Journal of Virus Eradication 9 (2023) 100330

Contents lists available at ScienceDirect

Journal of Virus Eradication

journal homepage: www.sciencedirect.com/journal/journal-of-virus-eradication

Original research

Antiviral activity of silver nanoparticles against the influenza A virus

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ARTICLE INFO

Keywords:
Influenza A virus
Silver nanoparticles
Antiviral activity

ABSTRACT

Viral infections occupy an essential place in modern medicine, particularly a large group of diseases caused by the influenza viruses. They are rapidly transmitted and mutate quickly, which can lead to significant socio-economic consequences. Silver nanoparticles (AgNPs) are considered to be an effective antimicrobial agent. This study shows that they have strong antiviral properties against the influenza A virus infection. Their absence of cytotoxicity at inhibitory concentrations demonstrates that they could be an effective antiviral agent against this virus. As AgNPs inhibit the influenza A virus replication and spread, they could also be successfully used as a post-infection virostatic agent.

1. Introduction

Viruses are one of the most numerous taxonomic groups in the world. They all caused various infections in humans, animals, and even bacteria.[1] The rapid appearance of new treatment-resistant strains is a serious global health problem.[2] The Influenza A viruses (IAV) are one of the causes of infection of the human airways, seasonal epidemics and severe health threats worldwide and have significant socio-economic impact. According to the latest World Health Organization (WHO) reports, seasonal influenza epidemics lead to about 3–5 million disease cases and about 500,000 deaths annually.

Based on the characteristics of viral proteins (viral nucleoprotein and major matrix protein), influenza viruses are classified as types A, B, C, and D. Only influenza A and B viruses are primary culprits in human diseases. The influenza virus is very variable, with new strains appearing each season due to the basic mechanisms inherent to the family of orthomyxoviruses. According to the literature, influenza viruses have two ways to ignite a pandemic. First, by direct transmission (called «shift») from animals to humans (one of the most significant pandemics in the world in 1918 with the «Spanish influenza») and second, via reassortment or «drift» of an avian virus with a human influenza virus (that is how the «Asian influenza» came about).[3]

Due to the characteristics of respiratory viruses, such as high genetic variability, rapid emergence of new strains, and drug resistance, the fight against the influenza viruses is a serious challenge. Thus, safe and new effective antiviral drugs are urgently needed, especially now that the global pandemic of COVID-19 has changed people's view of "flu-like" symptoms. COVID-19 is not caused by a flu virus but by a coronavirus, SARS-CoV-2. COVID-19 and influenza may have similar symptoms but present risks for complications in different at-risk groups and also require additional tests, treatment, and prevention measures.[4]

Over the last few decades, nanomaterials have attracted much attention in many fields, such as biomedicine, catalysis, energy storage, and sensors, due to their unique physico-chemical and biological properties. Silver nanoparticles (AgNPs) have been among the most engaging materials in medicine due to their low price, high efficiency, and unique optical and electronic properties, leading to potential industrial applications.[5] The most studied aspect of silver metal is its inhibitory activity against bacteria, fungi, and viruses. Silver nanoparticles are well-known antimicrobial materials that have proven their effectiveness against many types of bacteria[6–8] and fungi.[9] According to the literature, the mechanism of action of silver depends on Ag positive ions, which strongly inhibit the growth of bacteria, inhibiting respiratory enzymes and components of electronic transport and interfering with DNA function.[10]

Recently, the antiviral activity of AgNPs against viruses such as HIV-1,[11,12] hepatitis B,[13] herpes simplex,[14] respiratory syncytial,[15] and monkeypox[16] has also been studied. Their primary antiviral mechanism is the physical inhibition of binding between the virus and the host cell. The dependence of antiviral activity on the size of AgNPs was observed for all the above viruses. For example, AgNPs less than 10 nm specifically inhibit HIV-1 infection.[11] They exhibit anti-inflammatory,

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https://doi.org/10.1016/j.jve.2023.100330
Received 26 April 2022; Received in revised form 30 March 2023; Accepted 1 June 2023
Available online 10 June 2023

antiplatelet, and antiangiogenic activity[17–19] and generally have a broad biological activity spectrum. Thus, AgNPs could potentially be used as antiviral drug with a wide range of action, minimum toxicity, and be capable of altering their mode of action during their interaction with various viruses.

