Original article

Silver nanoparticles inhibit hepatitis B virus replication

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Background: Silver nanoparticles have been shown to exhibit promising cytoprotective activities towards HIV-infected T-cells; however, the effects of these nanoparticles towards other kinds of viruses remain largely unexplored. The aim of the present study was to investigate the effects of silver nanoparticles on hepatitis B virus (HBV).

Methods: Monodisperse silver nanoparticles with mean particle diameters of ~10 nm (Ag10Ns) and ~50 nm (Ag50Ns) were prepared from AgNO3 in HEPES buffer. The in vitro anti-HBV activities of these particles were determined using the HepAD38 cell line as infection model.

Results: Ag10Ns and Ag50Ns were able to reduce the extracellular HBV DNA formation of HepAD38 cells by >50% compared with the vehicle control (that is, HepAD38 cells in the absence of silver nanoparticles). Silver nanoparticles had little effect on the amount of HBV covalently closed circular DNA (cccDNA), but could inhibit the formation of intracellular HBV RNA. Gel mobility shift assays indicated that Ag10Ns bound HBV double-stranded DNA at a DNA:silver molar ratio of 1:50; an absorption titration assay showed that the nanoparticles have good binding affinity for HBV DNA with a binding constant ($K_b$) of $(8.8 \pm 1.0) \times 10^5$ dm$^3$mol$^{-1}$. As both the viral and Ag10Ns systems are in the nanometer size range, we found that Ag10Ns could directly interact with the HBV viral particles as revealed by transmission electronic microscopy.

Conclusions: Silver nanoparticles could inhibit the in vitro production of HBV RNA and extracellular virions. We hypothesize that the direct interaction between these nanoparticles and HBV double-stranded DNA or viral particles is responsible for their antiviral mechanism.

Introduction

Hepatitis B virus (HBV) is a partially double-stranded (ds) DNA virus that chronically infects more than 400 million people worldwide. The persistence of HBV is associated with the development of liver cirrhosis and hepatocellular carcinoma [1,2]. After the entry of HBV virions into the hepatocyte, viral core particles migrate to the nucleus where the viral genome is repaired to form a covalently closed circular DNA (cccDNA), that forms the template for the transcription of viral messenger RNA (mRNA) and 3.5 kb pregenomic RNA (pgRNA) [3,4]. The pgRNA serves as the template for the reverse transcription and for the synthesis of viral genomes [5]; thus, the transcription of pgRNA from cccDNA is a key event in HBV replication.

By 2007, six agents were registered for the treatment of chronic HBV infection. Two are immunomodulatory agents, namely conventional interferon (IFN)-α2b and pegylated IFN-α2a, which aim to restore host immune control on HBV and thus lead to sustained off-treatment disease remission. The other four agents are nucleos(t)ide analogues (lamivudine, adefovir, entecavir and telbuvudine) with direct antiviral activity [6]. IFN-α can promote the lysis of infected hepatocytes by both CD8+ cytotoxic T-lymphocytes and natural killer cells [7]; moreover, IFN-α can directly inhibit the synthesis of viral proteins by modulating the action of antiviral cytokines [8]. However, the use of IFN-α is hampered by the serious side effects and low success
rates [9]. Nucleos(t)ide analogues act mainly as specific inhibitors of the viral polymerase reverse transcriptase. Although the treatment of chronic hepatitis B (CHB) with nucleos(t)ide analogues produces rapid suppression of HBV replication in the short-term, this effect is not often sustainable due to the emergence of drug-resistant HBV strains [10]. It is therefore important to develop new antiviral strategies to combat wild-type and mutant HBV infections.

Although the use of metal nanoparticles in catalysis and optoelectronic devices has been extensively investigated [11–13], there have been relatively few studies on their biological properties and potential therapeutic applications [14,15]. In this regard, the antimicrobial activities of silver nanoparticles have received the most attention [16,17], and proteomic and biochemical studies on the antibacterial and/or fungal activities of silver nanoparticles have been reported [16]. In the literature, there are only two published studies on the antiviral activities of silver nanoparticles: both reporting their anti-HIV-1 properties [18,19]. It was found that silver nanoparticles could bind to HIV viral particles with a regular spatial arrangement and were able to inhibit the virus from binding to host cells [18]. Also, silver nanoparticles can inhibit the replication of HIV and exhibit promising cytoprotective activities toward HIV-infected T-cells [19]. Indeed, studies on the interaction of metal nanoparticles with viruses are sparse [18–20], despite both systems falling in the nanometer range.

In this study, we prepared monodisperse silver nanoparticles with mean particle diameters of ~10 nm (Ag10Ns) and ~50 nm (Ag50Ns) from AgNO3 in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. The in vitro anti-HBV activities of these particles were determined using the HepAD38 cellular model [21]. We found that silver nanoparticles could interact with HBV dsDNA and viral particles, and were able to inhibit the in vitro production of HBV RNA and extracellular virions.

**Methods**

Preparation and characterization of silver nanoparticles Ag10Ns (~10 nm) silver particles were prepared by adding AgNO3 solution (1 ml, 50 mM) to a HEPES buffer (48 ml, 10 mM, pH 7.4). The mixture was then refluxed for 2 h with vigorous stirring. A resultant golden yellow solution with a surface plasmon resonance (SPR) absorption peak at ~400 nm was obtained, revealing the formation of silver nanoparticles. Likewise, Ag50Ns (~50 nm) particles were obtained by adding AgNO3 (1 ml, 50 mM) solution and sodium citrate (0.01 g) to a HEPES buffer (48 ml, 10 mM, pH 7.4). The mixture was heated under reflux for 2 h and a resultant golden yellow solution with an SPR absorption peak at ~400 nm was obtained, revealing the formation of silver nanoparticles. Ag800Ns (~800 nm) were prepared by adding AgNO3 (1 ml, 50 mM) solution and ascorbic acid (0.05 g) to a HEPES buffer (48 ml, 10 mM, pH 7.4). After stirring for 10 min, the mixture was heated at 40°C with stirring for 2 h. Silver nanoparticles (Ag800Ns) were obtained.

The X-ray diffraction (XRD) spectra of silver nanoparticles were recorded with a Philips PW1830 powder X-Ray diffractometer (Philips, Madrid, Spain). The UV-vis absorption spectra were recorded using a Perkin-Elmer Lambda 900 UV-vis spectrophotometer (Perkin-Elmer, MA, USA). Transmission electronic microscopy (TEM) images of the silver nanoparticles were obtained with JEM-JEM2000 transmission electromicroscopy (accelerating voltage of 200 kV), Philips Tecnai 20 equipped with Oxford incax-sight EDX attachment (accelerating voltage of 200 kV) or Philips EM208s (accelerating voltage of 80 kV).

