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Room-temperature, atmospheric plasma needle reduces adenovirus gene expression in HEK 293A host cells

Z. Xiong,[1] X. Lu,[1,a)] Y. Cao,[2] Q. Ning,[3] K. Ostrikov,[4] Y. Lu,[5] X. Zhou,[2] and J. Liu[3]
[1]*State Key Laboratory of Advanced Electromagnetic Engneering and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430030, People's Republic of China*
[2]*Department of Stomatology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, People's Republic of China*
[3]*Laboratory of Infectious Immunology, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, People's Republic of China*
[4]*Plasma Nanoscience Centre Australia (PNCA), CSIRO Materials Science and Engineering, P. O. Box 218, Lindfield, New South Wales 2070, Australia and Complex Systems, School of Physics, The University of Sydney, Sydney, New South Wales 2006, Australia*
[5]*School of Physics, Anhui University, Hefei, Anhui 230039, People's Republic of China*

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Room-temperature, atmospheric-pressure plasma needle treatment is used to effectively minimize the adenovirus (AdV) infectivity as quantified by the dramatic reduction of its gene expression in HEK 293A primary human embryonic kidney cells studied by green fluorescent protein imaging. The AdV titer is reduced by two orders of magnitude within only 8 min of the plasma exposure. This effect is due to longer lifetimes and higher interaction efficacy of the plasma-generated reactive species in confined space exposed to the plasma rather than thermal effects commonly utilized in pathogen inactivation. This generic approach is promising for the next-generation anti-viral treatments and imunotherapies. © *2011 American Institute of Physics*. [doi:10.1063/1.3669534]

A very large number of serious human and animal diseases such as AIDS, SARS, common cold, human and avian influenza, chickenpox, and several others are caused by viral infections.[1] The infectious agents of these diseases are virus nanoparticles (virions) that contain DNA/RNA strands encased in a capsid protein coat and in some cases also in an outer lipid envelope.[2] The virions are usually transmitted from the infected cases through airborne dissemination or diffusion during nosocomial (occurring in a hospital) transfusion of blood or body liquids.[3] The onset of the viral infection is usually heralded by the entry of the virion's genetic material into the intracellular space followed by the uncontrollable integration with the host DNA and virus replication.[4] Despite decades of research, virion detection, pre-emptive and post-infection anti-viral treatments, and immunotherapies remain ineffective and the morbidity and mortality rates due to the associated infections remain high.

This problem has recently escalated because adenovirus (AdV), one of the most common and treatment-resistant pathogens of respiratory, ophthalmic, and enterogastritic diseases, has very recently been identified as a major obstacle on the way towards the next-generation, potentially revolutionary stem cell therapies.[5–7] Indeed, the risk of life-threatening systemic AdV infections (affecting the entire body and biologically defined by positive adenoviraemia) during hematopoietic stem cell transplantation may exceed 50%, and even higher in cord blood transplant recipients.[8] Since pre-emptive anti-viral treatments are very complicated (e.g., are based on multiple drug infusions and/or adoptive immunotherapy based on transfer of virotoxic, virion-specific T-cells) and ineffective,[7,9] the quest for reliable AdV inactivation methods and infectivity quantification protocols are presently on the agenda of multidisciplinary virology, microbiology, hematology, biochemistry, genetics, and applied physics research.

Here, we report on the effective AdV inactivation using low-temperature, atmospheric-pressure plasma (APP) needle treatment and also provide reliable quantification of the drastically reduced AdV infectivity by monitoring the AdV gene expression and the associated emission from the green fluorescent protein in host HEK 293A primary human embryonic kidney cells. The treatment relies on the physical effect of confinement of the APP-generated reactive radical species (RRSs) in a limited space which in turn increased the lifetime and eventually the efficacy of the RRS interaction with the AdV. Our combinatorial technique advances the highly topical plasma health care and medicine approaches. These approaches have recently been very successful in inactivation of a range of microorganisms including bacteria, fungi, spores, as well as control of reproduction, proliferation, and apoptosis of a range of prokaryotic and eukaryotic cells.[10–15] However, successful applications of APPs to virus inactivation are mostly limited to bacterial viruses, e.g., bacteriophages,[16] while mammalian viruses such as the AdV represent a major challenge.[3,10]

In this work, an APP needle discharge driven by direct current (DC) voltage is used to inactivate the envelope-free AdV, with a double-strand DNA, a diameter of 70–90 nm, and a genome size of approximately 36 kb. The schematic of the APP setup is shown in Fig. 1. The device is driven by a custom-made DC power supply with the variable voltage up to 20 kV.[17] The main part of the device is a syringe needle with the inner diameter of about 0.3 mm. The needle is connected to the power supply through a 130 MΩ resistor, which is used to limit the discharge current to the safe range for a human body. Helium mixed with 1% O_2 is used as working

a)Electronic mail: luxinpei@hotmail.com.