The current study investigates the effects of AgNPs at different stages of the H1N1 influenza virus replicative process, one of the most widespread viruses that endanger human health.

2. Material and methods

2.1. Characteristics of AgNPs

The stock stable experimental aqueous dispersion containing 20 mg mL silver nanoparticles coated with natural resins (AgNPs) was obtained from Noble Elements LLC, USA. The nanoparticles were well dispersed and highly uniform in size (10 ± 1.5 nm). Diluted nanoparticle samples were prepared directly in a cell growth medium and used immediately after preparation.

2.2. Viruses and cells

The MDCK (Madin-Darby canine kidney cell line) and influenza A virus (IAV) H1N1, strain A/FM/1/47 were obtained from the Gromashevskogo Institute of Epidemiology and Infection Diseases of NAS of Ukraine. Cells were grown in a medium that consisred of 46% DMEM (Biowest, France), 46% RPMI 1640 (Biowest, France), 8% bovine serum inactivated by heating (Biowest, France), and antibiotic gentamicin (100 μg/ml) in the atmosphere 5% CO_2 at 37 °C. Cultivation of the cells was performed according to standard procedures.[20] The IAV with titer $1*10^7$ $TCID_{50}$/ml was used in the study.

2.3. Determination of AgNP cytotoxicity

Cytotoxicity of AgNPs to MDCK cells was determined in terms of their concentration causing 50% of cells to die (cytotoxicity concentration, CC_{50}) by using the MTT assay (BioFroxx, Germany) and the neutral red cell cytotoxicity assay[20] (BioFroxx, Germany). The MTT test was performed in a standard manner with minor changes.[21] A concentration of NPs ranging from 2000 to 0.4 μg/ml was used for the study. All assays were performed in triplicate.

The 50% cytotoxic concentration (CC_{50}) was calculated from the concentration-effect curves. The percentage of cytotoxicity was calculated as [(A - B)/A] x 100, where A and B are the OD 540 nm of untreated and treated cells, respectively.

2.4. Assessment of antiviral activity

2.4.1. Virucidal activity

To evaluate the direct effects of AgNPs on H1N1 influenza particles, equal volumes of the viral suspensions and nanoparticle suspensions in the non-toxic concentration of 100 μg/ml were mixed. They were incubated at 37 °C for 5, 15, and 30 min in a humidified 5% CO_2 atmosphere. After incubation, samples were prepared in 10-fold dilutions and added in triplicate wells of the confluent monolayer of MDCK cells. After three days, crystal violet staining was performed standardly.[22] Viral titers were calculated and determined as 50% of the infective dose in tissue culture ($TCID_{50}$/ml).[23] Virucidal activity was then determined as described by Kohn LK et al.[24]

2.4.2. Antiviral acti according to the preventive scheme (pre-exposure)

The confluent monolayer of MDCK cells was pre-incubated with different concentrations of AgNPs in non-toxic concentration ranges in triplicates for 24 h at 37 °C with a humidified 5% CO_2 atmosphere. The concentration of AgNPs 0,032; 0,16; 0,8; 4; 20; 40; 80, and 100 μg/ml were studied. Virus (infected but untreated cells) and cell controls (uninfected untreated cells) were kept in each plate prepared throughout the experiment. After three days, cells were stained by crystal violet,[22] and the percentage of inhibition of the cytopathic effect (CPE) of the influenza virus was calculated by the following formula:

$$\%\ \text{inhibition of CPE} = (\text{OD exp.}) - (\text{OD vc}) \,/\, (\text{OD cc}) - (\text{OD vc}) \times 100\%$$

where optical density (OD) exp. - average OD of treated infected cells, OD vc - average OD of untreated infected cells, and OD cc - average OD of treated uninfected cells. The obtained EC_{50} value was defined as the effective concentration that reduced the uptake of infected cells by up to 50% compared to control cells and viruses.