Stability evaluation

The UV-vis absorption spectra of both Ag10Ns and Ag50Ns in aqueous solution were monitored over 48 h and 3 months. Their stabilities in physiologically relevant medium (HEPES buffer, pH 7.4) were also monitored by UV-vis spectrophotometry.

Cell culture

The HepAD38 cell line was maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, CA, USA) supplemented with fetal bovine serum (10% v/v), 1-glutamine (2 mM), penicillin (50 μg/ml), streptomycin (100 μg/ml), kanamycin (50 μg/ml), geneticin (400 μg/ml) and tetracycline (0.3 μg/ml). Cells were incubated at 37°C in a 5% CO2 humidified atmosphere.

Antiviral and cytotoxicity assays

To assess the antiviral activity of silver nanoparticles, HepAD38 cells were seeded into 24-well microtitre plates (2×10^5 cells/well) and grown for 3 days in the presence of tetracycline (0.3 μg/ml). The cells were washed twice with phosphate-buffered saline (PBS) and treated with tetracycline-free medium that contained either a test or control compound. The cell cultures were incubated at 37°C in a 5% CO2 humidified atmosphere for 48–96 h. The cell medium was then collected and total DNA was extracted from the medium using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). A quantitative real-time polymerase chain reaction (PCR) assay for the detection of HBV DNA was performed as described previously [22].
To assess the cytotoxic effects of silver nanoparticles, HepAD38 cells were seeded into 96-well plates at a density of 2x10^4 cells/well and exposed to silver nanoparticles with a treatment schedule identical to that described above for the antiviral assays. Following drug treatment, cell viability was assessed by a modified method of the Mosmann-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[23]

HBV RNA and cccDNA detection
HepAD38 cells were seeded into 24-well microtitre plates (2x10^5 cells/well) and grown for 3 days in the presence of tetracycline (0.3 μg/ml). The cells were washed twice with PBS and treated with tetracycline-free medium with or without Ag10Ns (1–10 μM). The cell cultures were incubated at 37°C in a 5% CO2 humidified atmosphere for 24, 48, 72 or 96 h. At the end of the incubation, adhesive HepAD38 cells were trypsinized, washed with PBS and divided into two aliquots for DNA extraction and RNA extraction. Total cellular DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN), and used for HBV cccDNA quantification by real-time PCR as described[24]. Total RNA was extracted from the cells using the RNAeasy Mini Kit (QIAGEN); HBV RNA was detected by real-time reverse transcription PCR (RT-PCR) with the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, CA, USA) according to the manufacturer's instructions, using the 5′ HCR primer (5′-GGCTCTTTGGAAGTGTGGATTGC nt2258–2280) and 3′ antisense HCR primer (5′-TCGTCTCTACAACACAGTATTTCC nt2356–2332). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize the quantity of total human RNA in the template, and the Human GAPDH Endogenous Control™ (VIC/TAMRA Probe, Primer Limited, Applied Biosystems) was used for GAPDH mRNA amplification and detection. The relative quantity of HBV RNA was calculated through comparison with the quantity of GAPDH by 2^-ΔΔCT method[25].

Cellular uptake experiments
Cellular uptake experiments were conducted according to the method described in the literature[26] with some modifications. HepAD38 cells (2x10^5 cells/well) were seeded in 24-well plates with culture medium (1 ml/well) and incubated at 37°C in an atmosphere of 5% CO2 for 24 h. The culture medium was removed and replaced with culture medium containing silver nanoparticles at a concentration of 5 μM. After exposure to the silver nanoparticles for 2, 4 and 24 h, the medium was removed and the cell monolayer was washed four times with ice-cold PBS. Milli-Q water (1 ml) was added and the cell monolayer was scraped off the well. Samples (300 μl) were digested in 70% HNO3 (500 μl) at 70°C for 2 h then diluted 1:100 in water for analysis by inductively coupled plasma mass spectrometry (ICP-MS).

Gel mobility shift assay
Full-length HBV dsDNA was produced according to a previously described method[27]. HBV dsDNA treated with Ag10Ns at different molar ratios (dsDNA:Ag10Ns 1:50, 1:5, 1:0.5 and 1:005) as well as the vehicle control (that is, HepAD38 cells in the absence of silver nanoparticles) were resolved by 3% agarose gel electrophoresis.

Absorption titration
A solution of the silver nanoparticles in PBS (95% v/v) was placed in a thermostatic cuvette in a UV-vis spectrometer and an absorption spectrum recorded. Aliquots of a millimolar stock DNA solution were added to the solution of silver nanoparticles and absorption spectra were recorded after equilibration for 10 min per aliquot until saturation point was reached. The binding constant K_s can be determined from the following equation:

$$K_s = \frac{[\text{DNA}] - [\text{DNA}]_{\text{abs}}}{[\text{Ag10Ns}] + 1/(\Delta e + K_b)}.$$  

where Δe is the A obs/[silver nanoparticles] and Δe=|e_A−e_B| with e_A and e_B corresponding to the extinction coefficients of the DNA-bound and unbound silver nanoparticles, respectively[28]. A plot of [DNA]/Δe versus [DNA] will have a slope of 1/Δe and a y intercept equal to 1/(ΔeK_b). K_s is given by the ratio of the slope to the y intercept.

Measuring the interaction between silver nanoparticles and HBV virions
Ag10Ns were coated on the surface of 96-well plates and serum samples of CHB patients, containing 1x10^7 HBV virions/ml, were incubated in treated or untreated (control) wells for 0–60 min. At 10 min intervals, the serum samples in the treated and control wells were collected and subjected to quantitative PCR following total DNA extraction.

Electron microscopy
HepAD38 cells were treated with Ag10Ns (5 μM) for 96 h at 37°C and were pelleted in a 1.5 ml centrifuge tube at 2,000 rpm for 10 min. Supernatant was removed and the cell pellet fixed in glutaraldehyde (2.5%) for 12 h at 4°C. The pellet was then washed three times in PBS, and incubated in OsO4 (2%) and K2Fe(CN)6 (1%) for 1 h. After three further PBS...
washes, pellets were dehydrated and infiltrated in a 1:1 mixture of propylene oxide and embedded in resin. Ultrathin (50 nm) sections were collected on 200-mesh copper grids and stained with uranyl acetate (6%) in double-distilled water for 10 min followed by lead citrate (1%) for 15 min. Sections were viewed using a Philips EM208s transmission electron microscope at 80 kV.

