment plans and unspecific toxic effects on host cells [10-12].
Macrocyclic polyenes such as Amphotericin B and Nystatin, and Fluconazole target the ergosterol pathway to rupture plasma membrane. As ergosterol is a component of the Acanthamoeba plasma membrane, it is a logical target to develop drugs against Acanthamoeba infections, however owing to their cytotoxicity to host cells; ergosterol-targeting drugs are in limited use clinically [13-15]. In our recent report, silver nanoparticles conjugated with these drugs were shown to exhibit significant amoebicidal effects, while their host cells cytotoxicity was also reduced as compared to drugs alone [16].

In our present study, Fluconazole, Nystatin, and Amphotericin B were conjugated with gold nanoparticles (AuNPs). These nanoparticles were produced by silver nitrate reduction through sodium borohydride alongside the drugs. Fourier-transformation infrared (FT-IR) spectroscopy, atomic force microscopy (AFM) and ultraviolet-visible (UV-vis) spectrophotometry were accomplished to characterise drugs, and drugs joined together with nanoparticles. Subsequently, their antiamoebic activity against A. castellanii were determined.

Materials and methods

Chemicals

For drug synthesis, chemicals of analytical grade were utilised. Sodium borohydride and tetrachloroauric acid were acquired from Merck chemicals. Nystatin, Fluconazole and Amphotericin B were obtained from Sigma-Aldrich.

Synthesis of AuNPs

AuNPs were produced by using sodium borohydride as reducing agent in the presence of drugs [17]. Nystatin, Fluconazole and Amphotericin B coated tetrachloroauric acid were reacted
at 0.1 mM concentration in different volume ratios to obtain stable gold nanoparticles. Briefly, Amp-AuNPs were obtained at 1:1 volume ratio, followed by addition of 30 μL of 4 mM Sodium borohydride aqueous solution (prepared freshly). A colour change from light yellow to ruby red of the reaction concoction signified reduction of gold ions and the establishment of Amp-AuNPs. This reaction mixture was further agitated for 1 h until no change in colour was detected. The process was repeated for Nys-AuNPs and Flu-AuNPs, by enhancing various v/v ratio of gold solution in presence of the drugs. To attain stable Nys-AuNPs and Flu-AuNPs, respective volume-ratio of gold to drug at 5:1 was utilised. AuNPs alone were produced using the same method but with a lack of drugs or stabilizing agent. Subsequently, to remove large aggregates, any additional unbound drugs or side products from the colloidal suspension, centrifugation at 10000 x g for 10 min was done. Following nanoparticle synthesis, FT-IR spectroscope (Vector 22, Bruker) and UV-vis spectrophotometer (Evolution 300, Thermo Scientific) analysis was achieved. For morphological examination an AFM (Agilent 5500) was utilised as described previously [16].

**Acanthamoeba cultures**

*Acanthamoeba castellanii* from the T4 genotype (ATCC 50492) were cultivated in in 75cm² tissue culture flasks encompassing 10 mL PYG medium or proteose peptone 0.75% (w/v), Glucose 1.5% (w/v), Yeast extract 0.75% (w/v) and maintained at 30°C [18]. Trophozoites adhered to the surface of the flask were procured by washing with fresh PYG media followed by resuspension in 10 mL RPMI-1640 and placing on ice for 15 min along with gentle knocking for 5 min. Next, the *A. castellanii* RPMI-1640 suspension was relocated to a 50 mL tube, and centrifuged at 2500 x g for 10 min and the pellet was resuspended in 1 mL RPMI-1640. *A. castellanii* were enumerated by means of a haemocytometer and subsequently utilised for assays.
Amoebicidal assay

106 Amoebicidal activity of drugs alone, drugs joined together to AuNPs, and AuNPs alone were elucidated as reported earlier [18]. Briefly, $5 \times 10^5$ *A. castellanii* cells were incorporated with various (5 and 10 µM) concentrations of drugs alone or drugs conjugated with AuNPs, nanoparticles alone, and solvent controls at 30 °C for 24 h. Following this Trypan blue cell exclusion assay was carried out. For controls, RPMI-1640 alone served as a negative control and 10 µM of the anti-amoebic drug: Chlorhexidine was utilised as a positive control. To elucidate the viability of amoebae 0.1% Trypan blue was added and living (non-stained) *A. castellanii* were enumerated with a haemocytometer. Results obtained were presented as percentage inhibition: mean inhibition ± standard error of three independent experiments performed in duplicate. The percentage of inhibition was calculated as follows: % inhibition = 100 – (no. of viable *Acanthamoeba* in sample treated / no. of viable *Acanthamoeba* untreated) × 100.