2.4.3. Cell co-treatment assay

The co-treatment assay was performed to evaluate AgNP inhibition of viral binding. The six ten-fold dilutions from 20 to 0,0002 μg/ml and 100 μL of 0,01 $TCID_{50}$/ml viral suspensions were used as described by Ghaffari H. et al.[25]

2.4.4. Antiviral action according to the treatment scheme (post-exposure)

The MDCK cells were incubated with 50 μL of H1N1 virus suspensions for 1 h at 37 °C in a humidified 5% CO_2 incubator. Different non-cytotoxic concentrations (six ten-fold dilutions from 20 to 0,0002 μg/ml) of Ag NPs suspended in the infection medium were then added in triplicate to the wells.[25] After three days, cells were stained by crystal violet,[22] and the percentage of inhibition was calculated as described at 2.4.2.

2.4.5. Determination of the infective titer of influenza synthesized de novo (the amount of total progeny virus)

The MDCK cells were treated according to the preventive and treatment schemes described above. After incubation for three days, the supernatant was collected, and the infectious titer of influenza virus de novo was determined. The MDCK cells were infected with 50 μL of ten-fold serial dilutions of the virus-containing suspension (treated or not treated with different concentrations of the NPs). After three days, the crystal violet staining was performed standardly.[22] Finally, the viral titers were calculated as 50% of the infective dose in tissue culture ($TCID_{50}$/mL).[24]

2.5. Statistical analysis

Statistical data was processed using standard approaches to calculate statistical errors (standard deviation) using Microsoft Excel 2010. Results were expressed as the mean ± S.D. for three independent experiments.

3. Results

The cytotoxic effect of AgNPs on the MDCK cell line was determined by using MTT. The concentrations in the 0.4–2000 μg/ml range were studied. The results obtained in the MTT assay showed that significant cytotoxicity occurred at the highest studied concentration (2000 μg/ml). As shown in Fig. 1, cell viability percentage was less than 2%. Cell viability increased with decreasing NP concentration. Using the linear regression model in Microsoft Excel, it was estimated that the CC_{50} index of AgNPs was 80 μg/mL.

We analyzed the effect of the studied preparation on the activity of lysosomes using neutral red in the concentration range from 80 to 2000 μg/ml, which inhibited cellular mitochondrial activity by 50% or more (Fig. 2). Results show that the maximum concentration lead to cell death in both cases, while the level of viable cells at lower concentrations was, on average 30% higher. Based on the correlation between the neutral red indices and enzymatic activity of lysosomes, this may indicate the absence of negative activity of the studied precursor on the functioning of lysosomes.

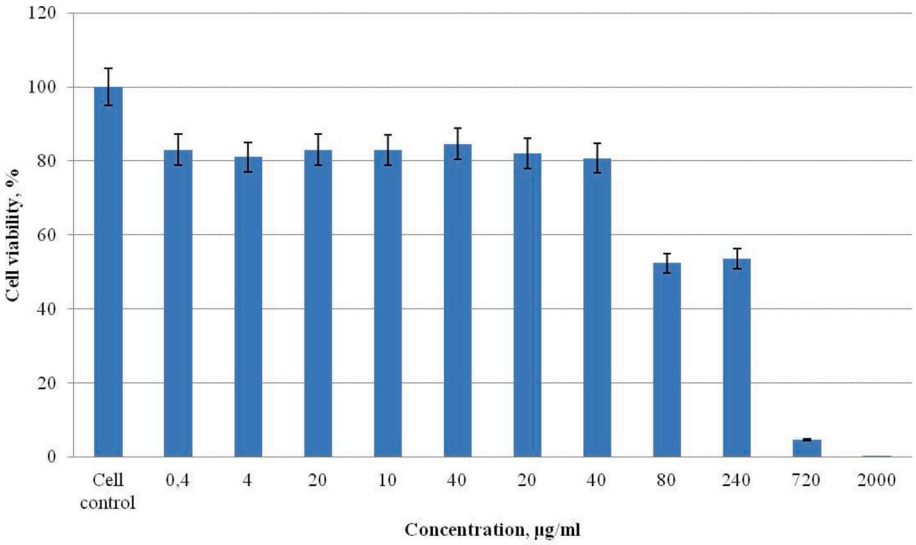

Fig. 1. Cytotoxic effect of silver nanoparticles on MDCK cell line (MTT-test).