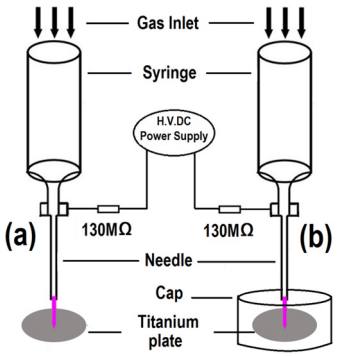

FIG. 1. (Color online) Schematic of the plasma needle adenovirus treatment setup: without (a) and with (b) a cap.

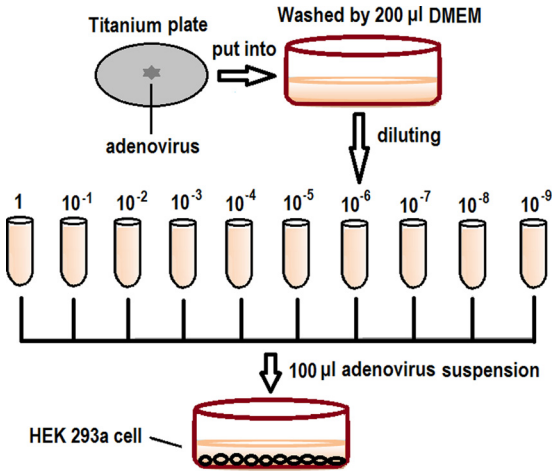

FIG. 2. (Color online) Process flow of the adenovirus titer measurements.

gas mixture and is let through the syringe with a flow rate of 0.5 l/min. In this study, two treatment methods are adopted and their AdV inactivation effects are compared. Figure 1(a) shows the first method where no cap is used to cover the sample. Figure 1(b) shows the second method where a cap with a center hole for the needle access is used to cover the sample on a titanium plate. It was expected, and later proven experimentally, that the cap significantly improves the inactivation efficacy due to the effective confinement and hence, longer lifetimes of the RRSs in a limited space. The adenovirus suspension (20 μl for each sample) is placed on the center of the titanium plate (diameter of 3 cm), and the distance between the needle tip and the plate is about 5 mm. The samples are divided into five groups and they are treated for 0, 2, 4, 8, and 16 min, respectively. Each group contains 3 samples.

Figure 2 shows the protocol of the titer testing after the plasma treatment. After the plasma exposure, the titanium plate is transferred into a sterile Petri dish with a diameter of 3.5 cm and washed by 200 μl high-glucose Dulbecco's modified eagle medium (DMEM, Gibco BRL, Grand Island, NY) for the titer testing. Then, the 200 μl adenovirus suspension is collected in a test tube for the next step. The 150 μl of the initial suspension is added into another 1350 μl DMEM to achieve the 10 times dilution. Similar procedures are carried out for the other dilutions as shown in Fig. 2. Afterwards, 100 μL suspension is sucked from each diluted sample and added to each well of a 96-well plate, which contains the HEK 293 A host cells. These cells feature the E1A and E1B regions of the adenoviral genome and represent an excellent packaging cell line for the generation of high-titer adenovirus. The host cells have been incubated for 24 h in a CO_2 incubator. The cells show good adhesion to the wells. For each diluted concentration (10^{-1}–10^{-9}), 10 samples are taken. Eighteen samples are used for blank control. After adding all the different concentration suspension to the host cells, the host cells are incubated in the CO_2 incubator for 10 days. Thereafter, the cell lesions of each cell wells are observed under a fluorescence microscope (Olympus, I × 500, TH4-200, U-RFL-T). If the adenovirus is active, the cells are expected to be infected. Since AdVs express green fluorescent proteins,[18] green fluorescence could be detected as long as the cells are infected. This indicator of the AdV infectivity was used in the titer calculations.

Figure 3 shows the fluorescence images of the infected cells (10^{-3} dilution of the initial virus concentration) incubated in the 96-well plate for different treatment time with the samples covered by the cap. Figures 3(a)–3(e) correspond to the treatment time of 0, 2, 4, 8, and 16 min, respectively. One can clearly see that the number of infected cells decreases with the plasma treatment time. In other words, the number of active AdV virions is dramatically reduced after the APP exposure. This effect was quantified using the median tissue culture infective dose (TCID50), which is defined as the amount of the pathogenic agent (AdV) that will produce pathological change in 50% of cell cultures inoculated and is calculated using the Karber method. The number of infectious viruses per ml (plaque forming units (PFU)/ml) was calculated as PFU/ml = 0.69 × TCID50/ml and expressed in PFU/ml, according to standard protocols.