Henrietta Lacks cervical adenocarcinoma cells (HeLa) cells culture

118 Henrietta Lacks (HeLa) cells were typically cultivated using 75 cm² culture flasks in RPMI-1640 comprising 10% Nu-serum, 10% FBS, 1 mM Pyruvate, 2 mM Glutamine, Streptomycin (100 µg/ml), Penicillin (100 Units/ml), non-essential amino acids and vitamins. Prior to experiments, media was removed and 2 mL trypsin was added to detach cells from confluent flasks, next cells were centrifuged at 2000 x g for 5 min. The resulting pellet was resuspended in 30 mL media, and harvested into 96-well plate, incubated at 37 °C, 95% humidity and 5% CO₂ for at least 24 h. Plates were scrutinized using a light microscope until a visible uniform monolayer of HeLa cells was observed for use in the cytotoxicity assay.
**Cytotoxicity assays**

To determine *in vitro* cytotoxicity of drugs conjugated AuNPs, cytotoxicity assays were performed using HeLa cells. Briefly, cells were developed until confluent in 24-well plates and different concentrations of drugs nanoconjugates were added. The plates were placed at 37°C in a 5% CO$_2$ incubator for 24 h. Supernatants were collected and percentage cell cytotoxicity was elucidated by measuring lactate dehydrogenase release (Roche Applied Science). Cells alone represented the negative control, whereas HeLa monolayers completely lysed with 1% Triton X-100 represented the positive control corresponding to 100% cell death. Absorbance of each sample was recorded on a plate reader at 490 nm. The percentage cytotoxicity was obtained: 

\[
\text{% cytotoxicity} = \left( \frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}} \right) \times 100.
\]

*Acanthamoeba* facilitated HeLa cell cytotoxicity was assessed as formerly depicted [19,20]. Concisely, experiments were accomplished in 96-well plates containing uniform HeLa monolayers. *Acanthamoeba* ($1 \times 10^4$) were treated with 5 and 10 μM concentrations of drugs alone, drugs conjugated AuNPs, AuNPs alone, chlorhexidine (10 μM), and incubated for 2 h at 37 °C. Moreover, centrifugation at 5000xg for 1 minute was completed to eliminate any extracellular constituents, and the resulting pellet was resuspended in fresh RPMI. These test samples and controls were then added to HeLa cell monolayers and incubated in a 5% CO$_2$ incubator for 24 h at 37 °C. Negative control values were obtained for monolayers in RPMI-1640 alone. Positive control values were acquired by completely lysing cells with 1% Triton X-100. Subsequently, the supernatants were carefully collected and cytotoxic activity elucidated by using the Lactate dehydrogenase (LDH) (Roche Applied Sciences) kit followed by absorbance estimation on a plate reader at 490 nm. The percentage
cytotoxicity was determined: \[
\% \text{ cytotoxicity} = \left( \frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}} \right) \times 100.
\]

Results

Drugs coated with gold nanoconjugates were characterised using FT-IR spectroscopy, UV-vis, and AFM

To characterise drugs coated AuNPs and to confirm the formation of nanoparticles, UV-vis spectrophotometry was performed. The characteristic surface plasmon resonance bands are shown in Fig. 1. Amp-AuNPs, Nys-AuNPs and Flu-AuNPs corresponding to successful synthesis. The UV-vis spectra of Amp-AuNPs produced maximum absorption at 555 nm in comparison to Amphotericin B alone which reveals various absorption bands in the array of 280-400 nm \[21\], implying its’ stabilizing interactions with AuNPs. On the other hand, Nys-AuNPs and Flu-AuNPs presented a characteristic surface plasmon resonance (SPR) band for AuNPs at 561 and 525 nm respectively, associated with bands approximately 280-330 nm and 260 nm correspondingly for Fluconazole and Nystatin alone \[22,23\]. FT-IR analysis was achieved to report interaction between drugs and AuNPs (Fig. 2). Amphotericin B also showed strong stretching band at 3400 \text{ cm}^{-1} for hydrogen bonded molecules and 1645 \text{ cm}^{-1} for carbonyl, whereas, in Amp-AuNPs these signals were shifted to 3427 and 1633 \text{ cm}^{-1} respectively. Since, Nystatin and Amphotericin B both have structural analogies, same effects were observed in the case of Nyst-AuNPs. Fluconazole alone displayed an absorption band at 1413 \text{ cm}^{-1} portraying triazole ring stretching, which on the formation of Flu-AuNPs, shifted to 1384 \text{ cm}^{-1}. These results indicate that the interaction of hydroxyl groups for Amphotericin B and Nystatin, and the
triazole ring for Fluconazole are responsible for the stabilization of AuNPs. Morphology and size of the nanoparticles was assessed by AFM. Nanoparticles including some larger aggregates were observed under AFM imaging (Fig. 3). Nyst-AuNPs provided the smallest particle size lying in the series of 10-50 nm, while particle size was highly polydispersed for Amp-AuNPs and Flu-AuNPs ranging from 50-200 nm.