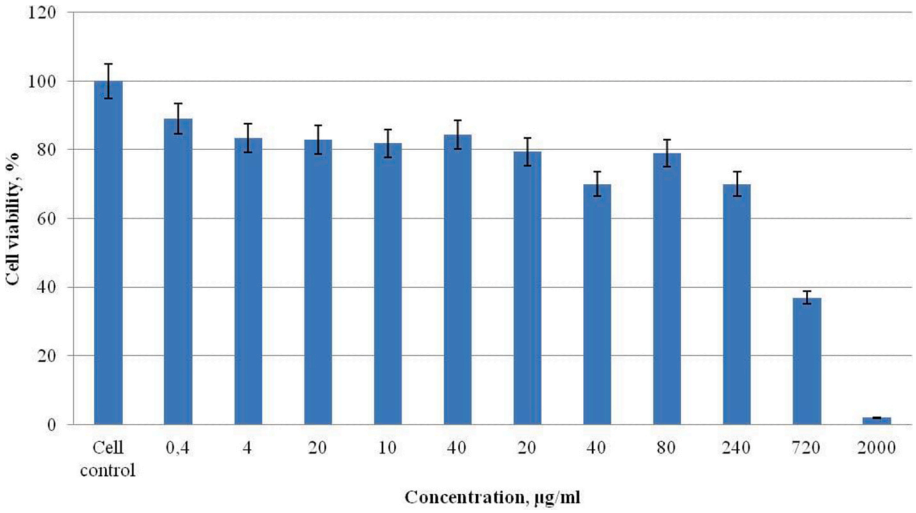

Fig. 2. Cytotoxic effect of silver nanoparticles on MDCK cell line (neutral red test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

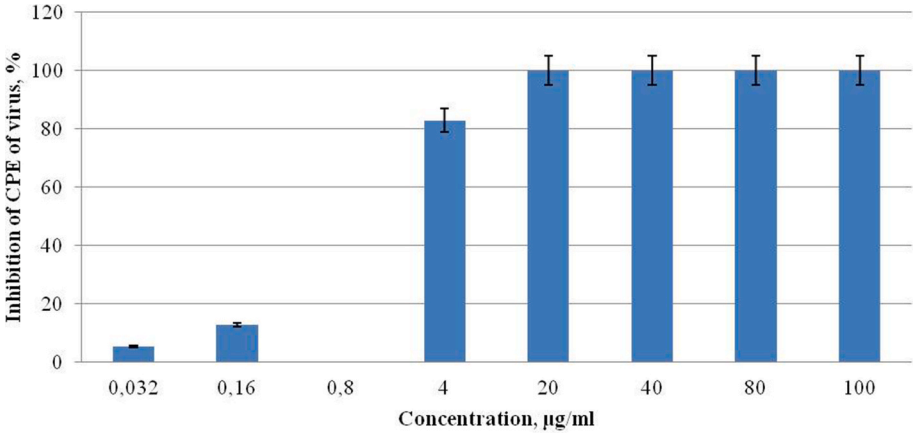

Fig. 3. Pre-exposure treatment of Ag NPs.

The AgNPs show a significant antiviral effect on the pre- and post-exposure treatment of MDCK cells. It was established that nanoparticles' cytotoxic effect on the cellular mitochondrial system was more powerful than on the lysosomal one. For further studies, we chose a wide range of concentrations from 0.0002 μg/ml to cytotoxic 80–100 μg/ml. Thus, we have investigated the antiviral effect of AgNPs at high concentrations and in minimal doses since there is an assumption that metal nanoparticles can work effectively in small amounts. The antiviral effect was detected at higher concentrations from 4 to 100 μg/ml (Fig. 3) on pre-exposure treatment. The percentage of inhibition of viral cytopathic effect (CPE) was in the range of 82–100%. Lower concentrations did not lead to a reduction of the CPE of the influenza virus. Results indicate that AgNPs showed a high protective effect.

