

Antiviral Colloidal Silver Composition

5 **Cross-reference to Prior Applications**

[0001] The present application is based on and claims priority from United States Patent Application Serial No. 11/538,262, filed 03 October 2006. The present application is a continuation-in-part of application 10/641,938, filed August 15, 2003, now U.S. Patent No. 7,135,195 which in turn is a continuation-in-part of application 09/946,834, filed September 4, 2001, now U.S. Patent No. 6,743,348 and a non-provisional of and claiming priority from
10 provisional application 60/475,657, filed June 3, 2003, which application is incorporated by reference herein; application 09/946,834, filed September 4, 2001, now U.S. Patent No. 6,743,348 is itself a continuation of application 09/323,310, filed June 1, 1999, now U.S. Patent No. 6,214,299.

U.S. Government Support

[0002] Not Applicable

15 **Background of the Invention**

Area of the Art

[0003] The present invention generally relates to colloidal silver, and more particularly to a composition of colloidal silver and a method for using said composition as an agent against organisms harmful to the health of humans—in particular avian influenza virus ("bird 'flu").

Description of the Background Art

20 [0004] It is well known that certain preparations of silver have germicidal properties. Silver was employed as a germicide and an antibiotic before modern antibiotics were developed. In previous centuries, users would shave silver particles into their drinking water, or submerge whole silver pieces in the drinking water, for the purpose of ingesting the silver by drinking the water. It seems likely that the practice of eating with silver utensils (i.e., silverware) resulted from a belief in the healthful properties of silver.

25 [0005] There may be many reasons why administering silver suspended in solution would enhance an individual's health. It is possible that such a solution operates to inhibit the growth of bacteria, viruses, and other unwanted organisms, as well as eradicating such existing bacteria, viruses, and other organisms. It is also possible that a solution of silver can have an anti-inflammatory effect, sufficient to reduce symptoms of asthma.

[0006] The present invention describes the use of a silver composition in water to treat certain human ailments. An embodiment of the invention is a silver composition comprising small particles of silver which comprise an interior of metallic silver and an exterior of ionic silver which particles are suspended in water. A preferred embodiment of the invention is a silver composition comprising particles of silver wherein more than 50% of the particles are less than 0.015 micrometers in size and the particles are colloidally suspended in water.

Summary

[0007] The present disclosure is generally directed to the use of silver, at a level of 5 to 40 ppm in water, to kill or to disable microorganisms, such as avian influenza virus, which are hazardous to human beings. The present disclosure specifically is directed to compositions comprising silver particles, said particles comprising an interior of elemental silver and an exterior of ionic silver oxide, and water, wherein the silver particles are placed in colloidal suspension in the water at a level of 5-40 ppm total silver. An embodiment of the present disclosure comprises silver particles in water, at a concentration of 5-40 ppm, wherein more than 50% of the silver particles have a maximum dimension less than 0.015 micrometers. The composition of silver in water of this disclosure is an effective antimicrobial agent. This disclosure is directed to silver compositions, of 5-40 ppm silver in water, which are effective antimicrobial agents, and to methods of using said silver compositions as antimicrobial agents.

[0008] A preferred embodiment of the present disclosure is directed to compositions of silver in water made using a modification of the device and methods described in U.S. Patent No. 6,214,299, which is a parent of the instant application and is incorporated herein by reference to the extent permissible.

[0009] The device and process of Patent No. 6,214,299 have been modified and improved to provide the silver composition of the present disclosure. Essentially, the eight-silver/one common electrode device as disclosed in the patent has been modified and scaled to fit a 75-gallon water chamber. To start the process approximately 70 gallons of high purity water are placed in the chamber. To this is added approximately five gallons of silver composition produced in a prior production run. This is necessary because the high purity water is insufficiently conductive for the process to occur properly. The water chamber is equipped with an air input that allows a stream of air bubbles to be streamed through the liquid during the processing. It has been discovered that this approach gives improved mixing as compared to the impeller mixer described in the patent.

[0010] The electrode device is operated at approximately ten thousand volts alternating current (with each silver electrode having an individual voltage supply) as described in the

patent. It has been found that voltages significantly lower than this produce a composition with larger particles not having the optimal properties described herein. Voltages significantly higher
5 tend to produce a solution with significant ionic silver dissolved therein. The present composition comprises in excess of 97% metallic silver with essentially no free ionic silver in solution.

[0011] The silver concentration is determined according to the methods explained below. Essentially, the device is operated continuously and samples are analyzed until the desired silver concentration is attained. The 10 ppm composition requires approximately one and one
10 half days of operation. The 22 ppm solution requires approximately three days, and the 32 ppm composition requires approximately six days. The rate of the process appears to slow as the higher concentrations are attained. Higher concentrations take a prohibitively long time with the ultimate highest concentration being about 50 ppm, at least within the current parameters.

