

Universal and reusable virus deactivation system for respiratory protection

Abstract

Aerosolized pathogens are a leading cause of respiratory infection and transmission. Currently used protective measures pose potential risk of primary/secondary infection and transmission. Here, we report the development of a universal, reusable virus deactivation system by functionalization of the main fibrous filtration unit of surgical mask with sodium chloride salt. The salt coating on the fiber surface dissolves upon exposure to virus aerosols and recrystallizes during drying, destroying the pathogens. When tested with tightly sealed sides, salt-coated filters showed remarkably higher filtration efficiency than conventional mask filtration layer, and 100% survival rate was observed in mice infected with virus penetrated through salt-coated filters. Viruses captured on salt-coated filters exhibited rapid infectivity loss compared to gradual decrease on bare filters. Salt-coated filters proved highly effective in deactivating influenza viruses regardless of subtypes and following storage in harsh environmental conditions. Our results can be applied in obtaining a broad-spectrum, airborne pathogen prevention device in preparation for epidemic and pandemic of respiratory diseases.

Introduction

Aerosols take a prominent role in airborne transmission of respiratory diseases. Droplets with aerodynamic size (d_a) $< 10 \mu\text{m}$ and $10 < d_a < 100 \mu\text{m}$ are known to infect the alveolar regions and upper respiratory tract, respectively^{1,2}. Notably, aerosols can also be a route of infection in diseases that, contrary to for instance influenza, do not specifically target the respiratory tract, as it could be the case of Ebola virus³. While vaccination can greatly reduce morbidity and mortality, during a pandemic or epidemic new vaccines matching the specific strain would be available, at the earliest, six months after the initial outbreak. Additionally, following development of an effective viral vaccine, several potential problems

would remain, such as limited supply due to insufficient production capacity and time-consuming manufacturing processes. As a result, individuals close to the point of an outbreak would be in imminent danger of exposure to infectious diseases during the non-vaccine period. In the absence of vaccination, respirators and masks can be worn to prevent transmission of airborne pathogenic aerosols and control diseases, such as influenza⁴.

The main alternative, the N95 respirator, requires training prior to use, must be expertly fitted to address the risk of face seal leakage at the face-mask interface, and must be disposed of as biohazard⁵. Due to these factors, the use of N95 respirators on a large scale is impractical and expensive during an epidemic or pandemic. Past experiences of severe acute respiratory syndrome (SARS), H1N1 swine flu in 2009, and Middle East respiratory syndrome (MERS) indicate that surgical masks have been most widely adopted by the public as personal protective measure, despite controversy on their effectiveness^{6,7,8,9}. Currently, among other factors, filtration in respirators and masks depends on filter characteristics, including fiber diameter, packing density, charge of fibers and filter thickness, as well as particle properties, such as diameter, density and velocity^{10,11,12,13,14}. However, in the lack of a system to deactivate the collected pathogens, safety concerns naturally arise about secondary infection and contamination from virus-laden filter media during utilization and disposal. Furthermore, since re-sterilization is not possible without causing damage, respirators and masks are recommended for single use only^{9,15,16}. Scientific efforts have been focused on treatment of filters with materials possessing well-known antimicrobial properties, such as iodine, chlorine and metals^{17,18,19,20,21,22,23,24,25}, although with limited effectiveness against virus aerosols^{26,27,28}. Therefore, a key challenge is the development of an easy-to-use, universal virus negation system, which is reusable without reprocessing and capable of deactivating pathogens, thereby reducing potential risk of secondary infection and transmission.

Here, we report a simple but efficient virus inactivation system exploiting the naturally occurring salt recrystallization. Our strategy is to modify the surface of the fibrous filtration layer within masks with a continuous salt film for virus deactivation via two successive processes: i) salt is locally dissolved by the viral aerosols and ii) supersaturation is followed by evaporation-induced salt recrystallization. Consequently, viruses are exposed to increasingly higher

concentrations of saline solution during drying and physically damaged by recrystallization.

