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Effects of cold atmospheric plasmas on adenoviruses in solution

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Abstract
Experiments were performed with cold atmospheric plasma (CAP) to inactivate adenovirus, a non-enveloped double stranded DNA virus, in solution. The plasma source used was a surface micro-discharge technology operating in air. Various plasma diagnostic measurements and tests were performed in order to determine the efficacy of CAPs and to understand the inactivation mechanism(s). Different stages of the adenovirus 'life cycle' were investigated—infectivity and gene expression as well as viral replication and spread. Within 240 s of CAP treatment, inactivation of up to 6 decimal log levels can be achieved.

(Some figures may appear in colour only in the online journal)

1. Introduction

Cold atmospheric plasmas (CAPs) have received a lot of attention recently for disinfection and sterilization [1–9], for wound care [10–12] and skin diseases [13]. The surprisingly high efficacy and skin compatibility [2, 14] have raised hopes that CAPs can become an effective tool in hospital hygiene, in particular with respect to resistant bacterial strains such as MRSA and others. Figure 1 illustrates the efficacy of CAPs in deactivating MRSA bacteria. The plasma technology for this experiment is the same as the one used in the current virus study. A further hope is that CAPs may even be useful to contain epidemics (e.g. the recent outbreak of EHEC O104 in Germany, [15]).

A different challenge for hospital hygiene is the inactivation of viruses. Common to all viruses is their dependence on a host organism for replication and spreading. They possess nucleic acids as genetic information, but do not have their own metabolism. The nucleic acid can either be RNA or DNA, which is generally protected and compacted by a protein shell, the capsid. In close co-evolution with their hosts, viruses have developed specific entry mechanisms to deliver their nucleic acids, e.g. to the nucleus for replication. Viruses have evolved mechanisms that manipulate the host cell metabolism in such a way that the infected cell dedicates all necessary resources to virus production. Whereas some viruses can persist for a long time in infected cells and are set free continuously without killing the host cell, others will cause rapid exhaustion of the metabolism of the host cell, eventually leading to death of the infected cell and subsequent virus release.

Among viruses affecting mammals, in particular humans, are types causing different diseases ranging from life threatening (e.g. SARS) to relatively mild diseases (adenovirus) or even symptom-less infections (adeno-associate virus). Many viruses have been well studied and some have been harnessed and can thus be used as molecular tools to unravel biological phenomena, and some are even used in therapeutic approaches of human diseases, e.g. in clinical trials for cancer treatment.

Adenoviruses are non-enveloped double stranded DNA viruses (figures 2 and 3(*a*)) usually causing mild diseases, ranging from respiratory tract to gastrointestinal infections, depending on the serotype. Adenoviruses lyse (destroy) their host cell and are concomitantly set free. Due to the protein capsid, adenoviruses are physically stable, can tolerate moderate increases in heat, deviations from neutral pH and are relatively resistant to UVC. For disinfection, either autoclaving (20 min at 121 °C at 1 bar) or chlorine bleach is used. The latter is prohibited in Germany due to

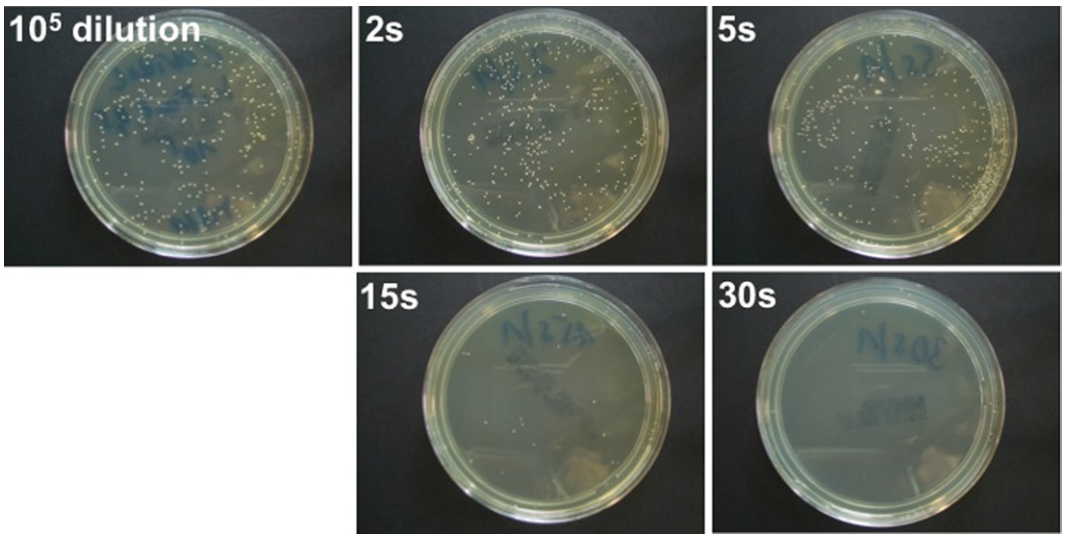

Figure 1. MRSA colony forming units after plasma treatment (on agar after 18 h of incubation). Each colony (bright spots) grew from a single bacterium, which survived the plasma treatment. Initial bacterial load on the plate was $\sim 10^7$. Plasma treatment times are indicated (2 s to 30 s). For comparison a log 5 dilution of untreated bacteria is shown (top left panel).