Results and discussion

Characterization and stability of silver nanoparticles
In this work, we prepared monodisperse silver nanoparticles with mean particle diameters of ~10 nm (Ag10Ns), ~50 nm (Ag50Ns) and ~800 nm (Ag800Ns) from AgNO₃ in HEPEs buffer. The silver nanoparticles were characterized by TEM (Supplementary material; Figure S1), X-ray powder diffraction (XRD; Figure S2) and energy dispersive X-ray analysis (EDX; Figure S3).

The silver nanoparticles Ag10Ns and Ag50Ns were found to be stable in water, with no significant spectral changes observed over a 48 h period at 37˚C based on UV-vis spectrophotometric measurements. In contrast, although we did not detect any spectral changes for solutions of Ag800Ns within the first few hours, after incubation at 37˚C for 48 h there was an approximate 38% decrease in absorbance at the \( \lambda_{\text{max}} \) (Figure S3).

Previously, human serum albumin (HSA) was reported to stabilize silver nanoparticles. In this work, we found...
that HSA was able to stabilize silver nanoparticles \(\text{Ag}10\text{Ns}\) and \(\text{Ag}50\text{Ns}\), with no significant UV-vis spectral changes observed over a 3-month period.

Antiviral effects and cytotoxicity of silver nanoparticles HepAD38 is a stably transfected hepatoblastoma cell line that secretes HBV-like particles and expresses high levels of HBV DNA into its surrounding medium (supernatant) \[21\]. To test the anti-HBV activity of silver nanoparticles, HepAD38 cells were separately treated with silver nanoparticles \(\text{Ag}10\text{Ns}\) and \(\text{Ag}50\text{Ns}\) at concentrations of 5–50 \(\mu\text{M}\). After 48 h incubation, total DNA was extracted from the supernatants, and the HBV DNA levels were quantified by quantitative real-time PCR. Parallel cytotoxicity evaluation was done by MTT assay \[23\]. Compared with the drug-free vehicle control, both \(\text{Ag}10\text{Ns}\) and \(\text{Ag}50\text{Ns}\) showed 38% inhibition at 5 \(\mu\text{M}\) and 80% at 50 \(\mu\text{M}\); \(\text{Ag}50\text{Ns}\) showed 53% inhibition at 5 \(\mu\text{M}\) and 92% at 50 \(\mu\text{M}\); the results are depicted in Figure 1A. Over 90% cell viability was observed for \(\text{Ag}10\text{Ns}\) and \(\text{Ag}50\text{Ns}\) at 5 \(\mu\text{M}\) (Figure 1B). In contrast, the viability of HepAD38 cells decreased in the presence of silver nanoparticles at a concentration of 50 \(\mu\text{M}\) for both \(\text{Ag}10\text{Ns}\) and \(\text{Ag}50\text{Ns}\). The induced cytotoxicity could be accounted for by the aggregation of silver nanoparticles at high concentrations. Silver nanoparticles (\(\text{Ag}800\text{Ns}\)) with particle diameters of 800 nm were found to induce severe cytotoxicity to the HepAD38 cells even at the 5 \(\mu\text{M}\) level; thus, their potential anti-HBV activity was not examined. For comparison, gold nanoparticles (10 nm, Au10Ns) prepared by sodium citrate reduction were found to exhibit relatively low anti-HBV activity (10–13%). Silver compounds with silver in different oxidation states, including \(\text{Ag}\text{NO}_3\), \([\text{Ag}^{III} \text{TPP}](\text{H}_2\text{TPP}=\text{meso-tetraphenylporphyrin})\) and \([\text{Ag}^{II} \text{TPC}](\text{H}_3\text{TPC}=\text{meso-triphenylcorrole})\), also showed no significant anti-HBV activities (Figure 1C). Taken together, the results indicate that the anti-HBV effects of silver nanoparticles are significant when compared with gold nanoparticles and other classes of silver complex.

Using \(\text{Ag}10\text{Ns}\) as an example, the dose-dependent (from 0.1 to 50 \(\mu\text{M}\)) anti-HBV effects of silver nanoparticles inhibit HBV replication.
nanoparticles were examined. Compared with the vehicle control, Ag10Ns inhibit HBV DNA replication in a dose-dependent manner after 48 h incubation (Figure 1D). However, Ag10Ns was found to achieve maximum inhibitory effect at a concentration of 5 μM with prolonged incubation (96 h), whereas the anti-HBV effect was reduced or reversed at higher concentrations (10, 20 and 50 μM; Figure 1F). The plateau effect of the antiviral activity could be explained by the release of intracellular HBV DNA at high concentrations of Ag10Ns. As mentioned, treatments of Ag10Ns at high concentrations were cytotoxic to the host HepAD38 cells (Figure 1E,G) and the intracellular HBV DNA might be released from the lysed cells. Prolonged incubation with high dosages of silver nanoparticles could have led to the sustained release of intracellular DNA to the surroundings and counteracted the antiviral effects of the silver nanoparticles.

The time-dependent anti-HBV activities of silver nanoparticles were further examined at various time points from 0 to 96 h. In this study, supernatants of HepAD38 cells were collected (0, 24, 48, 72 and 96 h) after the introduction of Ag10Ns. The time dependence of the percentage inhibition of extracellular HBV DNA production by Ag10Ns at a concentration of 5 μM level was first examined, with the results plotted in Figure 2A (solid line). Ag10Ns exhibited ~30% inhibition at t=48 and 72 h, and achieved over 50% inhibition at t=96 h. Notably, parallel MTT assays revealed that >95% of the Ag10Ns-treated cells remained viable (Figure 2A, dotted line). Ag10Ns exhibited ~50% inhibition at t=48 and 72 h, and achieved over 50% inhibition at t=96 h. Ag10Ns also inhibited HBV replication (~50% at t=48 and 72 h; Figure 2B, solid line), with over 95% viable cells detected (dotted line). At a concentration of 50 μM, both Ag10Ns and Ag50Ns exhibited potent inhibitory activities of extracellular HBV formation at 24 or 48 h of incubation (solid lines, Figures 2C and 2D, respectively). After these time points, the sustaining cytotoxic effects (dotted lines, Figures 2C and 2D, respectively) enhanced the release of intracellular HBV DNA to the surroundings.