**AuNP conjugated drugs display enhanced amoebicidal activity against A. castellani in comparison to drugs alone**

The amoebicidal potency of drugs alone and their gold nanoconjugates was determined by amoebicidal assay. All drugs exhibited significant amoebicidal activity at both 10 and 5 µM concentrations except for Fluconazole which only displayed amoebicidal results at 10 µM (P<0.05 by means of 2 sample T-test and two-tailed distribution) (Fig. 4). Drugs coated AuNPs on the other hand, displayed more pronounced killing effects as compared to drugs alone. Amp-AuNPs exhibited amoebicidal results at 10 µM with 76 % inhibition as compared to 56 % for Amphotericin B alone. Similarly, at 5 µM concentration, 11 % increase was found in percent inhibition of Amp-AuNPs as compared to Amphotericin B alone. Flu-AuNPs were also found to exhibit 11 % increased percent inhibition at 5 µM as compared to Fluconazole alone which only showed 22 % inhibition. However, no drastic changes were observed in the case of Nystatin alone vs Nyst-AuNPs. On the contrary, treatment with AuNPs alone had no effect on amoebae inhibition.

**Cytopathogenicity of drugs conjugated AuNPs**

To ascertain if AuNPs conjugated drugs inhibit microbial cytopathogenicity against host cells, microbial mediated cytotoxicity assays were completed. When *A. castellani* alone were
incorporated with host cells this caused 72% cell cytotoxicity (Fig. 5). On the other hand, pre-
treatment of *A. castellanii* with chlorhexidine resulted in minimal cells cytotoxicity. However,
only Amp-AuNPs and Nyst-AuNPs reduced the *A. castellanii* cytotoxicity (P<0.05 by means of
2 sample T-test and two-tailed distribution). The cytotoxicity profile of drugs joined together
with gold nanoparticles against HeLa cells was also evaluated. The results revealed that all drugs
coated nanoparticles produced less than 25% host cells death except Fluconazole.

**Discussion**

Nanotechnology has recently emerged as a tremendous tool in the arena of biomedicine,
particularly in diagnosis and drug delivery. Drug delivery carriers based on nanotechnology have
been developed and are being utilised in clinical applications for example nanotubes,
nanoparticles, and liposomes [24]. Nanomaterials hold a pivotal role in drug delivery and
provide numerous advantages including (i) augmented bioavailability; (ii) reduced side effects;
(iii) precise drug targeting. Moreover, the miniature proportions of nanoparticles delivers a
superior surface area for maximum drug packing in addition to elevated approachability for
precise targets [25]. Numerous nanoparticles conjugated with drugs have been produced and
utilized against several infectious diseases caused by resistant microbes and parasites [26]. In our
recent study, we reported the enhanced effects of antifungal drugs, Nystatin, Fluconazole
Amphotericin B conjugated with AgNPs on *A. castellanii* [16]. Since these drugs target the
ergosterol pathway which is an essential component of *A. castellanii* membrane, their
conjugation with nanoparticles resulted in increased bioactivity. Here, we determined the effects
of AuNPs conjugation with these drugs on protist and human cells. Drug coated AuNPs were
created by the one phase reduction method and categorised by UV-vis spectrophotometry, FT-IR
spectroscopy, and AFM. All drugs coated AuNPs showed surface plasmon resonance (SPR)
band in the series of 500-560 nm, characteristic of medium sized ligand stabilized AuNPs. FT-IR
analysis showed the involvement of hydroxyl groups and triazole ring for the stabilization of
nanoparticles. The sizes of nanoparticles were found to be in the range of 10-200 nm, as a result
of rapid reducing conditions. The conjugation of AuNPs to these drugs molecules enhanced the
antiacanthamoebic effects. Since the intravenous administration of these drugs lack specificity
and as a result causes host cells cytotoxicity. Conjugation with inert metal such as gold in the
form of nanoparticles was hypothesized to decrease these side effects along with enhancement in
bioavailability of drugs at low dosage.

The reason for enhanced cytotoxicity of drugs conjugated AuNPs is most probably due to
enhanced transport of drugs to the target site. Recent literature reports on antibacterial activity of
nanoparticles suggest that their biocidal effects are highly dependent on their size, shape and
surface modifications [27,28]. Moreover, their mode of bacteria killing is supposed to be a
combination of multiple factors including production of biocidal reactive oxygen species altering
the DNA, interaction with thiolated amino acids of enzymes etc. [29,30]. Hence nanoparticles
are speculated as next generation antibacterial agents due to their broad range of targets in the
microorganisms. In conclusion, these findings undoubtedly reveal that conjugation of drugs with
nanoparticles improved and enhanced the antiamoebic activity of existing drugs. Future studies
using in vivo models and administration of drugs coated with nanoparticles via different portals
of entry to assess their maximum biological potential need to be conducted.