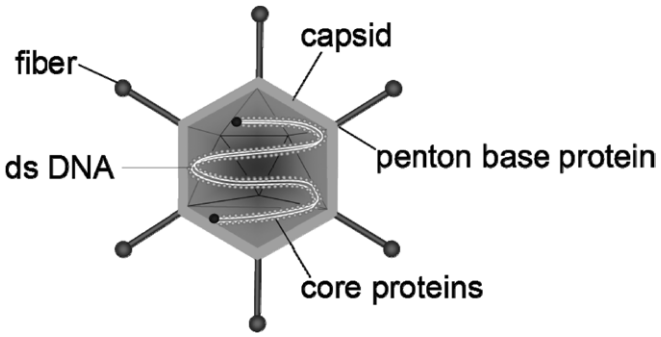

Figure 2. Schematic representation of an adenovirus. The protein capsid harbours the double stranded (ds) linear DNA genome, tightly packaged by core proteins. Fibres and penton base proteins are capsid structures necessary for infection of target cells.

environmental considerations, leaving autoclaving as the sole alternative for liquids. Accepted surface disinfectants contain, e.g., aldehydes like glutaraldehyde, and glyoxal as well as didecyldimethylammoniumchloride, or glucoprotamine, since adenoviruses cannot be efficiently inactivated by the use of 70% ethanol.

Due to their relative physical stability human adenoviruses were chosen as the model system for the plasma investigations. For initial proof-of-principle experiments a recombinant human adenovirus type 5 was used that allows the measurement of virus inactivation on the basis of infectivity as well as on the basis of gene expression of foreign genes. This recombinant adenovirus has the following main features: for safety considerations it is rendered replication defective by deletion of essential viral genes (E1 region, figure 3(*b*)). This defective virus can still infect different human as well as non-human cells, but will not replicate therein. For amplification, producer cell lines expressing the deleted viral E1 genes have to be used [16, 17]. Since these cell lines allow completion of the viral reproductive cycle they are used for determination of viral titers and virus-mediated cell killing (figure 3(*c*)). Additionally these recombinant viruses carry so-called 'reporter genes' that allow for expression of reporter proteins which can be traced by fluorescence or bioluminescence imaging of infected cells that do not support adenoviral replication (figure 3(*c*)). Reporter gene assays for quantification of reporter gene expression will only be possible if the infectivity of the virus is not impaired and the viral DNA is not damaged. Thus recombinant adenoviral vectors expressing reporter genes represent an alternative way of determination and quantification of virus inactivation in terms of infectivity/gene expression and titer.

2. Experimental setup and methods

2.1. Plasma dispenser

In this study, a cold atmosphere pressure plasma device (FlatPlaSter) similar to the HandPlaSter published by Morfill *et al* [18] was used under ambient conditions (temperature $\sim 20\,^{\circ}$C, relative humidity $\sim$40%). This device consists of one surface micro-discharge (SMD) plasma electrode ($10 \times 10\,\mathrm{cm}^2$) using an insulator plate made of Al_2O_3 (thickness 1 mm) sandwiched by an aluminium plate (10 mm thick) and an electrically grounded mesh grid (stainless steel, 6 mesh inch^{-1}) (figure 4). The wire of the mesh grid has a diameter of 0.5 mm. The plasma is produced homogenously on the mesh grid side by applying high sinusoidal ac voltage of 4.7 $\mathrm{kV_{pp}}$ at 10 kHz between the metal plate and the mesh grid. The power consumption of this electrode is 0.5 W cm^{-2} and was evaluated by the Lissajous figure method using a 1 μF capacitor.