Figure 2. The time-dependent anti-HBV activity of silver nanoparticles

(A) 5 μM Ag10Ns, (B) 5 μM Ag50Ns, (C) 50 μM Ag10Ns, (D) 50 μM Ag50Ns.
To determine whether or not binding to HSA would alter their biological activities, the anti-HBV activities of the silver nanoparticles were examined after incubation with HSA at a concentration 2 mM. Similar levels of potency were observed for both the HSA-free and HSA-bound nanoparticles (for example, 41% and 73% at 5 and 50 μM of HSA-Ag10Ns, respectively). This result suggests that HSA might also serve as a carrier for the silver nanoparticles to the biological targets, that is, HBV-infected cells.

Uptake of silver nanoparticles and effects on the HBV life cycle

The uptake of silver nanoparticles by HepAD38 cells was examined. After treatment with Ag10Ns (5 μM, 2 h incubation), the cells were collected and subjected to ICP-MS to analyse the silver content. Cell-free samples (100% silver content) were compared with cells treated with Ag10Ns absorbed (~15% of silver). The high level of initial cellular uptake implied that some silver nanoparticles could reach their potential intracellular target(s) (for example, HBV DNA, RNA and cccDNA, etc) by passing through the membranes of HepAD38 cells.

Using Ag10Ns as the model, the HBV replicative intermediates were examined at various time points in order to elucidate the effects of silver nanoparticles on the HBV life cycle. HepAD38 cells were treated with Ag10Ns at 5 μM for 0, 24, 48, 72 and 96 h. At each time point, the cells were collected and the total DNA and RNA were extracted. The HBV pgRNA and precore/core promoter directed RNA were relatively quantified through one step quantitative real-time PCR, and GAPDH was used as an endogenous control to normalize the quantity of total cellular RNA in the template. We found that Ag10Ns at 5 μM could inhibit the production of HBV RNA in a dose-dependent manner (Figure 3), and the percentage inhibition at 10 μM increased from 44.6% at 24 h to 67.9% at 48 h, reaching a peak of 72.5% at 72 h. After this time, inhibition dropped to 46.3% at 96 h.

HBV covalently closed circular DNA (cccDNA) levels were also quantified according to a previously described method [24]. However, no significant difference was observed between the cccDNA levels in treated HepAD38 cells and in the vehicle control at various time points (0 to 96 h; data not shown).

Silver nanoparticles had little effect on the cccDNA levels, but could inhibit the formation of HBV RNA. The reduction of HBV RNA levels suggested that transcription from the cccDNA or integrated HBV genome was impeded, which could result in the inhibition of HBV relaxed circular DNA (rcDNA) formation because pgRNA serves as the template of the reverse transcription. Most of the rcDNA is packaged into core particles and exported from the cells, whereas only a few cores need to be transported back to the nucleus where rcDNA is converted to cccDNA to maintain a stable intranuclear pool [3]. Thus, the inhibition of HBV rcDNA production had a greater influence on the amounts of extracellular virions than the intranuclear cccDNA levels [29,30]. In our study, we hypothesize that the inhibition of HBV RNA production by the silver nanoparticles might in turn reduce the formation of rcDNA, and subsequently decrease the number of extracellular virions. However, because the inhibitory effects of silver nanoparticles are moderate (maximum 72.5% inhibition of HBV RNA), there might be still enough rcDNA to complement the cccDNA pool in the nucleus. This hypothesis would explain why the silver nanoparticles had little effect on the HBV cccDNA levels, but still needs further investigation.

Interaction between HBV DNA and silver nanoparticles

Silver nanoparticles have special properties, such as a larger active surface area and increased porosity, which facilitate their interaction with other small particles and molecules [31]. On this basis, we hypothesized that Ag10Ns might impede the transcription of viral RNA by binding to directly or interacting with HBV DNA, which serves as the template of RNA synthesis. Gel mobility shift assays were employed to assess binding potential. HBV dsDNA was treated with Ag10Ns at different molar ratios (DNA:Ag10Ns 1:50, 1:5, 1:0.5 and 1:0.05) or with a vehicle control and the complexes formed resolved by agarose gel electrophoresis (Figure 4A). All samples treated with Ag10Ns were found to exhibit ‘shifting-up’ effects, even at the 1:0.05 molar ratio, and the 1:50 sample was found to exhibit a significant tailing effect. This effect resulted from the substantial binding of silver nanoparticles to the DNA, as revealed by their altered electrophoretic mobility compared to the control.

**Figure 3.** The inhibition of HBV RNA production by Ag10Ns

![Graph showing inhibition of HBV RNA production by Ag10Ns](image_url)
Figure 4. Interactions between HBV DNA and silver nanoparticles

(A) Gel mobility shift assay of hepatitis B virus (HBV) double-stranded (ds) DNA and silver nanoparticles. M, Marker; (lane 1) HBV dsDNA (vehicle control); (lane 2) dsDNA:Ag10Ns 1:50; (lane 3) dsDNA:Ag10Ns 1:5; (lane 4) dsDNA:Ag10Ns 1:0.5; (lane 5) dsDNA:Ag10Ns 1:0.05; (lane 6) HBV dsDNA (vehicle control).

(B) Electronic spectra of Ag10Ns (50 μM) in TBS buffer with (B) increasing HBV DNA concentrations or (C) increasing calf thymus DNA concentrations at 298K. Inset: plots of [DNA]/Δε_{ap} versus [DNA].
nanoparticles to the HBV DNA, which augmented the size of DNA and increased the viscosity.

Binding constants were determined using a UV-vis absorption titration assay. The addition of aliquots of HBV DNA (0–100 μM) to a solution of Ag10Ns induced an isosbestic-spectral change with hyperchromicity of ~15% at its λ_{max} at 409 nm. The binding constant (K_b) of Ag10Ns towards the HBV DNA was found to be (8.8 ± 1.0) × 10^5 dm^3mol^{-1}, which was determined from the plot of [HBV DNA]/{Δε}_{ap} versus [HBV DNA] (Figure 4B). Besides HBV DNA, we also found that Ag10Ns could bind to calf thymus DNA (ctDNA, Figure 4C) and the total RNA extracted from HepAD38 cells (Figure S4), with binding constants of (1.5 ± 0.3) × 10^6 and (3.1 ± 0.4) × 10^5 dm^3mol^{-1}, respectively. Given the similar binding affinity to the ctDNA and RNA, we reason that the binding of Ag10Ns to the HBV DNA is non-specific and thus could contribute to the cytotoxic activity of silver nanoparticles at high concentrations.