Abbreviations: AK: Acanthamoeba keratitis; GAE: Granulomatous amoebic encephalitis;
AuNPs: Gold nanoparticles; Amp: Amphotericin B; Nys: Nystatin; Flu: Fluconazole; LDH:
Lactate dehydrogenase; FT-IR: Fourier-transformation infrared spectroscopy; AFM: Atomic
force microscopy; UV-vis: Ultraviolet-visible spectrophotometry; PYG: Proteose peptone, yeast extract, and glucose.

**Declarations**

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**Availability of data and materials:** The datasets used and/or analysed during the current study are available from the corresponding author upon request.

**Authors contribution:** AA synthesized nanoparticles and carried out bioassays in the supervision of RS and NK. MRS supervised and participated in characterization of nanoparticles. RS and NK generated the idea. AA wrote the manuscript with corrections and improvements from RS and NK. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate:** Not applicable.

**Consent for publication:** The manuscript was submitted with the consent of all authors.

**Competing interests:** The authors report no competing interests in this work.
References


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Figure legends

Figure 1. UV-Vis spectra of drugs coated AuNPs showed surface plasmon resonance bands at 555, 561 and 525 nm for Amp-AuNPs, Nys-AuNPs and Flu-AuNPs, respectively which indicates the successful formation of drugs conjugated AuNPs.
Figure 2. FT-IR spectra of drugs conjugated AuNPs. Hydroxyl groups stabilized AuNPs in the case of amphotericin B and nystatin, whereas, triazole group chelated the AuNPs in Flu-AuNPs. Spectra were obtained by KBr disc method on Bruker Vector 22 instrument.

Figure 3. AFM images of Amp-AuNPs; A, Nyst-AuNPs; B, Flu-AuNPs; C. Extensive imaging was done to describe representative images. Nyst-AuNPs gave small particles size (10-50 nm), while Amp-AuNPs and Flu-AuNPs were found to be in the range of 50-200 nm.

Figure 4. *A. castellanii* viability was determined following incubation with various concentrations of drugs alone and drugs conjugated AuNPs as described in Materials and methods. All three drugs conjugated AuNPs and drugs alone exhibited amoebicidal effects. Notably, Amp-AuNPs and Flu-AuNPs showed most prominent amoebicidal effects at 10 µM compared with drugs alone (P<0.05 using 2 sample T-test and two-tailed distribution). The results are presented as the mean inhibition ± standard error of at least three independent experiments performed in duplicate. The percent inhibition was calculated as follows: % inhibition = 100 – (no. of viable *Acanthamoeba* in sample treated / no. of viable *Acanthamoeba* untreated) × 100.

Figure 5. Cytopathogenicity was determined by treating host cells with *A. castellanii* with and without pre-treatment with drugs and drugs coated AuNPs as described in materials and methods. Untreated microbes killed more than 70% cells, while microbes pre-treated with drugs and drugs coated AuNPs showed significantly reduced host cells cytotoxicity as compared to untreated microbes (P<0.05 using 2 sample T-test and two-tailed distribution). The data are presented as means standard errors from at least three independent experiments performed in duplicate. The percent cytotoxicity was calculated as follows: % cytotoxicity = (sample
absorbance – negative control absorbance) / (positive control absorbance – negative control absorbance) × 100.
Fig. 1

Absorbance vs. Wavelength (nm) for Nys-AuNPs, Amp-AuNPs, and Flu-AuNPs.
Fig. 2

Amp-AuNPs

Wavenumber (1/cm)

Percent Transmittance

Nyst-AuNPs

Wavenumber (1/cm)

Percent Transmittance

Flu-AuNPs

Wavenumber (1/cm)

Percent Transmittance
Fig. 3
Fig. 4

Percentage inhibition

Acanthamoeba alone
Chlorhexidine
AuNPs alone 10 µM
Amp alone 10 µM
Amp-AuNPs 10 µM
Amp alone 5 µM
Amp-AuNPs 5 µM
Nyst alone 10 µM
Nyst-AuNPs 10 µM
Nyst alone 5 µM
Nyst-AuNPs 5 µM
Flu alone 10 µM
Flu-AuNPs 10 µM
Flu alone 5 µM
Flu-AuNPs 5 µM
Fig. 5

Percentage cell cytotoxicity

- Acanthamoeba alone
- Acanthamoeba-CHX
- AuNPs alone 10 μM
- AuNPs alone 5 μM
- Amp-AuNPs 10 μM
- Amp-AuNPs 5 μM
- Nyst-AuNPs 10 μM
- Nyst-AuNPs 5 μM
- Flu-AuNPs 10 μM
- Flu-AuNPs 5 μM