[0012] The compositions all have the size characteristics described below and unlike conventional silver compositions are completely colorless and stable to light and temperature changes without use of any additives. The compositions are unreactive towards added
15 hydrogen peroxide.

[0012a] In a first aspect, there is provided a method of lowering infectivity of influenza A virus in vitro comprising the steps of: providing a composition of silver in water comprising a total concentration of silver of between about 5 and 40 parts per million, said silver in the form of colloidal silver particles having an interior of elemental silver and a surface of silver oxide, wherein a majority of the colloidal silver particles
20 have a minimum diameter greater than 0.005 micrometers and a maximum diameter less than 0.015 micrometers, said composition being free of cytotoxic properties; and contacting Influenza A virus with the composition.

[0012b] In a second aspect, there is provided the use of a composition of silver in water comprising a total concentration of silver of between about 5 and 40 parts per million, said silver in the form of colloidal silver particles having an interior of elemental silver and a surface of silver oxide, wherein a majority of the colloidal silver particles
25 have a minimum diameter greater than 0.005 micrometers and a maximum diameter less than 0.015 micrometers and wherein said composition is free of cytotoxic properties in the manufacture of a medicament for ameliorating infection by influenza A virus.

Detailed Description of the Invention

[0013] The following description is provided to enable any person skilled in the art to make and use the invention and sets forth the best modes contemplated by the inventor of carrying out his invention. Various modifications, however, will remain readily apparent to those skilled in the art, since the general principles of the present invention have been defined herein specifically to provide an improved colloidal silver product with significant abilities to kill or inhibit human pathogens both in vivo and in vitro.

[0014] Generally, the present invention represents a novel approach to killing or disabling microorganisms which are hazardous to human beings by the use of silver particles in water, at a concentration of 5 to 40 ppm silver. Depending upon the application, the silver composition may be used internally or externally.

[0015] PREFERRED EMBODIMENTS

[0016] Non-limiting preferred embodiments are presented in the following:

[0017] A composition comprising silver particles, colloidally suspended in water, wherein the total content of silver is between 5 and 40 ppm, which composition kills or disables virus which are hazardous to the human body.

[0018] A composition comprising silver particles, colloiddally suspended in water, wherein the total content of silver is about 10 ± 2 ppm, which composition kills or disables virus which are hazardous to the human body.

[0019] A composition comprising silver particles, colloiddally suspended in water, wherein the total content of silver is about 22 ± 2 ppm, which composition kills or disables virus which are hazardous to the human body.

[0020] A composition comprising silver particles, colloiddally suspended in water, wherein the total content of silver is about 32 ± 3 ppm, which composition kills or disables virus which are hazardous to the human body.

[0021] It will be appreciated that specifying the total amount of silver in a composition of particles does not completely specify the material. As the particles comprising the composition are made smaller, a given concentration of silver will represent a larger number of particles. In addition, the total surface area for a given silver concentration will increase. Therefore, particles size and range of particle size is an important parameter for defining an effective inventive composition.

[0022] A further class of embodiments is any of the above-described compositions, wherein more than 50% of the silver particles have a maximum dimension less than 0.015 micrometers.

[0023] A further class of embodiments is any of the above-described compositions, wherein the silver particles comprise both silver in the zero-valent, that is, metallic, oxidation state [Ag(0)] and a coating of silver in an ionic oxidation selected from the group consisting of Ag(I), Ag(II), and Ag(III).

[0024] A further class of embodiments is any of the above-described compositions, wherein the silver particles comprise both silver in the zero-valent, that is, metallic, oxidation state [Ag(0)] and a coating of silver in an ionic oxidation selected from the group consisting of Ag(I), Ag(II), and Ag(III).

[0025] Experimental evidence shows that AgO in the particles of the present invention is at least partially in the form of Ag_4O_4 —that is, silver II oxide. In a molecule of this material two of the silver atoms are in the 1+ state (silver I) while the other two silver molecules are in the 3+ state (silver III). Under certain conditions these molecules can give rise to silver atoms in the 2+ (silver II) state.

[0026] EXAMPLES

[0027] 1. FORMATION OF COMPOSITION

[0028] Compositions of silver in water can be made according to procedures set forth in U.S. Patent No. 6,214,299, incorporated by reference herewith.

5 [0029] A preferred method for producing a composition comprising silver according to this invention utilizes an electrochemical cell comprising electrodes and comprises the steps

[0030] (a) placing a silver electrode in contact with a quantity of high purity water;

[0031] (b) conveying electrical current through the silver electrode to thereby separate particles of silver from said silver electrode in a manner sufficient to cause production of suspended silver particles within the water; and

10 [0032] (c) agitating the water during said production of suspended silver particles to thereby disperse the silver particles into a more uniform concentration within said water such that a higher quantity of suspended silver particles can be produced per batch.