Discussion

Development of a universally applicable, low-cost, and efficient mechanism for virus negation is regarded as a major challenge in public health against general airborne biological threats. This led us to propose a new concept of personal/public preventive and control measures using salt-recrystallization against pathogenic aerosols based on two hypotheses. The salt-coating can enhance adsorption of virus on the filter fibers and inactivate virus by the increase of osmotic pressure followed by the crystallization of salts. As shown in [Fig. 2b](#), salt-coated filters exhibited significantly higher levels of filtration efficiency than bare filters. Notably, the bacterial filtration efficiency (BFE) reported by the mask manufacturer is 99%. The different value of filtration efficiency for bare filters obtained under our experimental conditions may be partially due to the use of aerosols with different biological origins. The FDA-recognized ASTM F2101 – 14 standard for evaluation of BFE exposes surgical masks to *Staphylococcus aureus* aerosols, by employing *S. aureus* ATCC 6538³⁴, which has an average diameter of about 1 μm . In this study, filtration efficiency was calculated following exposure of bare and salt-coated filters to influenza virus, which exhibits a smaller diameter than that of *S. aureus* by one order of magnitude. Additionally, whereas during BFE evaluation all three layers of surgical masks are used, in this work filtration efficiency refers to mask filters (middle layer). It is worth noting that the conditions for BFE standard evaluation (such as flow rate and time of application of flow) do not coincide with the experimental procedure we used for measurement of the filtration efficiency, which may further contribute to the different result. The enhanced filtration efficiency of salt-coated filters against influenza virus aerosols as compared to bare filters can be explained by the observed wetting of aerosols, favoring greater adhesion to salt-coated filters. Furthermore, the significant improvement in filtration efficiency resulted in complete protection of mice against lethal influenza aerosols, which demonstrates the high level of protection provided by salt-coated filters, outperforming currently used bare filters.

Rapid loss of HA activity and viral infectivity on salt-coated filters can be explained by physical destruction of virus during recrystallization of coated salts. When the salt-coated filter is exposed to virus aerosols, salt crystals below the aerosol droplet dissolve to increase osmotic pressure to virus. Due to evaporation,

the salt concentration of the droplet significantly increases and reaches the solubility limit, leading to recrystallization of salt. As a consequence, virus particles are exposed to increasing osmotic pressure during the drying process and are physically damaged by crystallization. As shown in Fig. 3e,f, the superior advantage of physically destroying the virus adsorbed to the salt-coated PP filters through natural salt crystallization process was further confirmed *in vivo*. According to previous reports, hyperosmotic stress (>541 mOsm) and crystallization induce membrane perturbation with irreversible deformation of the viral envelope and structural virus damage, respectively, resulting in infectivity loss of virus^{30,31}. Therefore, our data support that the extensive level of infectivity loss associated with a salt recrystallization process caused by physical contact between virus aerosols and salt coating can be used in developing virus negation systems that are reusable without reprocessing.

Similarly to CA/09 H1N1 aerosols, increased protection *in vivo* due to higher filtration efficiency of salt-coated filters compared to bare filters and deactivation of virus on salt-coated filters were observed following exposure to PR/34 H1N1 and VN/04 H5N1 (Fig. 4a,b). This suggests that salt-coated filters prevent virus penetration and destroy virus attached to the filter in a non-specific way. Furthermore, the performance of salt-coated filters was not degraded by storage at 37 °C and 70% RH, demonstrating that salt recrystallization-based filters can ensure protection even under harsh environmental conditions. Notably, for demonstration of the concept of salt-recrystallization based virus deactivation system, NaCl salt was used, which has a critical RH of 75% at 30 °C³⁵. However, salts with higher critical RH can be easily used, such as ammonium sulfate, potassium chloride and potassium sulfate, which have critical RH of 80%, 84% and 96.3% at 30 °C, respectively³⁵. This suggests that salt-coated filters may be developed for specific environmental conditions.

In conclusion, we demonstrated that the developed salt-recrystallization based filtration system provides high filtration efficiency and successfully deactivates multiple subtypes of adsorbed viruses. Moreover, we have shown that stability of the salt coating is not compromised by high temperature and humidity, which suggests safe use and long-term storage/reuse at such environmental conditions. Although our tests are based on exposure to different types of influenza virus, the significance of these results for personal and public protective measures may be generally extended to enveloped respiratory viruses where infection and transmission can occur by aerosol. Our salt-coated filter unit can promise the development of long-term stable, versatile airborne pathogen negation system,

without safety concerns. In fact, the destruction mechanism of viruses solely depends on the simple, yet robust naturally occurring salt recrystallization process, combining the destabilizing effects of salt crystal growth and concentration increase during drying of aerosols. This idea can be easily applied to a wide range of existing technologies to obtain low-cost, universal personal and public means of protection against airborne pathogens, such as masks and air filters in hospitals. Therefore, we believe that salt-recrystallization based virus deactivation system can contribute to global health by providing a more reliable means of preventing transmission and infection of pandemic or epidemic diseases and bioterrorism.

