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Effective Multistrain Inhibition of Influenza Virus by Anionic Gold Nanoparticles

Matias Sametband[a], Sourabh Shukla[a], Tal Meningher[b,c], Shira Hirsh[b,c], Ella Mendelson[c], Ronit Sarid[b], Aharon Gedanken[*a], Michal Mandelboim[c]

[a] Department of Chemistry, Kanabar Laboratory for Nanomaterials, Institute of Nanotechnology and Advanced Materials, Bar-Ilan University, 52900.

[b] The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel.

[c] Central Virology Laboratory, Public Health Service, Ministry of health, Sheba Medical Center, Tel Hashmer, Israel, 52621.

* Fax: +972 3 7384053; Tel: +972 3 5318315; E-mail: gedanken@mail.biu.ac.il

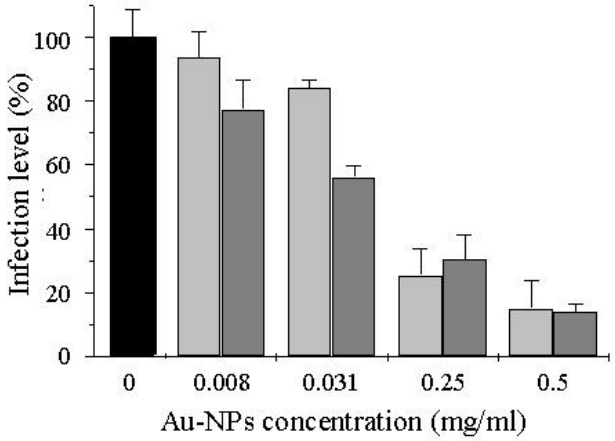

Fig. S1 Effect of Au-NPs-MES virus pre-incubation (gray) vs. no pre-incubation (light gray) on infection inhibition. The results indicated that more efficient inhibition was achieved when the Au-NPs-MES and the virus were incubated for an hour prior to their introduction to the MDCK cell lines. This difference was noticed for low Au-NPs-MES concentrations (0.031 and 0.008 mg/ml). No affect was noticed at high Au-NPs-MES concentrations (Mann-Whitney Test was applied for statistical analysis, using SPSS data editor software).

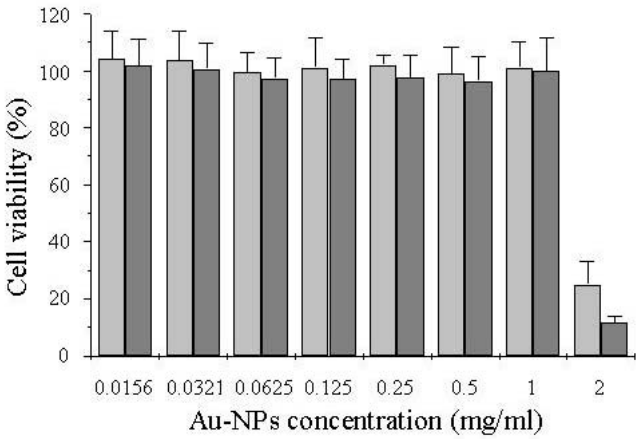

Fig. S2 Au-NPs crystal violet toxicity tests indicated that cell viability was not affected by the Au-NPs-MES (gray) and Au-NPs-MSA (light gray). Only at high Au-NPs concentration (2 mg/ml) the particles were toxic towards the MDCK cells. This concentration is well above working concentrations. Test was repeated twice, triplicates in each test.

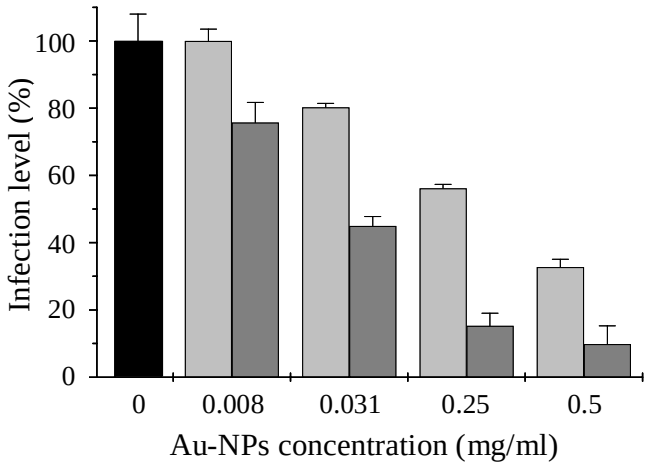

Fig. S3 Swine A/Israel/119/2009 (H1N1) inhibition by Au-NPs-MES (gray) and Au-NPs-MSA (light gray). The Au-NPs-MES were more effective, similar to the influenza A H5N1 tests.