[0033] Another preferred method for producing a composition comprising silver utilizes an electrochemical cell and comprises the steps of:

15 [0034] (a) establishing an electrical circuit comprising a current source, and a first conductor electrically connected to said current source and a second conductor electrically connected to said current source, wherein said first conductor is disposed spaced apart from said second conductor, and wherein at least one of the conductors is made of elemental silver;

[0035] (b) closing the circuit by placing the first conductor and the second conductor in communication with a fluidic resistor;

20 [0036] (c) operating the current source to supply alternating current simultaneously to the first conductor and the second conductor such that voltage is increasing and decreasing within the first and second conductors in alternating tandem to thereby cause silver particles to separate from the first electrode and enter the fluidic resistor and become disposed in suspension within said fluidic resistor; and

25 [0037] (d) selectively adjusting the electrodes by moving them toward the fluidic resistor to compensate for decrease in electrode length due to gradual separation of silver particles therefrom to thereby prevent arcing from occurring between the electrodes and said fluidic resistor.

[0038] The analysis of the silver content in the silver compositions of this invention may be done by atomic absorption (AA), inductively coupled plasma/atomic emission (ICP/AES), or other techniques known to one of ordinary skill in the art to be sensitive to silver in the appropriate concentration range. If the particles of the silver composition are small and uniformly sized (for example, 0.01 micrometers or less), a reasonably accurate assay may be

obtained by running the colloid directly by AA or ICP/AES. This is because the sample preparation for AA ionizes essentially all of the silver allowing its ready detection.

5 [0039] If the compositions comprise particles as large as 0.2 micrometers, it is preferred to use a digestion procedure. The digestion procedure is not necessarily ideal for silver compositions that may have been manufactured or stored in contact with halides or other anionic species that may react with finely divided silver, or combined with protein or other gelatinous material. An embodiment of the digestion procedure is as follows:

10 [0040] 1 Take a 10 ml aliquot of a thoroughly mixed or shaken silver composition to be analyzed, and place it in a clean polycarbonate bottle or other container of suitable material (generally, the bottle) with a tight fitting lid. A size of 30–100 ml is preferred.

[0041] 2 With a micropipette or dropper, add 0.1 ml of nitric acid, reagent grade to the silver composition in the bottle.

[0042] 3 With the lid of the bottle tightly in place, heat the silver composition to 80°C with mild agitation for a time sufficient to dissolve the silver—dissolution is essentially instantaneous.

15 [0043] 4 Allow the resulting mixture to cool to room temperature with the lid in place. Shake the bottle thoroughly.

[0044] 5 Utilize AA, ICP/AES, or equivalent means to analyze the silver content of the silver mixture. Preferably, one will utilize a freshly prepared standard or standards, preferably prepared according the equipment manufacturer's instructions, with appropriate dilution as needed.

20 [0045] 6 When reporting results, one must taken into account all dilutions during preparation, including the 1% dilution caused by addition of the nitric acid.

[0046] 1. ANALYSIS OF PHYSICAL/CHEMICAL FORM OF SILVER

[0047] A. Introduction

25 [0048] A sample of a composition, nominally containing 22 ppm silver in water, was analyzed by time-of-flight secondary ion mass spectrometry (TOF-SIMS) in order to determine the form of silver in the composition. The conclusion is that the bulk of the silver exists as silver (0) [that is, metallic silver] and that there is a surface coating which as on average a composition of silver (II) oxide [AgO]. As mentioned above silver (II) oxide is usually a stoichiometric combination of silver (I) and silver (III).

[0049] B. Experimental Procedure

[0050] A few drops of the 22 ppm inventive silver composition were evaporated to dryness on a silicon substrate at ambient temperature. The residue was analyzed by TOF-SIMS, and is denoted as the sample. A reference silver (II) oxide (AgO) material was analyzed by placing a few particles of the reference powder as received from the vendor on a silicon substrate, and is denoted as the reference.

[0051] The Time-of-Flight Secondary Ion Mass Spectrometry technique (TOF-SIMS) is based on the principle of bombarding a solid sample with a pulsed, finely focused beam of primary ions, and then analyzing the secondary ions produced from the surface of the sample via a time-of-flight mass spectrograph. This analytical technique is surface sensitive, deriving its information from a layer that extends to approximately 20 to 40 Å (one Angstrom = 1×10^{-4} micrometers) below the surface. The TOF-SIMS technique is normally used as a survey tool to identify the composition of unknown samples. It is capable of quantification if the appropriate microanalytical standards are available for calibration. This analysis was carried out using standard high mass-resolution conditions.

[0052] C. Results

[0053] Negative ion mass were obtained for the Ag(II)O reference material and the product sample, respectively. The mass spectral region for both spectra showed the presence of AgO⁻ species. The data suggest that silver (II) is the average oxidation state of the silver present on the surface of the sample particles. The silver oxide (AgO) signals exhibit significantly higher intensity in the reference sample compared to the product sample which is probably because metallic silver is dominant in the sample. It will be appreciated that as the average particle size in the sample is decreased the ratio of silver to silver oxide will also decrease as more silver oxide will be present.