Results obtained when studying the antiviral activity of NPs added after viral infection of cells (post-exposure treatment) showed a decrease in the development of the viral cytopathic a of the influenza virus. As shown in Fig. 4, almost all studied concentrations inhibited the CPE from 34 to 54%. The minimal concentrations (0,0002 and 0,002 μg/ml) did not reduce the development of the CPE. It should be noted that the concentration-activity curve initially seemed to be dome-shaped. However, a significant inflection occurred at a concentration of 20–40 mcg/mg, at which point the solution showed an increase from 35% to 100% of virus inhibition. Analysis of the results showed that the concentration range from 0.2 to 2 μg/l was optimal because, with increasing concentrations, activity decreased. At low concentrations, the antiviral effect was not detected.

Using the linear regression model in Microsoft Excel, the EC_{50} index of AgNPs was estimated with a different timing of therapy (Table 1). The selective index (SI) was defined as CC_{50}/EC_{50}. As shown in Table 1, the SI of AgNPs during post-exposure was significantly higher than that of the pre-exposure antiviral activity assay. AgNPs showed significant antiviral activity at the studied pre-exposure and post-exposure times, and the SI was 88 and 667, respectively. Tamiflu (oseltamivir), manufactured by Hoffman-La Roche Ltd. (Switzerland), was used as a reference drug. It was shown that the comparative drug was less effective than the nanoparticles in these treatment experiments.

We have studied the influenza virusinfection titer to determine the total viral progeny virus in the preventive scheme. Our results showed that NP inhibition of the influenza virus in the concentration range from 0.16 to 100 μg/ml was complete after the preventive scheme. The absence of cytopathic activity in the titration of viral progeny (cells in the test samples were similar to intact ones) does not make it possible to determine the titer of the newly synthesized virus and indicates its complete loss of infectivity. The effect of maximum concentrations, 4–100 μg/ml, confirms the results of antiviral activity in the preventive scheme of nanoparticles. It should be noted that the study of antiviral activity at concentrations of 0,16 and 0,8 μg/ml showed no significant inhibition of the cytopathic effect of the virus. However, in a further study of the infectious progeny, complete inhibition of the cytopathic activity of the inflluenza virus was shown. This is important because even though lower doses of Ag NPs (0,16 and 0,8 μg/ml) were not directly virucidal, they can be described as virostatic, meaning that viral replication was blocked, and infectivity neutralized. It can be assumed that AgNPs affect the late stages of viral replication and interact with newly-formed viral offspring. We have detected a violation of the monolayer of cells, however, the development of cytopathic effect wasnot observed during further titration of samples.

Table 1
Antiviral activity of silver nanoparticles.

Treatment scheme	Effective concentration (EC_{50}), μg/ml	Selective index (SI)
Pre-exposure (Preventive scheme)	4,5	88
Post-exposure (Treatment scheme)	0,6	667
Oseltamivir (Treatment scheme)	15	41

We have studied the infection titer after post-exposure treatment to show the influenza virus cytopathic effect at all studied concentrations (Fig. 5). The development of AgNP concentrations in the range from 0,02–20 μg/ml confirmed results of the antiviral activity of the nanoparticles at post-exposure treatment. Inhibition of influenza CPE ranged from 1,63 to 3,63 lg.

It should be noted that AgNPs at concentrations of 0,0002 and 0,002 μg/ml inhibit the cytopathic activity of the virus at 2,83 and 2,08 log. The antiviral activity of AgNPs showed a higher level of inhibition at a lower dose which might be due to their small size easily interacting with the virus and the distance between nanoparticles, and the suggestion that nanoparticles interact with the virus via preferential binding to the surface protein of the influenza A virus. Thanks to this interaction, nanoparticles inhibit the virus from binding to host cells, as demonstrated *in vitro*.[11] These results also suggest that these nanoparticles affect the late stages of virus replication, leading to the disruption of the cell monolayer and a loss of infectivity by the newly formed viruses. According to the treatment scheme, several viral replicative stages occur during that experiment. It can therefore be assumed that nanoparticles affect not only the late stages of replication but also newly synthesized viruses. However, all interaction mechanisms between the virus and nanoparticles require more detailed studies. The result of the antiviral assay showed that the co-exposure of cells to AgNPs did lead to a reduction of the influenza virus titer above a concentration of 0.03 μg/ml; however, higher concentrations had apparent virucidal activity and that the amount of total viral progeny after post-exposure was