Interaction between HBV viral particles and silver nanoparticles

Ag10Ns silver nanoparticles showed good binding affinity to HBV virions, as only 54% and 12% unbound viral particles (versus silver-nanoparticle-free control) were detected after incubation for 10 and 60 min, respectively (Figure 5A). In addition, the distribution of Ag10Ns in HepAD38 cells was investigated using TEM. Ag10Ns was distributed mostly in the cell cytoplasm and the TEM image showed that several Ag10Ns bound to the HBV-like particles (Figure 5B). However, because the HepAD38 cell line is an HBV-integrated cellular model, whether this binding activity of silver nanoparticles can prevent HBV virions from entering into the host cells requires further investigation.

In summary, we report that silver nanoparticles possess high binding affinity for HBV dsDNA and extracellular virions, and could inhibit in vitro production of HBV RNA and extracellular virions.

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Disclosure statement

The authors declare no conflict of interest.

Additional file

An additional file 'Supplementary material' containing Figures S1–S4 can be accessed via the Volume 13 Issue 2 contents page for Antiviral Therapy, which can be found at www.intmedpress.com (by clicking on ‘Antiviral Therapy’ then ‘Journal PDFs’).

References

Abstract

The interaction of nanoparticles with biomolecules and microorganisms is an expanding field of research. Within this field, an area that has been largely unexplored is the interaction of metal nanoparticles with viruses. In this work, we demonstrate that silver nanoparticles undergo a size-dependent interaction with HIV-1, with nanoparticles exclusively in the range of 1–10 nm attached to the virus. The regular spatial arrangement of the attached nanoparticles, the center-to-center distance between nanoparticles, and the fact that the exposed sulfur-bearing residues of the glycoprotein knobs would be attractive sites for nanoparticle interaction suggest that silver nanoparticles interact with the HIV-1 virus via preferential binding to the gp120 glycoprotein knobs. Due to this interaction, silver nanoparticles inhibit the virus from binding to host cells, as demonstrated in vitro.

Background

Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for nanomaterials[1]. In the biological sciences, many applications for metal nanoparticles are being explored, including biosensors[2], labels for cells and biomolecules[3], and cancer therapeutics[4].

It has been demonstrated that, in the case of noble-metal nanocrystals, the electromagnetic, optical, and catalytic properties are highly influenced by shape and size [5-7]. This has driven the development of synthesis routes that allow a better control of morphology and size [8-13]. Noble-metal nanomaterials have been synthesized using a variety of methods, including hard-template[14], bioreduction[9] and solution phase syntheses[8,10-13].

Among noble-metal nanomaterials, silver nanoparticles have received considerable attention due to their attractive physicochemical properties. The surface plasmon resonance and large effective scattering cross section of individual silver nanoparticles make them ideal candidates for molecular labeling[15], where phenomena such as surface enhance Raman scattering (SERS) can be exploited. In addition, the strong toxicity that silver exhibits in various chemical forms to a wide range of microorganisms is very well known [16-18], and silver nanoparticles have recently been shown to be a promising antimicrobial material[19].
For these reasons, and based upon our previous work regarding interactions of noble metal nanoparticles with biomolecules[20], we decided to study the interaction of silver nanoparticles with viruses. Herein, we present the first findings of our investigation, the discovery that silver nanoparticles undergo size-dependent interaction with HIV-1.

Findings

Characterization of the tested silver nanoparticle preparations

The physicochemical properties of nanoparticles are strongly dependent upon their interactions with capping agent molecules[21]. Indeed, the surface chemistry of the nanoparticles can modify their interactions with external systems. For this reason we tested silver nanoparticles with three markedly different surface chemistries: foamy carbon, poly (N-vinyl-2-pyrrolidone) (PVP), and bovine serum albumin (BSA).

PVP-coated nanoparticles were synthesized by the polypol method using glycerine as both reducing agent and solvent. In this method, a metal precursor is dissolved in a liquid polypol in the presence of a capping agent such as PVP[22]. PVP is a linear polymer and stabilizes the nanoparticle surface via bonding with the pyrrolidone ring. Infrared (IR) and X-ray photoelectron spectroscopy (XPS) studies have revealed that both oxygen and nitrogen atoms of the pyrrolidone ring can promote the adsorption of PVP chains onto the surface of silver[23]. The sample size distribution was obtained from high angle annular dark field (HAADF) images. The nanoparticles exhibited an average size of 6.53 nm with a standard deviation of 2.41 nm. (Figure 2d–e)

Silver nanoparticles directly conjugated to BSA protein molecules were synthesized in aqueous solution. Serum albumin is a globular protein, and is the most-abundant protein in blood plasma. Bovine serum albumin (BSA) is

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Figure 1

Transmission electron microscopy (TEM) of the foamy carbon-coated silver nanoparticles. a) TEM image of the sample prepared by dispersing the as-received powder in deionized water by ultra-sonication. The agglomeration of particles inside the foamy carbon matrix is observed. b) TEM image of nanoparticles outside of the carbon matrix. The broad distribution of shapes can be observed. c)-f) TEM images of nanoparticles with different morphologies. c) Icosahedral. d) Decahedral. e) Elongated. f) Octahedral. g) High Resolution TEM image of the foamy carbon matrix.
a single polypeptide chain composed of 583 amino acid residues [24]. Several residues of BSA have sulfur-, oxygen-, and nitrogen-bearing groups that can stabilize the nanoparticle surface. The strongest interactions with silver likely involve the 35 thiol-bearing cysteine residues. By using sodium borohydride, a strong reducing agent, BSA stabilizes nanoparticles via direct bonding with these thiol-bearing cysteine residues, and provides steric protec-

**Figure 2**

**Silver nanoparticle preparations.** a) TEM image of free surface silver nanoparticles released from the foamy carbon matrix by dispersing the as-received powder in deionized water by ultra-sonication. b) Size distribution of free surface nanoparticles measured by TEM analysis. c) UV-Visible spectrum of carbon-coated silver nanoparticles. d) HAADF image of PVP-coated silver nanoparticles. e) Size distribution of PVP-coated nanoparticles measured by TEM analysis. f) UV-Visible spectrum of PVP-coated silver nanoparticles. g) HAADF image of BSA-coated silver nanoparticles. h) Size distribution of BSA-coated nanoparticles measured by TEM analysis. i) UV-Visible spectrum of BSA-coated silver nanoparticles.
tion due to the bulkiness of the protein. The sample size distribution was obtained from HAADF images. Nearly 75% of the BSA-conjugated silver nanoparticles were 2.08 ± 0.42 nm in diameter, but a substantial fraction of larger particles was also observed, bringing the total average to 3.12 ± 2.00 nm (Figure 2g–h).