[0054] 2. SIZE ANALYSIS

[0055] It is likely that the unusual effectiveness of the silver preparations described herein is due to the relationship between the surface properties/inner properties (i.e., oxide/metal) of the particles and the size distribution of the particles. The smaller the average particle size, the greater the surface area and the greater the contribution of the particular surface chemistry. However, if the particles are excessively small there can be a loss of stability and/or other interactions that negatively affect the product. The silver compositions of the instant invention are remarkable because they are stable in essentially pure water without surfactants, etc. Also, the materials are essentially colorless while other colloidal silver preparations (particularly with larger particle sizes) usually show colors. These properties are a result of the exact manufacturing conditions as discussed above.

[0056] Digital analysis of the composition showed that there is an average particle diameter of 0.0106 micrometers with a range of 0.005 micrometer to 0.0851 micrometers.

5 However, size distribution analysis shows that more than 95% of the particles were between about 0.005 micrometers and about 0.015 micrometers in diameter.

[0057] 3. ANTIVIRAL PROPERTIES OF COLLOIDAL SILVER SOLUTIONS

[0058] In Vitro

[0059] The purpose of this study was to evaluate the antiviral properties of the inventive silver colloids (10 ppm and 32 ppm) against Influenza A (H1N1) virus or Avian Influenza A
10 (H3N2) virus ("bird 'flu") when exposed (in suspension) for a specified exposure period(s). The protocol used is a modification of the Standard Test Method for Efficacy of Virucidal Agents Intended for Special Applications (ASTM E1052).

[0060] This in-vitro virucidal suspension assay was designed to evaluate the antiviral properties of a product against Influenza A ((H1N1) and (H5N1)) virus or Avian Influenza A (H3N2) virus. The presence of virus (infectivity) was determined by monitoring the virus specific cytopathic effect (CPE) on the appropriate indicator cell line, Rhesus monkey kidney. The
15 indicator cell line chosen is capable of supporting the growth of the virus.

[0061] Protocol Summary

[0062] A suspension of virus was exposed to the use dilution of the product. At each pre-determined exposure time an aliquot was removed, neutralized by serial dilution, and assayed for the presence of virus. The positive virus controls, cytotoxicity controls, and neutralization controls were assayed in parallel. Antiviral properties of the test product was evaluated and
20 compared at the specified concentrations and time intervals.

[0063] Test Parameters

	<u>Dilutions to be assayed</u>	<u>Cultures/diln</u>
Cell Control		4
Virus Control (for each exposure time)	10^{-2} to 10^{-7*}	4
Test (for each exposure time and/or concentration)	10^{-2} to 10^{-7*}	4
Cytotoxicity Control (for each product concentration)	10^{-2} to 10^{-4*}	4
25 Neutralization Control (for each product concentration)	10^{-2} to 10^{-4*}	4

* Alternate dilutions may be assayed as determined by the stock virus titer.

[0064] The virus stocks were prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells were disrupted and cell debris removed by centrifugation. The supernatant was removed, aliquoted, and the high titer stock virus was stored at $\leq -70^{\circ}\text{C}$

until the day of use. Alternatively, virus propagated in 9-11 day old embryonated, fertilized eggs was utilized. On the day of use an aliquot of frozen virus was removed, thawed and kept under refrigeration until use in the assay. If an organic soil load challenge was required, fetal bovine serum (FBS) was incorporated into the stock virus aliquot and adjusted to yield the percent soil load requested.

[0065] Cell Cultures and Test Medium

[0066] Rhesus monkey kidney (RMK) cells were obtained from ViroMed Laboratories, Inc. Cell Culture Division. Cultures were maintained and used as monolayers in tissue culture lab ware at 36-38°C in a humidified atmosphere of 5-7% CO₂.

[0067] The test medium used for the virucidal assays was Minimum Essential Medium (MEM) supplemented with 1-10% (v/v) heat inactivated FBS. The medium may also be supplemented with one or more of the following: 10 µg/ml gentamicin, 100 units/ml penicillin, and 2.5 µg/ml amphotericin B.

[0068] METHOD

[0069] Preparation of Test Substance

[0070] The test substance was used directly after being equilibrated to the exposure temperature.

[0071] Treatment of Virus Suspension

[0072] A 4.5 ml aliquot of each concentration of the test substance was dispensed into separate tubes and each was mixed with a 0.5 ml aliquot of the stock virus suspension. The mixtures were vortex mixed for a minimum of 10 seconds and held for the remainder of the specified exposure times at the appropriate temperature. Immediately following each exposure period, a 0.1 ml aliquot was removed from each tube and the mixtures were titered by 10-fold serial dilutions (0.1 ml + 0.9 ml test medium) and assayed for the presence of virus. Note: to decrease the product cytotoxicity, the first dilution may be made in fetal bovine serum or other appropriate neutralizer with the remaining dilutions in test medium.