2.2. Plasma properties

The UV emission spectrum, produced by the plasma device was measured with the AvaSpec-2048 Fibre Optic Spectrometer (Avantes, USA) at a distance of 12 mm (figure 5). The acquisition time equalled 2 s. The small peaks in the UVC region of the spectrum refer to NO, the other peaks in the UVA

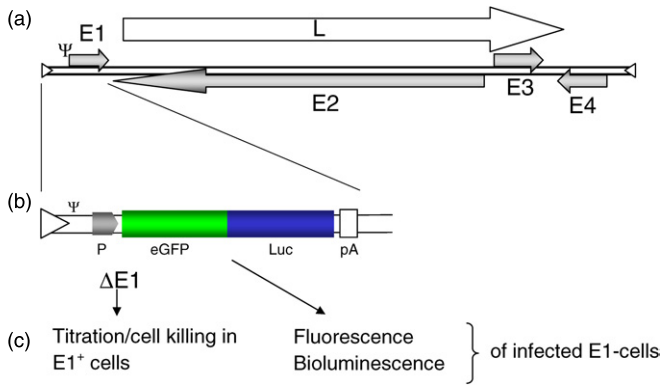

Figure 3. Schematic illustration of the genomic structure of adenoviruses. (*a*) Wild type genome. E1-4: early genes (E1: genes encoding for proteins that are functional in activation of gene expression of other early genes, manipulation of the host metabolism and anti-apoptotic function; E2: coding for proteins involved in viral DNA replication; E3: coding for proteins involved in modulation of immune response of host; E4: coding for proteins involved in the modulation of the host response to virus infection). L: late genes, coding for structural proteins; Ψ: packaging signal for encapsidation of DNA into the capsid; triangles: inverted terminal repeats are DNA sequences important for viral DNA replication; arrows indicate the direction of transcription. (*b*) ΔE1: deletion of the E1 sequence, which is replaced by an expression cassette for the eGFP-Luciferase fusion protein P: promoter (signal for start of transcription, which is to say gene expression), pA: polyadenylation signal (Signal for end of transcription); eGFP: enhanced green fluorescence protein gene; Luc: firefly luciferase gene. (*c*) Readout methods. Deletion of the E1 region leads to a replication deficiency of the virus. Replication and subsequent cell killing can only take place in $E1^+$ cells. Substitution of the E1 sequence in the recombinant virus by the cassette described in B leads to the expression of the eGFP-Luciferase fusion protein in infected E1-negative cells. The two halves of the fusion protein are functional and can be detected and quantified independently: after excitation with 490 nm light eGFP protein leads to the emission of green fluorescence (509 nm) in expressing cells. In the presence of O_2, cofactors and ATP, the luciferase enzyme leads to the conversion of luciferin to oxi-luciferin under the emission of light (bioluminescence).

region are from nitrogen molecules. In addition, the UV power density in the 170–340 nm wavelength range was measured by the HAMAMATSU UV-Power Meter C8026 (HAMAMATSU Photonics K.K., Japan). The measured power density at 12 mm was 1.8 μW cm^{-2} and the effective light power integrated with the ICNIRP weighting function was 0.43 μW cm^{-2}, almost two orders of magnitude below the WHO allowed limit of 30 μW cm^{-2}.

The non-equilibrium air chemistry initiated by the micro-discharge plasma has the dissociation of O_2 and N_2 molecules into atomic oxygen and nitrogen as a basic initial reaction. Consequently, from the reaction $O_2 + O + M \rightarrow O_3 + M$, ozone is a necessary by-product. The ozone production by the SMD electrode was measured using a photometric ozone analyser (TELEDYNE Advanced Instrumentation). With our experimental setup, the ozone concentration was measured around 1–2 ppm at a distance of 1 cm from the electrode when no plate was placed below the electrode (open volume), with a 96-well plate placed directly beneath the electrode, a maximum of 10–20 ppm of O_3 was measured (closed volume).

2.3. Cell cultures

For the experiments two different cell lines—HEK293 and CMS-5—were used. HEK293 cells (Microbix (Toronto, Canada)) carry the E1 region of the adenovirus integrated into their cellular genome and thus support replication of E1-deleted as well as wild type viruses (carrying the E1 region, e.g. the complete genome) [17]. The mouse fibrosarcoma cell line CMS-5 [19] can easily be infected with adenoviruses, but does not allow the replication of recombinant viruses, due to the lack of the E1 region in the genome. Both cell lines were cultured in DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (SERA plus, PAN Biotech GmbH, Aidenbach, Germany) and 1% L-glutamine (Biochrom AG, Berlin, Germany) at 37 °C, 5% CO_2 and 95% humidity.

For cultivation both cell lines were washed with phosphate buffered saline (PBS) (Biochrom) and detached with trypsin 0.25%/EDTA 0.02% (Biochrom AG, Berlin, Germany). Trypsin was inactivated by adding the complete medium and subsequently the samples were distributed to new tissue culture vessels and incubated as described above.

Twenty-four hours before infection with adenoviruses the cells were seeded in 24-well plates (TPP, Trasadingen, Switzerland) at a density of 5×10^5 cells per well in culture medium and cultivated as described above.