UV-visible spectroscopy is a valuable tool for structural characterization of silver nanoparticles. It is well known that the optical absorption spectra of metal nanoparticles are dominated by surface plasmon resonances (SPR), which shift to longer wavelengths with increasing particle size [25]. Also, it is well recognized that the absorbance of silver nanoparticles depends mainly upon size and shape [26,27]. In general, the number of SPR peaks decrease as the symmetry of the nanoparticle increases [27]. Recently, Schultz and coworkers [28] correlated the absorption spectra of individual silver nanoparticles with their size (40–120 nm) and shape (spheres, decahedrons, triangular truncated pyramids and platelets) determined by TEM. They found that spherical and roughly spherical nanoparticles, decahedral or pentagonal nanoparticles, and triangular truncated pyramids and platelets absorb in the blue, green and red part of the spectrum, respectively. In all the cases the SPR peak wavelength increases with size, as expected.

The UV-Visible spectra for all the nanoparticle preparations are shown in Figure 2. All samples presented a minimum at ~320 nm that corresponds to the wavelength at which the real and imaginary parts of the dielectric function of silver almost vanish [27]. The sample with carbon-coated silver nanoparticles exhibits four peaks at ~400, ~490, ~560 and ~680 nm, as shown in Figure 2c. The optical signature of this sample can be better understood in terms of the distribution of sizes and shapes observed in the TEM. As we previously mentioned, the distribution of shapes in the sample is broad, and a significant amount of nanoparticles are not spherical such as multi-twinned with five-fold symmetries. The presence of nanoparticles with pentagonal and triangular cross-sections could be responsible for the absorption at longer wavelengths. Thus, it is clear that the characteristic absorption of these nanoparticles arises from the contribution of different shapes and sizes, which agrees with the TEM observations.

On the other hand, the PVP-coated and BSA-coated silver nanoparticles present only one peak at ~450 and ~390 nm, respectively. These results indicate that both preparations are mainly composed by small spherical silver nanoparticles. It is also well know that for small particles a broadening of the plasmon absorption bands is expected, since there is a linear dependence of the full-width at half maximum (FWHM) with the reciprocal of the particle diameter [29]. The results for BSA-coated nanoparticles agree with the last statement, presenting just one broad symmetric peak at ~390 nm. On the other hand, the spectrum for the PVP-coated silver nanoparticles is not symmetric around the maximum absorption wavelength. In fact, this spectrum can be deconvoluted in two different curves, one centered at ~430 and another one at ~520 nm. The peak at ~430 nm could be assigned to the out-of-plane dipole resonance of the silver nanoparticles indicating the presence of spherical particles with small diameters. In addition, the synthesis of silver nanoparticles by the polyl method in presence of PVP promotes also the formation of multi-twinned nanoparticles (MTPs), being decahedra nanoparticles the most thermodynamically stable MTPs [23]. Therefore, the observed read shift is a consequence of both nanoparticles of larger size and the presence of decahedral nanoparticles with pentagonal cross sections.

**Interaction with HIV-1**

High angle annular dark field (HAADF) scanning transmission electron microscopy was employed to study the interaction of silver nanoparticles with HIV-1. In our previous works, HAADF has proven to be a powerful technique for analysis of biological samples, such as proteins [20] and bacteria [30], interfaced with inorganic nanoparticles. HAADF images are primarily formed by electrons that have undergone Rutherford backscattering. As a result, image contrast is related to differences in atomic number [31] with intensity varying as ~Z^2. Therefore, image contrast is strongly related to composition. As a good approximation, lighter elements appear dark and heavier elements appear bright. Due to a large difference in atomic number, silver nanoparticles are easily distinguished from the organic matter that composes the virus.

In Figure 3, we present HAADF images of the HIV-1 virus with (3a) and without (3b) silver nanoparticles. For complete experimental details, refer to Methods Section. The presence of silver was independently confirmed by Energy Dispersive X-ray Spectroscopy (EDS), shown in Figure 3c. Interestingly, the sizes of nanoparticles bound to the virus (Figure 3d) were exclusively within the range of 1–10 nm. In the case of the silver nanoparticles released from the carbon matrix, the fact that no nanoparticles greater than 10 nm in diameter were observed to interact with the virus is significant, since the size of ~40% of the overall population is beyond this range. This provides strong evidence for the size-dependence of interaction.

Additionally, the nanoparticles seen in Figure 3a are not randomly attached to the virus, as regular spatial relationships are observed among groups of three particles. Both the spatial arrangement of nanoparticles and the size dependence of interaction can be explained in terms of
HAADF images of the HIV-1 virus. a) HAADF image of an HIV-1 virus exposed to BSA-conjugated silver nanoparticles. Inset shows the regular spatial arrangement between groups of three nanoparticles. b) HAADF image of HIV-1 viruses without silver nanoparticle treatment. Inset highlight the regular spatial arrangement observed on the surface of the untreated HIV-1 virus. c) EDS analysis of image a) confirming the presence of Ag. The C signal comes from both the TEM grid and the virus, O, and P are from the virus, and Na, Cl, and K are present in the culture medium. Ni and Si come from the TEM grid, while Cu is attributed to the sample holder. d) Composite size distribution of silver nanoparticles bound to the HIV-1 virus, derived from all tested preparations.
the HIV-1 viral envelope, and can provide insight into the mode of interaction between the virus and nanoparticles.

The exterior of the HIV-1 virus is comprised of a lipid membrane interspersed with protruding glycoprotein knobs, formed by trimers consisting of two subunits: the gp120 surface glycoprotein subunit is exposed to the exterior, and the gp41 transmembrane glycoprotein subunit spans the viral membrane and connects the exterior gp120 glycoprotein with the interior p17 matrix protein[32]. The main function of these protruding gp120 glycoprotein knobs is to bind with CD4 receptor sites on host cells. Numerous cellular proteins are also embedded within the viral envelope[33]. However, the protruding gp120 glycoprotein knobs are more exposed to the exterior, and should be more accessible for potential nanoparticle interactions.

Leonard and coworkers[34] reported that the gp120 subunit has nine disulfide bonds, three of which are located in the vicinity of the CD4 binding domain. These exposed disulfide bonds would be the most attractive sites for nanoparticles to interact with the virus. As mentioned previously, the nanoparticles in Figure 1a appear to be located at specific positions, with regular spatial relationships observed among groups of three particles. The observed spatial arrangements correlate with the positions of the gp120 glycoprotein knobs in the structural model for HIV-1 proposed by Nermut and coworkers[32].