Methods

Bare and salt-coated filter samples preparation

The commercial surgical masks had a three-ply structure. The middle layer is the filter media, whereas the inner and outer layers provide support and protect the filter against wear and tear. The metal nose clips and elastic ear loops were removed and circular samples (radius: 3 cm) were cut from the masks. The PP filters (middle layer) were isolated by removing the inner and outer protective layers (bare filters, $\text{Filter}_{\text{bare}}$). The coating solution was prepared by dissolving sodium chloride (NaCl; Sigma Aldrich, St. Louis, MO) in filtered DI water (0.22 μm pore size; Corning, Tewksbury, MA) under stirring at 400 rpm and 90 °C, followed by the addition of Tween 20 (Fisher Scientific) to a final concentration of 29.03 w/v% of NaCl and 1 v/v% of Tween 20. To obtain the salt-coated filters, the mask bare PP filters were pre-wet to contain approximately 600 μL of coating solution by incubating overnight at room temperature. Any remaining dry areas were removed by applying gentle strokes with tweezers to the filters while immersed in the coating solution. Subsequently, the filters were deposited in the desired volume of coating solution (0, 100, 300, 600, 900 and 1200 μL , of which corresponding membranes are abbreviated as $\text{Filter}_{\text{wet}}$, $\text{Filter}_{\text{wet}+100\mu\text{L}}$, $\text{Filter}_{\text{wet}+300\mu\text{L}}$, $\text{Filter}_{\text{wet}+600\mu\text{L}}$, $\text{Filter}_{\text{wet}+900\mu\text{L}}$, and $\text{Filter}_{\text{wet}+1200\mu\text{L}}$, respectively) on petri dishes (60 \times 15 mm; Fisher Scientific) to control the amount of NaCl per unit area and dried in an oven (Isotemp Incubator, Fisher Scientific) at 37 °C for 1 day.

Influenza virus preparation

Influenza viruses A/California/04/2009 (CA/09, H1N1), A/Puerto Rico/8/34 (PR/34, H1N1) and A/Vietnam/1203/2004 (VN/04, H5N1) were grown in 10-day old embryonated hen eggs, in which H5N1 virus was derived by reverse genetics from HPAI A/Vietnam/1203/2004³⁶. Influenza viruses were purified from allantoic fluid using discontinuous sucrose gradient (15%, 30% and 60%) layers following the previously reported procedure³⁷.

Aerosols exposure to filters

For experiments involving aerosols exposure, an aerosol chamber ($L \times W \times H = 145 \times 145 \times 150$ mm; Emka Inc., Middletown, PA) was used (Fig. S11). It has a connection to the vacuum line and a circular aperture in the top wall (diameter: 22 mm) to exactly accommodate the cylindrical part (diameter: 20 mm, height: 20 mm) of the nebulizer unit that is below the aerosol generator (Aeroneb Lab Nebulizer System; Aerogen, Galway, Ireland). Bleach was used as trap between the chamber and the vacuum pump (Welch 2522C-10, 22 L/min; Niles, IL). The filters were placed on top of the chamber aperture and the nebulizer unit was inserted, ensuring the tight seal of the filters against the side of the aperture. 5 μ L of virus stock were added to the nebulizer unit, aerosols (VMD 2.5–4 μ m from manufacturer specifications) were generated for 30 sec and subsequently the desired vacuum level (3, 10 or 17 kPa) was applied, by manual control, three times in 1 sec cycles. Notably, in the case of bare filters, pressure was only applied for filtration efficiency tests.

For all assays and analysis, suspensions of the filters were prepared as follows, unless otherwise indicated. To reconstitute virus adsorbed onto filters, virus-laden filters were immersed in 400 μ L of sterilized DI water for about 5 min, and then removed after vortexing from the suspension. The virus suspension was centrifuged at 19,800 g and 4 °C for 10 min (Centrifuge 5810 R, Eppendorf, Hauppauge, NY), followed by resuspension of pellets in 70 μ L of DI water to eliminate any interference from materials in supernatant during assays.