2.4. Adenoviruses

A recombinant adenovirus (AdeGFPLuc) based on the replication defective human type 5 adenovirus was constructed to express a fusion protein consisting of the enhanced green fluorescent protein (eGFP) and the firefly luciferase (Luc) [20]. The expression of the fusion protein is regulated by the murine cytomegalovirus immediate early promoter (mCMV) and a downstream simian virus 40 polyadenylation signal (SV40pA). Gene expression mediated by the adenovirus can be detected in infected cells. The two halves (eGFP and Luc) of the fusion protein are functional and can be detected and quantified independently: (1) after exciting with light at a wavelength of 490 nm the eGFP protein emits green fluorescence (509 nm) in infected expressing cells. (2) In the presence of O_2, cofactors and ATP, the firefly luciferase enzyme converts luciferin to oxi-luciferin under the emission of light (bioluminescence). The recombinant virus was plaque purified and amplified on HEK 293 cells and further purified by double caesium chloride ultracentrifugation according to standard protocols [21]. Adenovirus stocks were titered using the end-point-dilution assay (see below).

2.5. Treatment of Adenoviruses

AdeGFPluc (stock concentration 2×10^9 *plaque forming units* (pfu) per ml) was diluted in PBS solution to obtain concentrations of 2.5×10^7, 5×10^7 and 2.5×10^8 pfu ml^{-1}. AdeGFPluc dilutions—equivalent to virus doses of 1, 5 and 10 *multiplicities of infection* (MOI) per cell—were added to 96-well plates in triplicates in volumes of 20 μl. Plates were stored at 4 °C until further use.

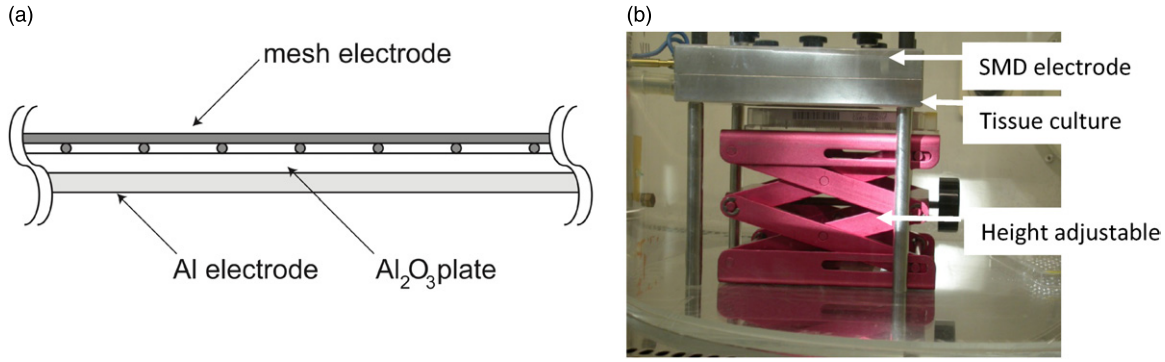

Figure 4. (*a*) Cross-sectional view of one SMD electrode. (*b*) Experimental setup for treating AdeGFPluc in solution with the FlatPlaSter. The 96-well tissue culture plate is placed underneath the SMD electrode with the help of a height adjustable stand.

For the plasma treatment, the 96-well plates containing the AdeGFPluc solution were placed in direct contact underneath the flat $10 \times 10\,\mathrm{cm}^2$ SMD electrode of the FlatPlaSter (figure 4(*b*)). Consequently the surface of the AdeGFPluc solution located at the bottom of the wells was approximately 12 mm away from the electrode. The AdeGFPluc solutions were exposed to the plasma (voltage = 4.66 kV; frequency = 10 kHz; power = $0.5\,\mathrm{W\,cm}^{-2}$) for 240 s. After the plasma treatment the 96-well plates were incubated for 1 h at room temperature to allow for diffusion of reactive species into the solution. The plates were stored on ice until time of infection or titration. The control plates were prepared using the same master mix of diluted adenovirus. They were handled as described above, except for the plasma treatment, during which time (240 s) they were stored on ice.

3. Analysis procedure

3.1. Infection of CMS-5 and HEK293 cells

CMS-5 cells and HEK293 cells (5×10^5 cells per well in a 24-well plate) were infected with plasma-treated and untreated virus (control) at a multiplicity of infection (MOI) ranging from 1 to 20 plaque forming units (pfu) per cell. For infection, the cell culture medium was aspirated and replaced by the adenovirus solution. Infection was allowed to proceed for 30 min at 37 °C in a humidified incubator, after which time fresh cell culture medium was added. CMS-5 cells were incubated for 24 h at 37 °C and 5% CO_2 followed by the reporter gene analysis of eGFP and luciferase. HEK 293 cells were incubated for 48 h under the same conditions for sulforhodamine B staining or were used in end-point dilution assays.