Regular spatial relationships are also found on the surface of the untreated virus, as seen in the inset of Figure 1b. The observed darker contrast at these sites could indicate the locations of the glycoprotein knobs. As mentioned previously, contrast in HAADF images is strongly dependent on differences in atomic number. However, this is not the only factor in determining the image contrast. If the material is composed of elements of similar atomic numbers, as is the case for the organic constituents of the pure virus, local variations in sample density will provide noticeable contrast. The majority of the viral envelope consists of a densely-packed lipid membrane. However, for the glycoprotein knobs, we would expect a localized region of lower density due to the presence of membrane-spanning gp41 glycoproteins rather than the densely-packed lipids. Hence, these areas should appear darker than the rest of the viral envelope.

It has previously been determined that the center-to-center spacing between glycoprotein knobs is ~22 nm[35]. In the inset of Figure 3a, the average measured center-to-center spacing between silver nanoparticles is ~28 nm, which correlates with the expected spacing between glycoprotein knobs. The average center-to-center spacing between the small darker regions on the untreated virus is ~22 nm, which again suggests these sites are the gp120 glycoprotein knobs. Thus, the observed spatial arrangement of nanoparticles, the center-to-center distance between nanoparticles, and the fact that the exposed sulfur-bearing residues of the glycoprotein knobs would be attractive sites for nanoparticle interaction strongly suggest that silver nanoparticles interact with the HIV-1 virus via preferential binding to the gp120 glycoprotein knobs.

Presuming that the most attractive sites for interaction are the sulfur-bearing residues of the gp120 glycoprotein knobs, there are only a limited number of bonds that a nanoparticle can form. This limited number of stabilizing sites can explain why larger nanoparticles are not observed to attach to the virus. Assuming that each nanoparticle interacts with a single glycoprotein knob, and that each nearest-neighbor knob is occupied by another nanoparticle, from geometric considerations the theoretical upper limit for the diameter for these nanoparticles would be ~20 nm. However, if a nanoparticle larger than the diameter of one knob (~14 nm[35]) were to be attached, only a small fraction of the total nanoparticle surface would be anchored, resulting in a less stable interaction. Thus, if the nanoparticles are interacting with HIV-1 via preferential binding at gp120 glycoprotein knobs, we would expect to find mostly nanoparticles less than 14 nm in diameter, as particles in this size range would have the most stable surface interactions. This corresponds closely with our experimental observation that particles greater than 10 nm were not attached to the viral envelope.

Although the mechanism by which HIV infects host cells is not yet fully understood, there are two steps that are broadly agreed to be critical. The first step involves binding of gp120 to the CD4 receptor site on the host cell. Then, upon binding to CD4, a conformational change is induced in gp120, resulting in exposure of new binding sites for a chemokine receptor, i.e. CCR5 or CXCR4 [36-38]. An agent that preferentially interacts with the gp120 glycoprotein would block the virus from binding with host cells. Therefore, we measured the inhibitory effects of silver nanoparticles against HIV-1 in vitro.

The capacity of silver nanoparticles to inhibit HIV-1 infectivity was determined by testing against CD4+ MT-2 cells and cMAGI HIV-1 reporter cells. For complete experimental details, refer to Methods Section. The cytopathic effects of CD4+ MT-2 infection were analysed by optical microscopy assessment of syncytium formation as described elsewhere[39,40], as well as by the HIV-1 infection of cMAGI cells using the Blue Cell Assay[41,42]. The cytotoxicity of all the nanoparticle preparations against MT-2 cells was determined using the Trypan Blue exclusion
assay [43]. For all three nanoparticle preparations, at silver concentrations above 25 µg/mL, viral infectivity was reduced to an extent that it could not be detected by syncytium formation, as shown graphically in Figure 4. For each nanoparticle preparation, we found a dose-dependent inhibition of HIV-1 infectivity, with an IC50 where only moderate cell toxicity was observed, as seen in Figure 4.

Although the findings regarding interaction with HIV-1 were congruent among nanoparticles with markedly different surface chemistry, the toxicity and inhibition results were not the same. The differences in the observed trends in HIV-1 inhibition can be explained in terms of the capping agents employed for each nanoparticle preparation. BSA- and PVP-protected nanoparticles exhibit slightly lower inhibition because the nanoparticle surface is directly bound to and encapsulated by the capping agent. In contrast, the silver nanoparticles released from the carbon matrix have a greater inhibitory effect due to their essentially free surface area. The fact that the carbon-coated nanoparticles present higher cytotoxicity can also be explained in terms of surface chemistry. Since a significant amount of these silver nanoparticles possess nearly

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**Figure 4**

**Inhibition of HIV-1 and toxicity data.** a) Assessment of HIV-1 mediated syncytium formation in MT-2 cells. b) Percentage of HIV-1 transmission in cMAGI cells. The toxicity of the nanoparticle preparations against MT-2 cells was determined using the Trypan Blue exclusion assay. The samples were incubated at 37°C, and the cells were evaluated via optical microscopy after c) 3 h and d) 24 h of exposure to silver nanoparticles.
free surfaces, they are able to interact stronger with the host cells, thus increasing their toxicity. Clearly, selection of capping agents will be crucial for future research on the interaction of nanoparticles with viruses, microorganisms, and more complex biosystems, and many more variables require further testing, including the long-term effects of the presence of nanoparticles, and the impact of traces of precursor molecules and reaction by-products.

In conclusion, we have found that silver nanoparticles undergo size-dependent interaction with HIV-1, and that the bound particles exhibit regular spatial relationships. These observations lead us to suggest that the nanoparticles undergo preferential binding with the gp120 subunit of the viral envelope glycoprotein. Silver nanoparticles inhibit the HIV-1 virus infectivity in vitro, which also supports our proposal regarding preferential interaction with gp120. These findings only provide indirect evidence for our proposed mode of interaction, and we are currently undertaking testing to determine conclusively if direct conjugation between gp120 and silver nanoparticles exist.

The interactions of inorganic nanoparticles with biosystems are just beginning to be understood, and potential applications are being discovered at an increasing rate. However, in order to realize the future promise of nanoscience, it is imperative that the toxicity and long-term health effects of exposure to nanomaterials be fully explored. The flexibility of nanoparticle preparation methods, the multitude of functionalization techniques, and facile incorporation of nanoparticles into a variety of media provide the incentive for further research on the interaction of metal nanoparticles with viruses.