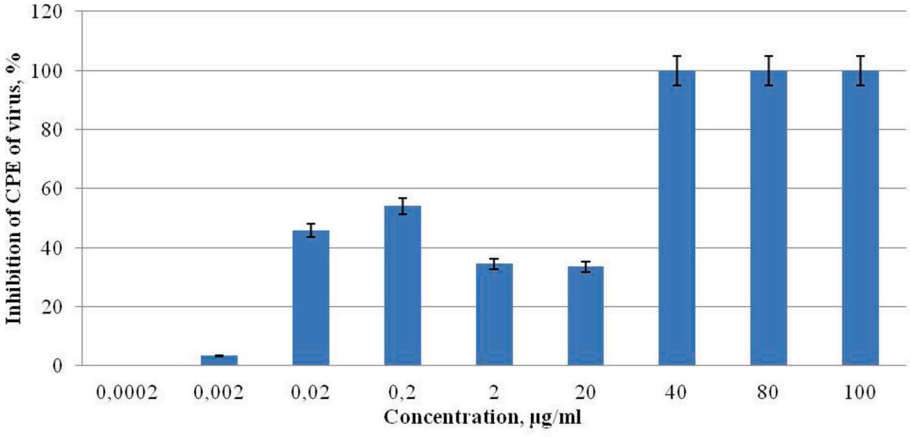

Fig. 4. Post-exposure treatment with silver nanoparticles.

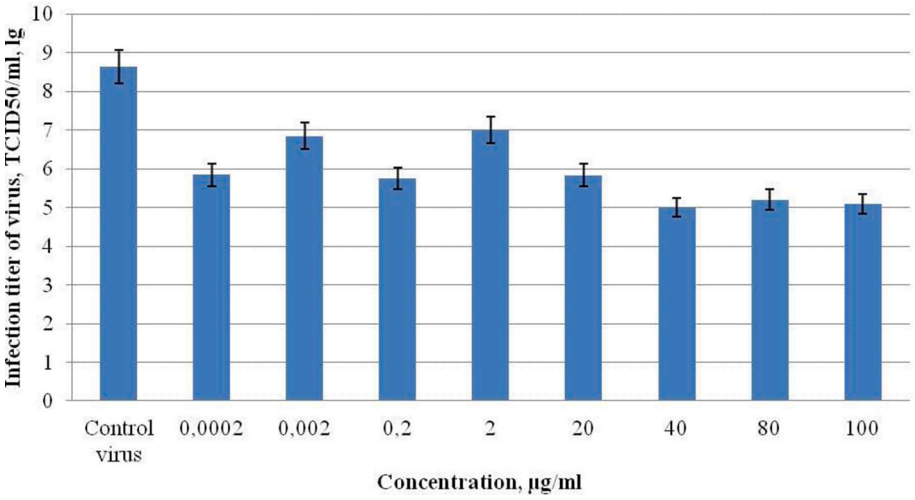

Fig. 5. Determination of the amount of total progeny virus after post-exposure treatment.

initially not inhibited; however, it seems that virucidal actiivity subsequently inactivated the progeny.

4. Discussion

Nanoparticles have attracted much attention due to their unique properties, such as physical and chemical ones, due to their large number of surface atoms and high area/volume ratio.[2] In recent years, nanosizes of different metals (zinc, titanium, gold, and silver) have been designed. Most have been effective against diverse microorganisms.[7] In particular, the antibacterial activity of AgNPs against *S. aureus*, *E. coli*, *P. aeruginosa*, V. cholera, and B. subtilis has been documented.[2,7,8,26]

The antiviral activity of AgNPs has also been documented by researchers worldwide, and several mechanisms of action have been identified. Researchers have demonstrated that small AgNPs coated with PVP can block the penetration of HIV by interacting with the surface glycoprotein of the virus[10] and that they mimic heparin sulfate and may compete for the binding of the glycoprotein of HSV-1.[27,28] Another group has shown the antiviral activity of PVP-coated AgNPs against the respiratory syncytial virus by interference with viral attachment.[15] Rogers et al. have documented that they may block virus-host cell binding and penetration of the monkeypox virus.[16] Speshock et al. have described the inactivation of the tacaribe virus (TCRV) particles before entry. They have recently analyzed the activity of two types of AgNPs against TCRV: uncoated (Ag-NP) and polysaccharide-coated silver nanoparticles (*PS*–Ag). Researchers have suggested that this type of NPs might bind to the viral membrane glycoproteins.[29] It demonstrates the antiviral activity of AgNPs directly at the virus particle or at adsorption levels, which includes viral binding, attachment, and entry into cells. Because of the above, it can be assumed that the main action of AgNPs is associated with the effect on the extracellular virus, lipid membrane, and block of surface proteins.