3.2. Bioluminescence measurement

A luciferase assay was performed for quantification of firefly luciferase expressed in AdeGFPLuc-infected CMS-5 cells. Twenty-four hours after infection, the CMS-5 cells were washed once with PBS and then incubated in 500 μl lysis buffer (0.1% Triton X-100 in 250mM Tris/HCl pH 7.8). 50 μl of cell lysate was transferred to wells of a black 96-well plate (Costar, Corning Life Sciences, Amsterdam, The Netherlands), mixed with 100 μl of luciferin buffer (60mM dithiothreitol, 10mM magnesium sulfate, 1mM ATP, 30μM D(-)-luciferin, in 25mM glycyl-glycine, pH 7,8) and assayed for bioluminescence using a TopCount instrument (Canberra Packard, Groningen, The Netherlands). The protein content of the cell lysates was determined using the Bio-Rad protein assay dye (Bio-Rad Laboratories, Munich, Germany) adapted for use in a 96-well plate format. Specific luciferase activity in picograms luciferase per milligram of total protein was calculated from a calibration curve, which was obtained from the luminescence of a serial dilution of purified recombinant firefly luciferase (Roche, Penzberg, Germany).

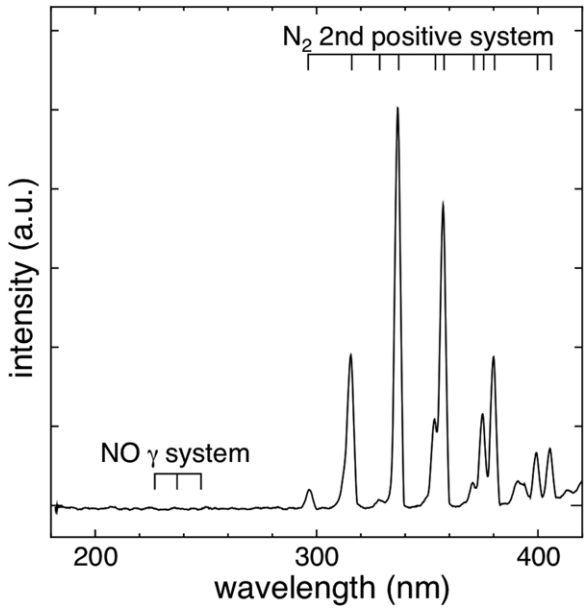

Figure 5. UV emission spectrum produced by the plasma of the FlatPlaSter, measured at a distance of 12 mm. The acquisition time equalled 2 s. The peaks between 290 and 400 nm result from the N_2 C–B (second positive system). The emission in the UVC range is from the NO γ system.

3.3. Fluorescence measurement

eGFP fluorescence of AdeGFPLuc-infected CMS-5 cells was quantified by flow cytometry. Twenty-four hours

post infection, CMS-5 cells were washed once with PBS, trypsinized and centrifuged for 3 min at 300 × *g* (Heraeus, Germany). The cell pellets were washed once with PBS and once with buffer (PBS, 1% FCS). After washing, each sample was fixed with 250 μl Cytofix fixation buffer (Becton Dickinson, Heidelberg, Germany) and incubated for 15 min on ice in the dark. Cell samples were then washed twice with buffer and finally each cell pellet was resuspended in 500 μl buffer for analysis by flow cytometry (FACS Vantage Becton Dickinson, Heidelberg, Germany). The percentage of eGFP-positive cells and the mean fluorescence intensity were determined using the CellQuest pro software (Becton Dickinson, Heidelberg, Germany).

3.4. Sulforhodamine B staining

Adenovirus inactivation was indirectly measured by determining the survival of AdeGFPLuc-infected HEK 293 cells that allow for replication of E1-deleted adenoviral vectors using sulforhodamine B staining (SRB). The attached cells appear dark red. The determination of surviving cell numbers is based on the measurement of cellular protein content [22]. Forty-eight hours after infection of HEK 293 cells with AdeGFPluc the cells were washed once with 500 μl ice cold PBS and then fixed with 300 μl 10% trichloroacid (wt/vol) overnight at 4 °C. The next day the samples were washed four times with deionized water and then stained with 200 μl 0.5% sulforhodamine B (Sigma, Taufkirchen, Germany) for 10 min at room temperature. Afterwards the cells were washed four times with 1% acetic acid and dried completely at room temperature. The attached cells will appear in dark red. For quantification 100 μl of 10mM basic Tris solution was added to each well. Dye was dissolved by shaking for 1 hour at room temperature. Subsequently dye absorption was determined spectrophotometrically at 590 nm.

