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Crucial roles of reactive chemical species in modification of respiratory syncytial virus by nitrogen gas plasma

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ABSTRACT

The exact mechanisms by which nanoparticles, especially those composed of soft materials, are modified by gas plasma remain unclear. Here, we used respiratory syncytial virus (RSV), which has a diameter of 80–350 nm, as a model system to identify important factors for gas plasma modification of nanoparticles composed of soft materials. Nitrogen gas plasma, generated by applying a short high-voltage pulse using a static induction (SI) thyristor power supply produced reactive chemical species (RCS) and caused virus inactivation. The plasma treatment altered the viral genomic RNA, while treatment with a relatively low concentration of hydrogen peroxide, which is a neutral chemical species among RCS, effectively inactivated the virus. Furthermore, a zero dimensional kinetic global model of the reaction scheme during gas plasma generation identified the production of various RCS, including neutral chemical species. Our findings suggest the nitrogen gas plasma generates RCS, including neutral species that damage the viral genomic RNA, leading to virus inactivation. Thus, RCS generated by gas plasma appears to be crucial for virus inactivation, suggesting this may constitute an important factor in terms of the efficient modification of nanoparticles composed of soft materials.

1. Introduction

Gas plasma, which is commonly referred to as the fourth state of matter, comprises >99% of matter in the universe [1]. Artificial generation of gas plasma is possible using various types of discharge including arc, corona, direct current, glow, high/low frequency, micro, pulse, and streamer. The practical application of gas plasma technology in industry is very broad and includes energy production, agriculture, medicine, dentistry, material processing, and environmental science [2–4].

Plasma based modification is particularly useful because it can deposit an ultra-thin coating to a depth of only a few nanometers, resulting in a change of biocompatibility such as adherence to cells. This modification can be achieved by several techniques including plasma sputtering and etching, plasma implantation, plasma deposition, plasma polymerization, laser plasma deposition and plasma spraying [5]. Plasma treatment can also induce changes to the morphology, structure and properties of polymers. For example, plasma treatment can facilitate coupling reactions between existing polymer chains, graft monomers onto polymers, immobilize proteins or antimicrobial drugs, and enhance cellular attachment [6,7] as well as mediate the immobilization of biopolymers, such as proteins, onto the surface of nanoparticles [8–10]. These techniques are a relatively simple means of producing highly effective bioactive surfaces with enormous potential utility.

Nanoparticles have recently been used in a broad range of applications [11–15]. To fully exploit these modified nanoparticles requires a thorough understanding of the mechanism of the changes brought about by the plasma treatment. However, although the usefulness of the plasma induced modification is now generally accepted, the exact mechanism of interaction between plasma and nanoparticles, especially in the case of soft materials composed of biomacromolecules such as DNA, RNA and proteins, remains unclear.

Respiratory syncytial virus (RSV) is a single-stranded enveloped RNA virus with a diameter of 80–350 nm [16]. The two major glycoproteins on the surface of the RSV virion, the fusion glycoprotein (F) and attachment glycoprotein (G), are involved in the initial phase of infection. RSV is clinically important because it causes severe lower respiratory tract infections in infants and young children [17] and is one of the most important pathogens in pediatric medicine. Interestingly, virus-like nanoparticles with a combination of RSV F and/or G [18–22] or DNA-chitosan nanoparticles encapsulating RSV genes [23] have been

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extensively studied as promising RSV vaccine candidates. These considerations prompted us to use RSV as a model nanoparticle composed of soft material.

We recently developed a nitrogen gas plasma instrument (BLP-TES) that delivers a short high-voltage pulse to nitrogen gas via a static induction (SI) thyristor power supply to generate gas plasma [24–26]. Employing this instrument, we aimed to reveal the mechanism of interaction between gas plasma and nanoparticles composed of soft material using RSV as a model system. Here, we focused on identifying the major factor responsible for modification of RSV by using a combination of biochemical and virological methods as well as simulation analysis.

2. Materials and methods

2.1. RSV

RSV Long strain [American Type Culture Collection (ATCC), VR-26; A subtype laboratory virus] was used in the present study. Human pharyngeal cancer cell line HEp-2 cells (ATCC, CCL-23) were cultured in minimal essential medium (MEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

2.2. Gas plasma

BLP-TES (NGK Insulators Ltd., Nagoya, Japan) was used as a nitrogen gas plasma device, which employs a short high-voltage pulse via a SI thyristor power supply [24–26]. A cathode electrode (earth electrode) was placed between the anode electrodes (high voltage electrodes). The cell culture medium containing RSV spotted onto glass coverslips and indicator strips were placed on the grid of the earth electrode. The operation procedure for nitrogen gas plasma production was as follows. Firstly, the chamber box containing the samples was decompressed and degassed, and then nitrogen gas (99.9995%; Okano, Okinawa, Japan) was introduced. The pressure in the box was maintained at about 0.5 atm during the electrical discharge at 1.5 kpps (kilo pulse per second). The nitrogen gas plasma treated and untreated RSV samples on the glass coverslips were then recovered for analysis.