1. Synthesis of gold nanoparticles:

All the chemicals were purchased from Sigma-Aldrich Co., unless stated otherwise.

Gold nanoparticles stabilized with mercatoethanesulfonate (Au-NPs-MES) were synthesizes according to a method developed in our group. The particles were synthesized in a simple one phase procedure: 0.059 g $HAuCl_4$ was dissolved in 1.5 ml double distilled water (DDW), and further diluted to a final volume of 150 ml (0.15 mmol). 0.0738 g MES was dissolved in 50 ml DDW (0.38 mmol) and added to the gold solution, followed by the addition of 0.01 g $NaBH_4$ in 10 ml DDW (0.26 mmol). The solution was mixed vigorously for another hour, resulting in a clear pale-brown solution. Additional 0.04 g $NaBH_4$ in 5 ml DDW (1.05 mmol) was added to complete the gold-ions reduction, and stirred over night at r.t. The solution turned dark brown, indicating on the creation of the Au-NPs-MES (small absorbance peak at λ_{max} ~ 520 nm, Cary 100 Scan UV spectrophotometer). The solution was further dialyzed against DDW and lyophilized, resulting in a dark-brown powder. The Au-NPs-MES powder was readily redispersed in DDW. High Resolution Transmission Electron Microscope (200 KV JEOL, JEM 2100) measurements indicated that the average particle size was 4 ± 1 nm.

The Au-NPs-MES charge was acquired using ζ-potential measurements (Malvern Zetasizer 3000 HAS), indicating that the particle's charge had a magnitude of ζ = -35 mV.

Au-NPs stabilized with mPEG thiol (n = 6, Polypure AS) where synthesized in a similar manner.

Au-NPs stabilized with mercaptosuccinic acid (Au-NPs-MSA) were synthesized as follows:[1] 0.197 g $HAuCl_4$ was dissolved in 100 ml methanol (0.5 mmol). To this solution, 0.150 g MSA (1.0 mmol) was added and stirred for 0.5 hours in an ice bath. 0.190 g $NaBH_4$ dissolved in cold 25 ml DDW (5 mmol) were introduced to the methalonic solution and stirred for another hour. The particles precipitated, and the precipitate was separated by centrifugation and filtration. The solid was washed repeatedly with methanol and dried, resulting in a dark-brown powder. The particles were redisperse in DDW, dialyzed against DDW and lyophilized resulting in a dark-brown powder. The average particle size was of 4 ± 1 nm, with a small absorbance peak at λ_{max} ~ 525 nm. The Au-NPs-MSA particles had a higher charge compared to the Au-NPs-MES of -50 mV. IR measurements indicated that carboxylic groups were present on the Au-NPs-MSA surface (Nicolet 400D Impact FTIR).

Inductively coupled plasma (ICP) [Spectroflame Module E, Spectro, Kleve, Germany] measurements were performed by dissolving the Au-NPs in 1 ml aqua regia, followed by the addition of 9 ml DDW.

2. Determination of Au-NPs antiviral activity:

Enzyme linked immunosorbent assay (ELISA) was applied for the study of influenza inhibition activity.

The ELISA procedure was performed as follows:

To a 96-well plate, 100 µl Madin-Darby canine kidney (MDCK, WHO) cells in 2% Dulbecco's modified Eagle's medium (DMEM) were added per well ($10x10^4$ cells/well), and incubated at 37ºC, 5% CO_2 for 3 hr. In a separate plate, 25 µl virus-suspension was incubated for an hour at r.t. in the presence of 25 µl of different Au-NPs concentrations [diluted with 2% DMEM or phosphate buffered saline (PBS)]. The Au-NPs

concentrations varied from 0.5 to 0.004 mg/ml. The virus-NPs solutions were introduces to the cells, and incubated for 72 hr at 37ºC, 5% CO_2. After incubation, the plate was washed three times with PBS-Tween (PBS-T, Tween 20, 0.05% (v/v), Bio-Rad Laboratories Ltd), and 100 µl of primary anti-influenza A/B antibody (WHO), diluted by 1:4000 with blocking buffer, was added to each well and the plates were incubated for 1 hr at room temperature. After three additional washings with PBS-T, 100 µl of peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 80351) diluted by 1:2000 with blocking buffer, was added to each well, and the plates were incubated for 1 hr at room temperature. 100 µl of o-phenylenediamine dihydrochloride (OPD) were added to each well after washing five times with PBS-T. The plates were read with a multichannel ELISA reader (Titertek, Multiskan MCC/340 MK II; Helsinki, Finland) at 492 nm.