3.5. Adenovirus titration by end-point-dilution assay

The infectious titer was analysed in an end-point dilution assay on HEK 293 cells and compared with titers of untreated virus in order to determine the overall reduction between plasma-treated and untreated adenovirus. Twenty-four hours before treatment of the virus, HEK 293 cells were seeded in a 96-well plate (2000 cells per well) and cultured overnight in 100 μl DMEM (10% FCS; 1% L-glutamine). The next day, 1 : 10 serial dilutions in DMEM (1%L-glutamine) of the treated and untreated virus followed. For untreated virus the dilutions 10^{-4} up to 10^{-11} were used, whereas the treated virus was diluted in a ratio 1 : 3 up to $1 : 3 \times 10^7$. 100 μl of each dilution was added to wells in columns 1 to 10. Wells 11 and 12 were non-infected controls. The plate was incubated in a humidified CO_2 (5%) incubator at 37 °C. After 10 days the wells were checked for cytopathic effect (CPE) using a light-microscope (Zeiss, Jena, Germany). The viral titer (pfu ml^{-1}) was calculated according to the following formula: Titer (pfu ml^{-1}) = $10^{(x+0.8)}$ where x is the sum of the fractions of CPE-positive (lysed) wells.

3.6. Measurement of pH

In accordance to the treatment of adenoviruses in solution, PBS (pH 7.21) was exposed to plasma (voltage = 4.6 kV, frequency = 10 kHz, power = 0.5 W cm^{-2}) to evaluate the change in pH after a treatment time of 240 s. After the plasma treatment the pH of PBS was reduced to 6.6, which corresponds to a reduction of 0.6.

In general a drop in pH to a value less than 6 is considered to induce capsid disassembly. However, Rexroad *et al* described an increase in capsid stability with decreasing pH in two Ad5 formulations containing high or low NaCl concentration [23]. A decrease in pH to as low as 3 did not trigger capsid disassembly in Ad5 at low temperatures, although the aggregation of particles was increased.

3.7. Measurement of temperature

After a plasma treatment time of 240 s an increase in the gas temperature of 0.8 °C above ambient was measured using a thermocouple. This temperature increase does not have any virucidal effect. For instance, adenovirus type 5 loses significant activity only upon heating above 50 °C [24].

3.8. Measurements with bacteria in solution

CAP treatment of bacteria on agar results in very fast and efficient inactivation (see figure 1). In order to compare bacteria data with the effect of CAP plasmas on adenoviruses, which have to be kept in solution, experiments with bacteria in solution were also performed. The procedure was to plasma treat the *Escherichia coli* bacteria in solution (as for the virus experiments) and then spread them on agar for incubation and quantitative measurements of the CFUs. By comparing the number of CFUs with the number of CFUs obtained from controls, we determined that the bacterial reduction in PBS was comparable to the virus data shown here [25].

4. Results

Adenoviral vectors were treated in small volumes of solutions with CAP in order to determine the achievable inactivation rates. Recombinant adenoviral vectors were used which can differentiate between different stages of the adenovirus 'life cycle': infectivity and gene expression as well as replication. A recombinant adenovirus expressing eGFP fused to firefly luciferase was treated with CAP or mock treated (control) prior to infection of cells. By infection of CMS-5 cells, gene expression, which is a pre-requisite for infectivity, can be monitored and quantified. The treatment of viruses with CAP leads to a reduction in eGFP expression in infected cells (figure 6(*c*)).

For the control, 74.1 ± 2.0% AdeGFPLuc-infected cells were positive with green fluorescence at MOI 1, increasing to 93.3 ± 0.7% eGFP-positive cells at MOI of 10 and 20 (figure 6(*a*)). In contrast, when AdeGFPLuc was plasma-treated for 240 s at MOI 1, 4.0±0.7% cells were eGFP positive and at MOI 10 and 20 only 0.04 ± 0.01% cells were positive for the reporter gene (figure 6(*a*)). Likewise, after infection of