[0073] If excessive cytotoxicity to the indicator cell cultures was caused by the test substance or suspected, the affected dilution(s) may be passed through individual Sephadex gel filtration columns following titration to aid in reducing the toxicity. In such a case identical dilutions of the controls must also be passed through individual columns.

[0074] Treatment of Virus Control

[0075] A 0.5 ml aliquot of the stock virus suspension was exposed to a 4.5 ml aliquot of test medium instead of test substance and treated as previously described under Treatment of
5 Virus Suspension. A virus control was performed for each exposure time tested. All controls employed the same neutralizer utilized in the test. The virus control titer was used as a baseline to compare the percent and log reduction of each test parameter following exposure to the test substance.

[0076] Cytotoxicity Controls

[0077] A 4.5 ml aliquot of each concentration of the test substance is mixed with a 0.5 ml
10 aliquot of test medium containing any requested organic soil load in lieu of virus and treated as previously described. The cytotoxicity of the cell cultures was scored at the same time as virus-test substance and virus control cultures. Cytotoxicity was graded on the basis of cell viability as determined microscopically. Cellular alterations due to toxicity were graded and reported as toxic (T) if greater than or equal to 50% of the monolayer is affected.

[0078] Neutralization Controls

[0079] Each cytotoxicity control mixture (above) was challenged with low titer stock virus
15 to determine the dilution of test substance at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity will not be considered in determining reduction of the virus by the test substance.

[0080] Neutralization

[0081] As previously described, 0.1 ml of each test and control parameter following the exposure period was added to a 0.9 ml aliquot of neutralizer followed immediately by 10-fold
20 serial dilutions in test medium to stop the action of the test substance. To determine if the neutralizer chosen for the assay was effective in diminishing the virucidal activity of the test substance, low titer stock virus was added to each dilution of the test substance-neutralizer mixture. This mixture was assayed for the presence of virus (neutralization control above).

[0082] Infectivity Assays

[0083] The RMK cell line, which exhibits cytopathic effect (CPE) in the presence of
25 Influenza A (H1N1) or Avian Influenza A (H3N2) virus, was used as the indicator cell line in the infectivity assays. Cells in multi-well culture dishes were inoculated in quadruplicate with 0.1 ml of the dilutions prepared from test and control groups. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. The cultures were incubated at 36-38°C in a humidified atmosphere of 5-7% CO₂ in sterile disposable cell culture lab ware. The cultures

were scored periodically for approximately seven days for the absence or presence of CPE, cytotoxicity and for viability.

5 [0084] Test Criteria

[0085] A valid test will require 1) that stock virus be recovered from the virus control, 2) that the cell controls be negative for virus, and 3) that negative cultures be viable.

[0086] Calculations

10 [0087] Viral and cytotoxicity titers will be expressed as -log₁₀ of the 50 percent titration endpoint for infectivity (TCID₅₀) or cytotoxicity (TCD₅₀), respectively, as calculated by the method of Spearman Karber.

$$\text{- Log of 1st dilution inoculated -} \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100} \right) - 0.5 \right) \times (\text{logarithm of dilution}) \right]$$

Percent (%) Reduction Formula

$$\% \text{ Reduction} = 1 - \left[\frac{\text{TCID}_{50} \text{ test}}{\text{TCID}_{50} \text{ virus control}} \right] \times 100$$

15 Log Reduction Formula

$$\text{TCID}_{50} \text{ of the virus control} - \text{TCID}_{50} \text{ of the test}$$

[0088] Results

[0089] Virus Controls

20 [0090] The titer of the virus control following a two hour exposure time at 37.0°C was 5.0 log₁₀. The percent and log reduction calculations were calculated from this result for all test substances exposed for two hours.

[0091] The titer of the virus control following a six hour exposure time at 37.0°C was 5.25 log₁₀. The percent and log reduction calculations were calculated from this result for all test substances exposed for six hours.

25 [0092] The titer of the virus control following a twelve hour exposure time at 37.0°C was 4.75 log₁₀. The percent and log reduction calculations were calculated from this result for all test substances exposed for twelve hours.

[0093] Silver10 ppm

[0094] Test substance cytotoxicity was not observed in any dilution assayed (≤ 1.5 log₁₀). The neutralization control demonstrated that the test substance was neutralized at ≤ 1.5 log₁₀.

[0095] Following the two hour exposure period at 37.0°C, test virus infectivity was detected in the virus-test substance mixture at 4.5 log₁₀. Under the conditions of this investigation, in the presence of no organic soil load, Silver 10 ppm demonstrated a 68.4% reduction in viral titer following a two hour exposure period to Avian Influenza A (H3N2) virus (Avian Reassortant). The log reduction in viral titer was 0.5 log₁₀.