2.3. Ultraviolet (UV) irradiation and heat treatment of RSV

Aliquots (20 μl) of cell culture medium containing RSV-infected HEp-2 cells were spotted onto glass coverslips and dried on a hot plate (AS ONE, HP-4530, Japan) at 35 °C. The dried samples were then subjected to heating on the hot plate at 35–60 °C for 5 min. The dried spots were also irradiated with long-wave UV radiation (UV-A) or short-wave UV radiation (UV-C) using a UV transilluminator UVGL-58 (UVP; Upland, CA) for 5 min. In all cases, the distance between the samples and UV transilluminator was 1.3 cm. Energy dose of UV was estimated on the basis of a colour change of UV label-H indicator (Nichiyu Giken Kogyo Co., Ltd., Tokyo, Japan). The treated samples were resuspended in 20 μl of phosphate buffered saline (PBS), which were then used for viral titration assays.

2.4. Hydrogen peroxide treatment of RSV

The minimum virucidal concentration of hydrogen peroxide (WAKO, Osaka, Japan) for RSV was determined as follows. Aliquots (20 μl) of cell culture medium containing RSV-infected HEp-2 cells were incubated with various concentrations of hydrogen peroxide at 37 °C for 5 min. The resultant samples were then subjected to a viral titration assay as described below.

2.5. Measurement of reactive chemical species (RCS)

A chemical indicator strip was used to determine the concentration of RCS produced during exposure to nitrogen gas plasma. Specifically, we used Quantofix Active oxygen test strips (Macherey-Nagel GmbH & Co. KG, Duren, Germany) to measure the concentration of RCS generated during operation of the nitrogen gas plasma instrument (BLP-TES). The test strips were placed on the earth electrodes prior to treatment with nitrogen gas plasma at 1.5 kpps for 0, 5 and 15 min. After nitrogen gas plasma plasma treatment, the strip was immediately dipped in distilled water and then scanned to generate an image. The change in the colour of the strip was converted to an RGB code and compared with a standard curve developed from the RGB code of a reference strip.

2.6. Immunochromatography

RSV antigens were detected using the Bionax NOW® RSV test (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. This immunochromatography kit specifically recognizes RSV F protein [27,28].

2.7. Viral titration assay

Plaque forming units (PFUs) per ml were determined by performing 10-fold serial dilutions of samples in 96-well plates containing 1×10^3 cells/well of HEp-2 cells. Cells were incubated for 24 h prior to infection. Infected cells were incubated for 5 days at 37 °C in a 5% CO_2 atmosphere. To determine the virus titer, wells containing plaques caused by the cytopathic effect of viral proliferation were counted. Viral titers were calculated from the value of the final dilution of wells containing individual plaques.

2.8. Viral RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR)

RSV RNA was extracted using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). Plasma-treated and untreated samples were extracted with the QIAamp Viral RNA mini kit and solubilized in lysis buffer. The extracted viral RNA was bound to a column and then eluted in 60 μl of nuclease-free water. Random primers were used for the RT-reactions. After incubation at 25 °C for 10 min, RNA was reverse-transcribed at 65 °C for 50 min, followed by denaturation of the enzyme at 85 °C for 5 min using a PrimeScriptII 1st strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan). The resultant cDNA was amplified in a reaction mixture containing primers, Ex Taq (Takara Bio Inc.), and Ex Taq buffer under conditions of 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. PCR was carried out using the following primers specific for the RSV F gene [27];

RSVfusion-F: 5′-TTAACCAGCAAAGTGTAAGA-3′,

RSVfusion-R: 5′-TTTGTTATAGGCATATCATTG -3′.

RT and PCR were performed on a PC320 instrument (ASTEC Co., Ltd., Fukuoka, Japan).

The amplified DNA products were analyzed by agarose gel electrophoresis using a 1.5% gel. The 243-bp product was confirmed to

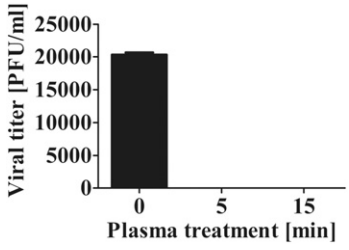

Fig. 1. Viral titration assays demonstrate that nitrogen gas plasma treatment of respiratory syncytial virus (RSV) results in a decrease in viral titer. RSV was treated with nitrogen gas plasma using BLP-TES for the indicated time and subjected to a viral titration assay as described in Materials and methods. The number of plaque forming units (PFU) per ml of RSV treated with nitrogen gas plasma at 1.5 kpps for 0, 5 or 15 min ($N = 3$) was then determined. No plaques were detected after treatment for 5 and 15 min.