The survival plots of the adenovirus after the plasma treatment are shown in Fig. 4. The titer decreases dramatically with the treatment time, which remarkably agrees with the observable changes in the fluorescence images. For the treatment time of 2 min, the adenovirus titer decreases in almost one order of magnitude without a cap and almost 2 orders of magnitude when the cap is used. In the latter case, more than 99% of the viruses are inactivated within 8 min, which shows the APP ability for the pre-emptive control of AdV infections. The difference in the inactivation efficacy between the two methods is due to the longer lifetime of the reactive oxygen species, which are restrained from diffusion into the surrounding air by the cap surfaces. This phenomenon is quite similar to the previous report on bacterial and fungal inactivation in APPs.[19]

To characterize the active species and the gas temperature in the discharge, optical emission spectra (OES) of the APPs were collected using a Princeton Instruments Acton SpectraHub 2500i spectroscope. In this way, one can accurately measure the vibrational and rotational temperatures of the plasma.[20] It is found out that the simulated spectra with a gas temperature of 300 K have a good fit to the measured spectra. Thus, thermal effect in the a denovirus inactivation appears to be rather insignificant. The measured optical emission spectra clearly show that excited O, OH, N_2, N_2^+, and He species are present in the plasma. O and OH species have previously been found particularly effective in inactivating microorganisms.[10] These species can enhance

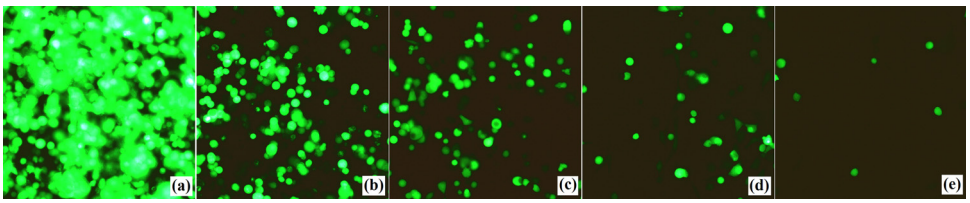

FIG. 3. (Color online) Fluorescence images of the adenovirus infected HEK 293A cells after the plasma needle treatment. Panels (a)-(e) show the fluorescence images from the cells infected with AdV after the plasma treatment of 0, 2, 4, 8, and 16 min, respectively. The images are taken on the 10th day after the treatment and correspond to the 10^{-3} dilution concentration of the infected cells.

permeability or even partially destroy the outer membranes of the microorganisms, which leads to the effective protein denaturation, lipid peroxidation, as well as major changes in the cell cytoplasm and nucleus. As mentioned above, the adenovirus consists of only a capsid shell and a double-strand DNA. The plasma-generated reactive species thus first damage the capsid, which mainly consists of proteins. After etching away the protein protective layer, the reactive species could directly act on the DNA of the adenovirus thereby destroying the AdV genetic material. This is consistent with the previous report on the APP effects on the plasmid DNA.[21]

The specific mechanism of the dramatic reduction of the AdV infectivity is related to either the much reduced ability of the damaged virion to permeate through the cell membrane or the effectiveness of the AdV DNA to interact with the DNA of the host HEK 293A cells, or both factors. The first effect mostly owes to the plasma-induced damage to the capsid. On the other hand, the second effect is most likely due to the structural damages (e.g., oxidation) to the AdV DNA during the plasma treatment. The extent of activity of these DNA molecules in the intracellular space may be quantified by antigen detection, immunochromatography or quantitative polymerase chain reaction (qPCR) analyses.[7] This analysis at the DNA level complemented with the analysis of the rates of AdV replication in the intracellular space is forthcoming and is expected to offer additional insights into the interaction of the APP-treated adenovirus nanoparticles with the host cells and the emerging adenoviremia pathology. Successful selective inactivation of virions in the intracellular space in turn would offer a major breakthrough in the presently elusive ability of post-infectious viral treatments.

In conclusion, successful adenovirus inactivation in cold atmospheric-pressure plasmas generated by a plasma needle device is demonstrated. The survival plots derived from the green fluorescence from the infected HEK 293A cells suggest that more than 90% of the AdV virions can be effectively inactivated during a short (~8 min) plasma exposure. Optical emission spectroscopy of the He + O_2 plasmas has been studied to examine the plausible roles of the reactive species and gas temperature in the pathogen inactivation. Subject to the discovery of the appropriate treatment protocols, APPs are expected to have quite similar effect on a range of other viral pathogens thereby significantly expanding the options and improving the therapeutic efficacy of pre-emptive anti-viral treatments.

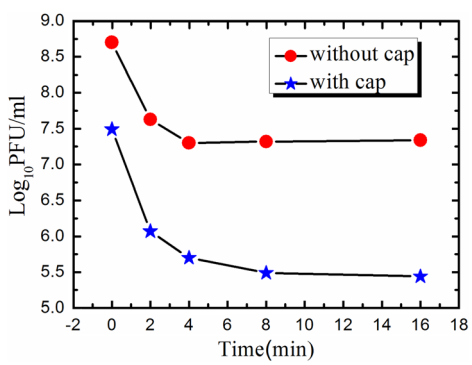

FIG. 4. (Color online) The survival plots ($Log_{10}PFU/ml$) for the two treatment techniques, without and with the cap, labeled by the filled circles and stars, respectively.

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