[0096] Following the six hour exposure period at 37.0°C, test virus infectivity was detected in the virus-test substance mixture at 4.75 log₁₀. Under the conditions of this investigation, in the presence of no organic soil load, Silver 10 ppm demonstrated a 68.4% reduction in viral titer following a six hour exposure period to Avian Influenza A (H3N2) virus (Avian Reassortant). The log reduction in viral titer was 0.5 log₁₀.

[0097] Following the twelve hour exposure period at 37.0°C, test virus infectivity was detected in the virus-test substance mixture at 2.75 log₁₀. Under the conditions of this investigation, in the presence of no organic soil load, Silver 10 ppm demonstrated a 99.0% reduction in viral titer following a twelve hour exposure period to Avian Influenza A (H3N2) virus (Avian Reassortant). The log reduction in viral titer was 2.0 log₁₀.

[0098] Silver 32 ppm.

[0099] Test substance cytotoxicity was not observed in any dilution assayed (≤ 1.5 log₁₀). The neutralization control demonstrated that the test substance was neutralized at ≤ 1.5 log₁₀.

[00100] Following the two hour exposure period at 37.0°C, test virus infectivity was detected in the virus-test substance mixture at 4.5 log₁₀. Under the conditions of this investigation, in the presence of no organic soil load, Silver 32 ppm demonstrated a 68.4% reduction in viral titer following a two hour exposure period to Avian Influenza A (H3N2) virus (Avian Reassortant). The log reduction in viral titer was 0.5 log₁₀.

[00101] Following the six hour exposure period at 37.0°C, test virus infectivity was detected in the virus-test substance mixture at 3.75 log₁₀. Under the conditions of this investigation, in the presence of no organic soil load, Silver 32 ppm demonstrated a 96.8% reduction in viral titer following a six hour exposure period to Avian Influenza A (H3N2) virus (Avian Reassortant). The log reduction in viral titer was 1.5 log₁₀.

[00102] Following the twelve hour exposure period at 37.0°C, test virus infectivity was detected in the virus-test substance mixture at 1.75 log₁₀. Under the conditions of this investigation, in the presence of no organic soil load, Silver 32 ppm demonstrated a 99.9% reduction in viral titer following a twelve hour exposure period to Avian Influenza A (H3N2) virus (Avian Reassortant). The log reduction in viral titer was 3.0 log₁₀.

[00103] Results for Avian Virus

Dilution	Test: Avian Influenza A (H3N2) virus (Avian Reassortant)+ Silver 10 ppm		
	Exposure Time Two Hours	Exposure Time Six Hours	Exposure Time Twelve Hours
Cell Control	0000	0000	0000
10 ⁻²	++++	++++	++++
10 ⁻³	++++	++++	000+
10 ⁻⁴	++++	++++	0000
10 ⁻⁵	0000	000+	0000
10 ⁻⁶	0000	0000	0000
10 ⁻⁷	0000	0000	0000
TCID ₅₀ /0.1 ml	10 ^{4.5}	10 ^{4.75}	10 ^{2.75}
Percent Reduction	68.4%	68.4%	99.0%
Log ₁₀ Reduction	0.5 log ₁₀	0.5 log ₁₀	2.0 log ₁₀

(+) = Positive for the presence of test virus

(0) = No test virus recovered and/or no cytotoxicity present

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Dilution	Test: Avian Influenza A (H3N2) virus (Avian Reassortant)+ silver 32 ppm.		
	Exposure Time Two Hours	Exposure Time Six Hours	Exposure Time Twelve Hours
Cell Control	0000	0000	0000
10 ⁻²	++++	++++	00+0
10 ⁻³	++++	++++	0000
10 ⁻⁴	++++	000+3	0000
10 ⁻⁵	0000	0000	0000
10 ⁻⁶	0000	0000	0000
10 ⁻⁷	0000	0000	0000
TCID ₅₀ /0.1 ml	10 ^{4.5}	10 ^{3.75}	10 ^{1.75}
Percent Reduction	68.4%	96.8%	99.9%
Log ₁₀ Reduction	0.5 log ₁₀	1.5 log ₁₀	3.0 log ₁₀

25 (+) = Positive for the presence of test virus

(0) = No test virus recovered and/or no cytotoxicity present

[00104] Summary Results for Avian virus:

Test Substance	Two Hour Exposure		Six Hour Exposure		Twelve Hour Exposure	
	Percent Reduction	Log Reduction	Percent Reduction	Log Reduction	Percent Reduction	Log Reduction

Silver 10 ppm	68.4%	0.5 log ₁₀	68.4%	0.5 log ₁₀	99.0%	2.0 log ₁₀
Silver 32 ppm	68.4%	0.5 log ₁₀	96.8%	1.5 log ₁₀	99.9%	3.0 log ₁₀

5 [00105] Cytotoxicity and Neutralization Controls

Dilution	Cytotoxicity Control		Neutralization Control	
	10ppm	32 ppm	10 ppm	32 ppm
Cell Control	0000	0000	0000	0000
10 ⁻²	0000	0000	++++	++++
10 ⁻³	0000	0000	++++	++++
10 ⁻⁴	0000	0000	++++	++++
10 TCID ₅₀ /0.1 ml	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}

(+) = Positive for the presence of test virus.