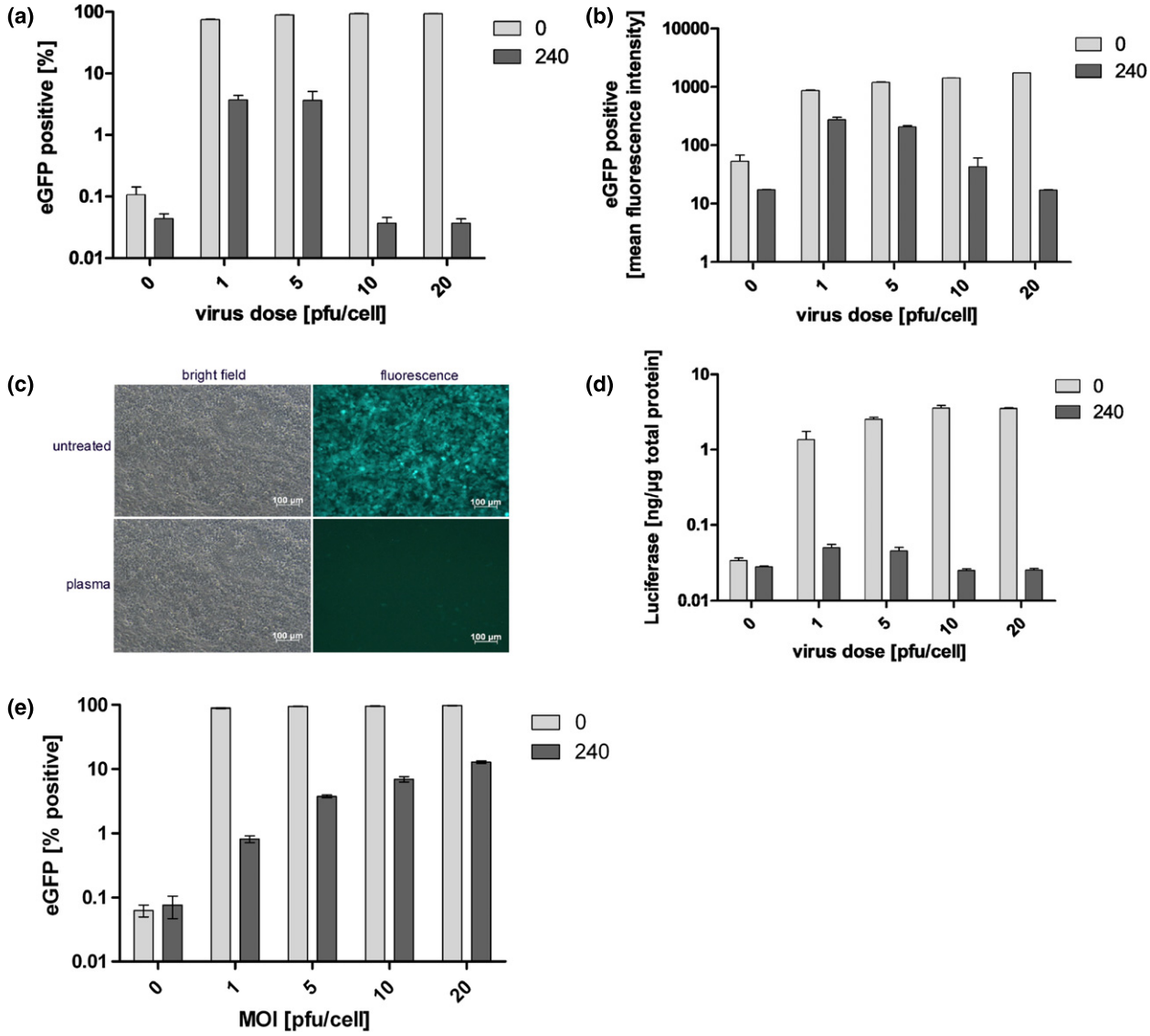

Figure 6. Inactivation of adenoviral vectors expressing eGFP and luciferase fusion protein. Diluted samples of AdeGFPLuc were treated with CAP (240 s) or control treated (0 s) prior to infection of CMS-5 cells. (*a*) Autofluorescence of expressed eGFP (in %) was inactivated by the treatment with CAP for up to three orders of magnitude with respect to the control cells. (*b*) Reduction up to two orders of magnitude regarding the mean fluorescent intensity within the whole population. (*c*) eGFP expression can be monitored on-line using a fluorescence microscope. Representative fluorescence images and corresponding fields of view in bright field, demonstrating the virus infected (fluorescent) cells in the untreated sample (top right) and the presence of non-infected cells in the plasma-treated sample (bottom right) are shown. These images refer to infection of CMS-5 cells with AdeGFPLuc at a MOI of 10. (*d*) Determination of luciferase activity indicated an up to two orders of magnitude reduction in infectivity or gene expression of AdeGFPLuc recombinant virus by plasma treatment. (*e*) Inactivation of AdeGFPLuc as measured by eGFP-positive CMS-5 cells. In this experiment the virus was inactivated in solution at high concentration (MOI 20) by cold plasma treatment (240) and subsequently diluted.

CMS-5 cells with AdeGFPLuc, the eGFP mean fluorescence intensity is decreased by a factor of 3.2 at MOI 1 and by a factor of 103 at MOI 20 (figure 6(*b*)). Similarly, the firefly luciferase activity was reduced in infected CMS-5 cells after treatment with CAP: reduction rates ranged from 26.8-fold at MOI 1 to 141.6-fold at MOI 20, i.e. they were again virus dose dependent (figure 6(*d*)).