Our results show that AgNPs have a pronounced virucidal effect, which is consistent with the work of the researchers cited above. Despite their small size, AgNPs inhibited the viral cytopathic effect. Therefore, on can assume that they interact with the influenza virus' surface proteins (hemagglutinin and neuraminidase). Since a decrease in virus titer was detected, nanoparticles disrupted the viral lipid envelope. It should be noted that coated AgNPs have antiviral activity at pre and post-exposure treatment. They are 10±1.5 nm in diameter, so we can assume that they can penetrate cells with pre-exposure treatment. These nanoparticles may inhibit the cellular replication, assembly, and release of virions. Under conditions of post-exposure treatment, they also penetrate cells and inhibit viral replication. These results correlate with publications from other researchers, such as Lu et al., which have shown the ability of AgNPs to inhibit hepatitis B virus (HBV), probably via a specific interaction with its double-stranded DNA.[13] Many other researchers have suggested that the size and charge of nanoparticles influence the interaction of viral particles and AgNPs. Their small size allows for a large surface area for cellular contact, and therefore a higher efficiency than larger particles.[30,31] Results of this study confirm this hypothesis. The NP size and their type of coating have a significant role in the interaction with viruses/cells. It should also be noted that AgNPs have antiviral activity due to their viral DNA and RNA blocking action.[2,32]

These results demonstrate that the preparation of AgNPs has a significant impact on their antifungal activity and that, when stabilized with polyvinylpyrrolidone (PVP), they could have potential practical applications due to the presence of bactericidal, sporicidal, and fungicidal activity.[33] Literature shows that AgNPs can inhibit different viral infections and affect prokaryotic and eukaryotic cells.[34,35] Their mechanisms of action on bacteria and viruses differ. Their antiviral activity is primarily due to competitive binding to cellular receptors and the destruction of the viral envelope. It is known that the development of a viral infection depends on the initial stages of its attachment to the surface of the host cells by binding the surface components with ligands and proteins on the cell membrane.[36,37]

Of particular interest are preparations of nanoparticles that show their effectiveness not only outside the cell, i.e., not a direct virucidal effect but activity on viral replication. Studies show AgNPs' inhibitory action inside cells at different stages of viral replication, indicating their promise as an effective anti-flu agent candidate.

5. Conclusion

Due to their unique properties, AgNPss have great potential in the fight against pathogenic microbial activity. This study shows that they have a high antiviral activity by completely suppressing the infectious titer of the influenza A virus. As indicated by the selectivity index, the absence of cytotoxicity at inhibitory concentrations demonstrated that AgNPs could be an effective antiviral agent against nfluenza A virus. Their antiviral properties can also be successfully used post-infection as a powerful viro-static agent because, as shown in this study, they inhibit further replication and spread of this virus. According to present findings, experimental AgNPs coated with natural resins are a promising agent against the influenza A virus infection, one of the most common viruses affecting the human population.

Funding

The research was funded by Noble Elements/NOBEL LLC, the developer of the experimental silver nanoparticles coated with natural resins.

Author contributions

N.K., Z.S., CP, and NR wrote the paper. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

N.K. and Z.S. authors declare no conflict of interest. Dr. Calin Pop and Nodari Rizun are the Noble Elements/NOBEL LLC shareholders. Nodari Rizun is the executive director of Noble Elements/NOBEL LLC.

Data availability

The authors do not have permission to share data.

Acknowledgments

The authors thank Dr. Dan V. Goia (Emeritus Professor at Clarkson University, Potsdam, NY, USA) for providing scientific advice and practical recommendations for the properties, characterization, and handling of silver nanoparticles.

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