(0) = No test virus recovered and/or no cytotoxicity present.

[00106] The colloidal silver also showed significant activity against human Influenza A strains.

[00107] Virucidal Efficacy against Influenza A (H5N1)

TIME INTERVAL		10 ppm	32 ppm	MEM CONTROL
2 hour	LOG ₁₀ CCID ₅₀	4.2	3.8	3.5
2 hour	LOG ₁₀ REDUCTION	0	0	n/a
6 hour	LOG ₁₀ CCID ₅₀	<1.9	1.5	3.5
6 hour	LOG ₁₀ REDUCTION	>1.6	2.0	n/a
12 hour	LOG ₁₀ CCID ₅₀	<1.5	<1.6	2.9
12 hour	LOG ₁₀ REDUCTION	>1.4	>1.3	n/a

[00108] The anti-Influenza results show that the broad antimicrobial properties of the inventive colloidal silver extend to influenza virus and especially to avian influenza virus. As demonstrated above the silver colloid is non-toxic and is thus an ideal product for disinfecting surfaces that might be contaminated with influenza virus.

[00109] In Vivo.

[00110] The foregoing establishes that the inventive colloidal silver is effective at killing influenza viruses on surfaces. The efficacy of the colloidal silver as an in vivo treatment was

explored in the following experiments. The goal of the experiments was to pre-treat mice with either 10 ppm or 32 ppm colloidal silver and then to challenge them with H5N1 avian influenza.

5 [00111] Pathogen-free female BALB/c mice each with a body weight of 18-21 g were obtained from Charles River Laboratories (Wilmington, MA). Influenza A/Duck/MN/1525/81 (H5N1) was adapted to mice by two passages through weanling animals. A pool of adapted virus was then grown in MDCK (Madin-Darby canine kidney) cells and titrated against young adult mice prior to use.

10 [00112] Arterial oxygen saturation (SaO_2) was determined using the ear probe attachment of an Ohmeda Biox pulse oximeter. The probe was placed on the thigh of the animal, and readings were made following a 30 sec stabilization period. The lungs of the experimental animals were removed, weighed and then placed in Petri dishes bearing pre-numbered sections (one lung per section). The lungs were then scored from 0 (normal) to 4 (maximal plum coloration over 100% of the lung). Then each lung was homogenized and dilutions of the homogenate were assayed in triplicate for infectious virus.

15 [00113] Groups of 19 mice were treated by oral gavage with either the 32 ppm or 10 ppm colloidal silver solution every 12 hr for seven days. Then the animals were infected intranasally with an LD70 dose of the infectious influenza virus (negative controls were "infected" with nasally administered sterile water) and maintained for 21 days. As a control a similar group of mice were treated with ribavirin, an anti-viral drug (75 mg/kg twice a day for five days, starting 4 hr pre-virus exposure). SaO_2 measurements were made on days 3-11 (which is the time over which this parameter usually declines in infected animals). Survivorship was statistically evaluated by chi square analysis with Yates' correction. Increases in mean day to death, differences in mean SaO_2 values, mean lung weight and mean virus titers were analyzed using a student's t-test. A Wilcoxon ranked sum analysis was used for lung scores.

20 [00114] The results of the experiment are summarized in the following table which shows that the virus challenge was lethal to 14 of the 20 placebo-treated animals, with the mean day to death being 8.4 days. Treatment with 32 ppm colloidal silver appeared to not affect the numbers of animals dying of influenza, although a half-day delay in mean day to death was seen. SaO_2 declines in this group of treated mice were almost at the same rate as those in the placebo controls, although it was interesting that on the first day this parameter was assayed, a highly significant ($P < 0.001$) difference was seen. SaO_2 declines are a manifestation of declining lung function, suggesting that the lung consolidation in the lungs did not progress as rapidly in animals treated with 32 ppm colloidal silver as seen in the placebo controls. The treatment appeared to moderately lessen lung consolidation as seen by lower lung scores at each time evaluated, the day 6 mean lung score being significantly ($P < 0.05$) less than the

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placebo treated controls. Lung weights, another indication of fluid developing in the lungs to cause pneumonia in the animal, were also less at each time point than seen in the placebos.

- 5 The mean lung virus titers in the mice treated with 32 ppm colloidal silver were lower than the placebo controls on days 3 and 6 of the infection. Treatment with 10 ppm colloidal silver also provided some intriguing results. There was a marked improvement in survival in that 60% of the infected mice treated with the 10 ppm solution survived compared to the 30% of the placebo-treated controls. Although not statistically significant because of the number which survived in the latter controls, this effect is strongly suggestive a disease inhibitory effect may have occurred. At two time points during the SaO₂ assays, days 3 and 6, the declines normally
- 10 seen were significantly lessened ($P < 0.01$), and there was a general lessening of decline throughout the times of assay. Modest inhibition in lung scores was seen in this treated group as well, especially on day 6. As with the 32 ppm treatment slight inhibition of lung virus titers were seen.