The MDCK cells were maintained in DMEM supplemented with heat-inactivated 10% fetal calf serum, 37ºc, 5% CO_2. Twice a week the media was removed, the cells were pealed from the growing flask using an EDTA trypsin solution, and the cells were brought to the desired dilution in 10% DMEM.

The monoclonal antibodies (mAbs) used in this work were: Mouse Monoclonal Antibody Influenza Type A (pooled), Lot 58685896 (Cat. No. FR-51), Mouse Monoclonal Antibody Influenza Type B (pooled), Lot 58685897 (Cat. No. FR-52).

3. Determination of viral titer:

Several virus strains were use in the ELISA experiments: (a) A recombination of a vaccine H5N1 virus. A/Puerto Rico/8/1934 (H1N1), in which the H1 and N1 proteins were exchanged with H5 and N1 proteins from a violent avian virus A/Vietnam/1194/2004 (H5N1), (b) Swine influenza A/Israel/119/2009 (H1N1), (b) A/Brisbane/10/2007 (H3N2), (c) A/Puerto Rico/8/1934 (H1N1), (d) B/Brisbane/60/2008 and (e) B/Shandong/7/1997.

The viruses were grown in the allantoic cavity of 11-day-old embryonated hen eggs (SHEBA, Israel). Virus growth and purification were performed according to standard methods as described by Barret and Inglis.[2]

Hemagglutination titration and tissue culture infectious dose ($TCID_{50}$) were applied for quantifying the virus and know the titer:

1) Hemagglutination titration: Turkey red blood cells were suspended in Alsever solution and the concentration was brought to 0.5% in PBS. Assay was performed in microtiter plates with 50 µl of sample containing the influenza virus and 50 µl of 0.5% Turkey red blood cells. Wells were inspected for agglutination, and the results were evaluated as hemagglutination units (HAU).

2) $TCID_{50}$: serial dilutions of the virus were added to a cell culture in a 96 well plate (100 µl/well, $5x10^4$ cells/well). The cells infected with different viral concentrations were incubated for 5-6 days at 37ºC, 5% CO_2. The crystal-violet staining method (procedure described below) was applied for the detection of cytopathogenic effect (CPE). Virus titer was expressed as the tissue culture infective doses leading to 50% infected cells ($TCID_{50}$/ml), calculated using the Reed-Münch method.

4. Cell toxicity assay

Cell viability after the introduction of Au-NPs was tested using two toxicity assays:

1) *Crystal-violet staining:* MDCK cells were grown in a 96 wells plate as described previously. Au-NPs were diluted by adding 100 µl particles in 2% DMEM to the cell's well. Further dilutions were done in serial dilutions of the particles. After 3 days incubation at 37ºc, 5% CO_2, 200 µl saline solution was added proceed by the removal of all the solvents from the wells. 50 µl of crystal-violet solution was added and incubated for an hour at r.t. The plate was washed with water, and 100 µl lysis buffer was added to the wells. The absorbance was measured using an ELISA reader at λ = 540 nm.

2) *MTT test:* 100 µl MDCK cells in 2% DMEM ($5x10^4$ cells/well) were added to a 96 well plate, and incubated for 3 hours at 37ºC, 5% CO_2. 50 µl Au-NPs at different concentrations were added and further incubated for 5 days. 5 µl of MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (4 mg/ml in PBS) were added to the wells, and incubated for another 3 hours. The solvent was removed from the wells, and the cells were suspended in 150 µl isopropanol (Merk). The optical density values were quantitated using an ELISA reader at 595 nm.

References

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(2) Barrett, T.; Inglis S.-C. *Growth Purification and Titration Influenza Viruses in Virology: A Practical Approach*; Mahy W.-J. (Ed.), IRL Press: Washington D.C., U.S.A, **1985**; pp. 119-151.