**Methods**

**a) HIV-1 strains and cell lines**

HIV-1<sub>IIIB</sub> laboratory strain of HIV-1 an X4 wild type (wt) virus was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CD4<sup>+</sup> MT-2 cell line was obtained from the American Type Culture Collection. The cMAGI HIV-1 reporter cells were a generous gift from Dr. Phalguni Gupta from the University of Pittsburgh. All other reagents used were of the highest quality available.

cMAGI cells were cultured in DMEM Dulbecco’s Modified Eagle Medium (DMEM) (1X) liquid without sodium phosphate and sodium pyruvate. The medium contained 4,500 mg/L D-glucose and L-glutamine (Invitrogen, Paisley, UK), with 10% fetal calf serum (FCS), 0.2 mg/mL gentamicin (G418), and 0.1 µg/mL puromycin. MT-2 cells were cultured in RPMI 1640, containing 10% fetal calf serum (FCS) and antibiotics.

HIV-1<sub>IIIB</sub> primary clinical isolates were propagated by subculture in MT-2 and cMAGI cells. HIV-1<sub>IIIB</sub> was reproduced according to the DAIDS Virology Manual for HIV Laboratories, version 1997, compiled by Division of AIDS of the National Institute of Allergies and Infectious Diseases and the National Institute of Health, and Collaborators. Aliquots of cell-free culture viral supernatants were used as viral inocula.

All the work related to HIV-1 cells, except for HIV-1<sub>IIIB</sub>, was done in a Biosafety Level 3 (BSL-3) Laboratory.

**b) Synthesis of the three different silver nanoparticle preparations**

Carbon coated silver nanoparticles tested in this study were obtained from Nanotechnologies, Inc. and used without further treatment. For more information about the synthesis of these nanoparticles, please visit [http://www.nanoscale.com](http://www.nanoscale.com)

PVP-coated silver nanoparticles were synthesized by the polyp method using glycerine as both reducing agent and solvent. Silver sulfate (Ag<sub>2</sub>SO<sub>4</sub>, reagent grade) and poly(N-vinyl-2-pyrrolidone) (PVP-K30, MW = 40,000) were purchased from Sigma Aldrich and 1,2,3-Propanetriol (Glycerin, >99%) was purchased from Fischer Chemicals, all the materials were used without any further treatment. Briefly, we added 0.2 g of PVP to a round bottom flask following by the addition of 30 mL of glycerin. Once PVP was dissolved, we increased the temperature to 140°C. After 30 minutes we added 2 mL of 0.015 M Ag<sub>2</sub>SO<sub>4</sub> and left to react for 1 h.

Silver nanoparticles directly conjugated to bovine serum albumin (BSA) protein molecules were produced as following described. Silver nitrate (AgNO<sub>3</sub>, 0.945 M), sodium borohydride (NaBH<sub>4</sub>, 99%) and 200 proof spectrophotometric-grade ethanol were purchased from Aldrich. Bovine serum albumin (BSA) was purchased from Fisher and was used without further treatment. Briefly, sodium borohydride was added to an aqueous solution of silver nitrate and BSA under vigorous stirring. The molar ratio of Ag<sup>+</sup>:BSA was 28:1, and the molar ratio of Ag<sup>+</sup>:BH<sub>4</sub> was 1:1. The reaction volume was 40 mL, and contained 13.50 µmol BSA. The reaction was allowed to proceed for 1 h, and the product was purified by precipitation at -5°C, followed by cold ethanol filtration.

**c) Characterization of the different silver nanoparticle preparations**

Transmission electron microscope was conducted in a HRTEM JEOL 2010F microscope equipped with Schottky-type field emission gun, ultra-high resolution pole piece (Cs = 0.5 mm), and a scanning transmission electron microscope (STEM) unit with high angle annular dark
field (HAADF) detector operating at 200 kV. Briefly, a droplet of each different solution of silver nanoparticles was placed on a Cu grid with lacey carbon film (Ted Pella), and allowed to evaporate. Size distributions for each nanoparticle preparation were obtained from TEM analysis based on the measurement of 400 particles, and 600 particles in the case of BSA-coated nanoparticles.

UV-visible spectra were obtained at room temperature using a 10 mm path length quartz cuvette in a Cary 5000 spectrometer. All the solutions were diluted 30 × in deionized water before acquiring the spectra.

d) Electron microscopy of HIV-1 and silver nanoparticles
Samples were prepared for electron microscopy as follows: 10⁵ TCID₅₀ samples of HIV-1₂HIV cell free virus were treated with solutions of different silver nanoparticles at a concentration of 100 µg/mL. After 30 seconds, a 10 µL droplet was deposit on a carbon coated nickel TEM grid and exposed to a 2.5% solution of PBS/glutaraldehyde vapors for 30 minutes. Microscopy was done using a JEOL 2010-F TEM equipped with an Oxford EDS unit, at an accelerating voltage of 200 kV and operated in scanning mode using an HAADF detector.

e) Inhibition of HIV-1 with silver nanoparticles
RPMI medium only or containing varying concentrations of silver nanoparticles were mixed with samples 10⁵ TCID₅₀ of HIV-1₂HIV cell free virus. The highest concentration of silver nanoparticles used was 100 µg/mL. After 30 seconds, sequential 2-fold dilutions of the solutions were added to cultures of target cells (2 × 10⁵ MT-2 and 2 × 10⁵ cMAGI HIV-1 reporter cells with 0.2–0.5 multiplicity of infection (m.o.i) of HIV-1₂HIV virus) prepared as previously mentioned. Each dilution was exposed to four replicate wells. After that, the cells were incubated in a 5% CO₂, humidified incubator at 37 °C for 3–5 days. Assessment of HIV-1 mediated syncytium formation was performed for the MT-2 cells, while for cMAGI cells, the percentage of transmission was estimated as follows: the number of blue-stained cells obtained from the supernatant of each of the tested wells was divided by the number of blue-stained cells obtained from the culture supernatant in the well of the positive control.

f) Cytotoxicity of silver nanoparticles against MT-2 cells
The cytotoxicity of the nanoparticle preparations against MT-2 cells was determined using the Trypan Blue exclusion assay. In all cases, the initial concentration of silver nanoparticles was 50 µg/mL and sequential two-fold dilutions were made and mixed with 2 × 10⁵ MT-2 cells. The samples were incubated at 37°C, and the cells were evaluated via optical microscopy after 3 h and 24 h of exposure to silver nanoparticles. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% Trypan Blue and the cells were counted using a haemocytometer. Viability was expressed as the percentage of number of unstained treated cells to that of the total number of cells.

Additional material

Additional File 1
Supporting information. The file is a word document that contains the complete size distribution of the carbon-coated silver nanoparticles evaluated by TEM
Click here for file [http://www.biomedcentral.com/content supplemental/1477-3155-3-6-S1.doc]

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