[00115] Experimental Results on Influenza Infected Mice

Treatment	Survival	Mean Day to Death	Day 11 SaO ₂ (%)
15 Normal (not infected)	5/5	—	88.8±3.0
Ribavirin (positive control)	10/10	>21	86.6±2.5
Water (negative control)	6/20	8.4 ±1.8	76.0±1.9
32 ppm Colloidal Silver	2/10	8.9±1.4	75.4±1.0
10 ppm Colloidal Silver	6/10	7.3±1.0	76.7±2.1

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[00116] There were no demonstrated adverse side effects from either of the colloidal silver solutions. It is difficult to attribute the effects seen in this experiment wholly to viral inactivation, since both test materials were administered orally to animals infected by direct nasal inhalation. Because the treatments began one week before virus exposure, it is possible that some portion of the colloidal silver may have reached the vicinity of the virus-exposed lung tissue.

- 25 There are a number of hypotheses for the mechanism of action and effectiveness of colloidal silver including the possibility that this material is exerting a mild immunomodulatory effect on the animals, which would provide modest protection against the infection. If such a mechanism is indeed associated with the activity seen, then a different treatment schedule, perhaps limiting the number of treatments to one per day or once every other day, might enhance any immunomodulatory effects, since it is recognized that too-frequent dosing may overtax the

immune system. The greater protection seen by the 10 ppm material could be explained by such immunomodulation, since the greatest immunologic effect is not necessarily at the highest dose used.

[00117] Another mechanism whereby the silver materials may have inhibited the influenza virus infection in these studies may simply be one of coating the virion with colloidal silver to prevent attachment and penetration. Again, the silver material would need to be in the vicinity of the exposed lung tissues at the time infection was initiated. Perhaps the amount administered or the general dosage scheme affects the ability of the colloidal silver to redistribute within the animal's body. The colloidal silver material could also play a role in limiting apoptosis of the epithelial lining of the lung induced during acute lung inflammation. Apoptosis plays a causative role in acute lung injury in part due to epithelial cell loss. Consideration of combined use of oral administration of the silver materials and intranasal instillation at near the time of virus exposure would determine whether the effects seen were indeed associated with direct virucidal effects of the silver materials.

[00118] The observation that the colloidal silver is highly effective against the influenza virus *in vitro* while apparently less effective *in vivo* brings into question the dosage and route of *in vivo* administration. Because the colloidal silver is essentially non-toxic, it seems likely that attaining sufficiently high concentrations of the material at the site of influenza virus entry or replication will be efficacious without causing side effects. At this time it seems likely that periodic treatment of the nasal passages and lungs with a mist containing the colloidal silver would be a good treatment option.

[00119] The following claims are thus to be understood to include what is specifically illustrated and described above, what is conceptually equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention. Those skilled in the art will appreciate that various adaptations and modifications of the just-described preferred embodiment can be configured without departing from the scope of the invention. The illustrated embodiment has been set forth only for the purposes of example and that should not be taken as limiting the invention. Therefore, it is to be understood that, within the scope of the appended claims, the invention may be practiced other than as specifically described herein.

We claim:

1. A method of lowering infectivity of influenza A virus in vitro comprising the steps of:
providing a composition of silver in water comprising a total concentration of silver of between about 5 and 40 parts per million, said silver in the form of colloidal silver particles having an interior of elemental silver and a surface of silver oxide, wherein a majority of the colloidal silver particles have a minimum diameter greater than 0.005 micrometers and a maximum diameter less than 0.015 micrometers, said composition being free of cytotoxic properties;
and
contacting Influenza A virus with the composition.
2. The method according to claim 1, wherein the Influenza A is Avian Influenza A.
3. The method according to claim 2, wherein the Avian Influenza A is Avian Influenza A (H3N3).
4. The method according to claim 1, wherein the Influenza A is Human Influenza A.
5. The method according to claim 4, wherein the Human Influenza A is Human Influenza A (H5N1).
6. The use of a composition of silver in water comprising a total concentration of silver of between about 5 and 40 parts per million, said silver in the form of colloidal silver particles having an interior of elemental silver and a surface of silver oxide, wherein a majority of the colloidal silver particles have a minimum diameter greater than 0.005 micrometers and a maximum diameter less than 0.015 micrometers and wherein said composition is free of cytotoxic properties in the manufacture of a medicament for ameliorating infection by influenza A virus.
7. The use according to claim 6, wherein the Influenza A is Avian influenza A.
8. The use according to claim 6, wherein the Avian Influenza A is Avian Influenza A (H3N3).