Filtration efficiency tests

The filters were exposed to the virus aerosols at 3, 10 and 17 kPa and suspensions of the filters were obtained, as described above. The filtration efficiency was calculated as the ratio of the amount of virus (i.e., total proteins measured from the virus) reconstituted from the filter to that from the virus in the exposure aerosols. The concentration of virus in aerosols was determined by generating

viral aerosols into a 15 mL centrifuge tube, containing 1 mL of DI water. After vortexing, virus concentrations (i.e., total protein concentration) were measured with bicinchoninic acid assay (BCA protein assay kit; Thermo Fischer scientific, Waltham, IL) with bovine serum albumin as a standard. In the case of virus reconstituted from salt-coated filters, virus-laden filter suspension was replaced with DI water prior to BCA assay.

In vivo infection tests

Lethal infectivity of influenza viruses (CA/09 H1N1) was examined in 8 week old female inbred BALB/c mice (Nara Biotech; Seoul, Korea) by using the intranasal route. For bare and salt-coated filters, 12 mice per group were infected with individual penetration dosage of influenza virus through each filter. The penetration dosage of the virus through the filters ($\text{Filter}_{\text{bare}}$, $\text{Filter}_{\text{wet}}$, $\text{Filter}_{\text{wet}+600\mu\text{L}}$, and $\text{Filter}_{\text{wet}+1200\mu\text{L}}$) was calculated from the filtration efficiency at 10 kPa (near breathing pressure) using the relationship: penetration dosage = virus dosage in lethal aerosol \times penetration efficiency (%) / 100, where penetration efficiency (%) = 100 – filtration efficiency (%). To examine the effects of the aerosolization process on the viral infectivity change, two mice groups were infected with a lethal dose of virus before and after aerosol formation, which served as negative control groups. Body weight changes and survival rate of mice were monitored daily for 15 days. Mice with body weight loss greater than 25% were euthanized. All animal protocols were approved by the Kyung Hee University (KHU) Institutional Animal Care and Use Committee (IACUC). All animal experiments and husbandry involved in this work were conducted under the approved protocols and guidelines of KHU IACUC. KHU IACUC operates under National Veterinary Research and Quarantine Service (NVRQS), and animal welfare law and regulations of the WOAHOIE (World organization for animal health).

To test strain-dependent lethal infection behavior, mice (12 per group) were infected with the penetrated dosage of viral aerosols (PR/34 H1N1 and VN/04 H5N1 viruses) through $\text{Filter}_{\text{wet}+600\mu\text{L}}$ at 10 kPa. Time-dependent body weight change was monitored in the same manner described above.

Lung viral titer and lung inflammatory cytokine assays after infection

On day 4 after infection 6 mice of each group were sacrificed for the collection of lung samples. Lung virus titers were measured on six-well plates containing confluent MDCK cell monolayers. Inflammatory cytokines (IFN- γ) were determined using BD OptEIA mouse IFN- γ ELISA kit (BD Biosciences, San Jose, CA) following the manufacturer's procedure.

Test of viral infectivity change on filters

To investigate the effects of salt-coating on viral infectivity loss, lethal influenza aerosols were exposed to four different types of filters (Filter_{bare}, Filter_{wet}, Filter_{wet+600 μ L}, and Filter_{wet+1200 μ L}). Since Filter_{bare} exhibited almost complete penetration upon pressure application, aerosols were exposed to the bare filter in the absence of pressure and samples were carefully handled to prevent mechanical agitation. To measure time-dependent stability change of virus, virus-laden filters were incubated at ambient conditions for 0, 5, 15, and 60 min after aerosol exposure, and suspended in DI water to reconstitute virus at each time point. *In vitro* stability of virus was characterized by measuring hemagglutinin activity (HA) and virus titers at the same concentration as lethal dose³⁰. The conformational stability of antigenic proteins was characterized by measuring intrinsic fluorescence using 0.1 mg/mL of virus suspension³⁸. To investigate morphological change of virus, lipid stability of viral wall was characterized by Nile red fluorescence (Sigma Aldrich), a fluorescent lipid stain, following manufacturer's protocol³⁹. A decrease in fluorescence intensity can be used to examine the level of disintegration of the virus. Both intrinsic and Nile red fluorescence were measured by using a fluorimeter (LB 50B; PerkinElmer, Waltham, MA). Intensity changes of fluorescent spectra were compared relative to those of a control from virus stock.

To test infectivity difference observed from *in vitro* findings, *in vivo* study was performed for the virus reconstituted from the filters (Filter_{bare}, Filter_{wet}, Filter_{wet+600 μ L}, and Filter_{wet+1200 μ L}) after incubation for 60 min at RT (aerosol exposure at 10 kPa, except for Filter_{bare}). 12 mice per group were infected with a lethal dose of virus collected from each type of filter. Body weight change and lung virus titers were measured as described above.

Effects of environmental conditions on the performance of salt-coated filter

Salt-coated filters (Filter_{wet}, Filter_{wet+600 μ L}, and Filter_{wet+1200 μ L}) were stored at 37 °C, 70% RH in an incubator (Maru Max; Rcom, Gyeonggi-do, South Korea) for 15 days. Every day, the filters were collected and incubated at ambient conditions for 5 min. At 1-day incubation, filtration efficiency was measured at 10 kPa from Filter_{wet+600 μ L}, followed by *in vivo* infection test. Lethal infectivity between two different filter groups (before and after incubation at 37 °C, 70% RH) was compared by measuring body weight change and survival rate of mice after exposure to lethal CA/09 H1N1 aerosols. XRD analysis was performed to salt-coated filters incubated for 1 and 15 days, and SEM/EDX mapping analysis for 15-day incubated samples.

Contact angle measurements and imaging of aerosols

The bare and salt-coated filters were fixed with carbon tape (Ted Pella, Inc., Redding, CA) to a metal, flat substrate and 3 μ L of DI water were added on the surface of the filters. The contact angles were measured from images collected with an optical microscope (10 \times lens, Motic SMZ-140; Motic, Richmond, Canada) at RT. Images of aerosols on filter fibers were obtained using a dispersive Raman microscope (Nicolet Almega XR; Fisher Scientific).

Aerosol drying time on filters

The bare and salt-coated filters were fixed with carbon tape to a metal, flat substrate and exposed to aerosols generated from 5 μ L of Sulforhodamine B Dye solution (1 mM, Sigma-Aldrich). Aerosol drying time was determined with timer by observation with optical microscope.

Electron microscopy analysis

For virus stability tests, bare and salt-coated filters were exposed to CA/09 H1N1 aerosols and, after 5 and 60 min incubation, virus was recovered by suspension of the filters, as described above. To study the effects of the coating formulation during aerosol drying independently from crystal growth, bare and salt-coated filters were immersed in DI water and removed after 60 min. Subsequently, virus was incubated in the obtained suspension for 60 min. Additionally, the virus suspension was centrifuged at 19,800 g and 4 °C for 10 min to collect the samples and suspend them in DI water. For TEM analysis (200 kV, JEOL JEM 2100; JEOL, Peabody, MA), samples were deposited on copper grid (Electron Microscopy Sciences, Hatfield, PA) and negatively stained with solution

comprised of phosphotungstic acid hydrate (1.5 w/v%, pH = 7.0; Sigma-Aldrich, Oakville, Canada).

To identify the morphology of salt-coated filters and recrystallized salts, SEM/EDX analysis was performed for bare and salt-coated filters after coating with 10 nm thick gold layer. Scanning electron microscopy analysis (Hitachi S-3000N; Hitachi, Toronto, Canada) was operated in secondary electron mode at 20 kV and EDX analysis was obtained with EDX detector (Oxford Instruments, Concord, MA).

XRD analysis

To confirm the formation of crystalline NaCl coating during drying process and its stability during storage at 37 °C and 70% RH, XRD analysis (BRU-1098; Bruker, Billerica, MA) was performed at different coating conditions. Filters (1 × 1 cm) were mounted on a slide glass for XRD analysis (θ – 2θ mode) using a CuK α radiation.

Statistical analysis

To compare multiple conditions, Student's t-test, One-way analysis of variance (ANOVA), and general linear model were used (Minitab release 14; Minitab, State College, PA). *P* value of less than 0.05 was considered to be significant.

Additional Information

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Contributions

H.J.C. conceived and designed the experiments. F.S.Q., I.R., S.H.L., B.K., and H.J.C. performed the experiments. F.S.Q., I.R., S.H.L., B.K., and H.J.C. analyzed the data. I.R. and H.J.C. wrote the manuscript. F.S.Q. and B.K. edited the manuscript.

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Ethics declarations

Competing interests

The authors declare no competing financial interests.

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