When adenoviral vectors were inactivated in solution at high concentration (MOI 20) by CAP treatment and diluted immediately prior to infection of cells (CMS-5) a linear relation (figure 6(*e*)) rather than a bell shaped curve between virus dose and gene expression was detected, also indicating that the likelihood of virus inactivation is higher at higher concentrations.

We also analysed the virus replication after treatment with CAP. A SRB staining assay was performed on HEK 293 cells, which support replication of E1-deleted adenoviral vectors. The SRB assay allows the determination of surviving cells—cells that have not been lysed by the adenovirus. After the pretreatment of adenovirus with CAP, cells were completely protected, indicating that under the tested conditions virus replication associated cell lysis is completely abolished (minimum 97.6 ± 1.6 surviving cells at MOI 10), whereas control treated AdeGFPLuc resulted in a dose-dependent

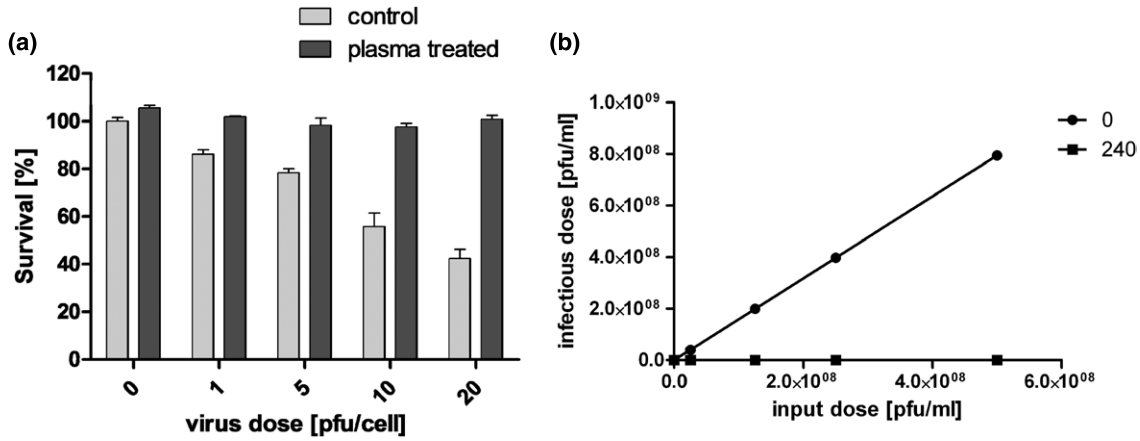

Figure 7. Adenovirus replication can be effectively impaired by CAP treatment. Shown are the results from AdeGFPLuc dilutions in buffer, which were treated with cold plasma compared with the control, which was treated prior to infection of HEK 293 cells that allow for adenoviral replication and thus cell lysis. (*a*) SRB staining was performed to determine cell survival. Control treated vectors lead to virus dose-dependent killing of cells. Pretreatment with cold plasma restored ∼100% survival of cells indicating that virus replication was effectively inhibited. (*b*) Determination of infectious virus titer by end-point-dilution assay indicated an up to six orders of magnitude reduction in adenovirus by treatment with CAP (results are representative for two independent experiments).

reduction of survival of up to 60% at MOI 20 (figure 7(*a*)). When analysing the infectious titer in an end-point dilution assay on HEK 293 cells we almost achieved complete inactivation of adenovirus ranging from 7.5×10^2 pfu ml^{-1} at MOI 1 to 30 pfu ml^{-1} at MOI 10 (figure 7(*b*)). Titers of untreated virus were determined to be 4.0×10^6 pfu ml^{-1} at MOI 1 and 6.3×10^7 pfu ml^{-1} at MOI 20. Thus a reduction of about six orders of magnitude of infectious adenovirus was achieved.

5. Discussion

For the analysis of inactivation of viruses by CAP we chose adenoviruses as a surrogate virus. Due to their protein capsid, adenoviruses are among the most resistant viruses to inactivation. This is especially true for inactivation by UVC, changes in pH and moderate heat. For instance, for UV C alone, Walker and Ko [26] and Nwachuku *et al* [27] found an inactivation of 60–70% of Ad2 viruses with a dose of 2.6 mW s cm^{-2}. For four log inactivation Nwachuku *et al* estimated a dose of 137.9–217.1 mW s cm^{-2}, depending on the used serotype. By comparison, the plasma device used here has a UV emission mainly in the UVA and UVB region. The emission in the wavelength range 180–400 nm (UV C) is 1.8 μW cm^{-2} resulting in an integrated dose of only 0.43 mW s cm^{-2} for a 240 s plasma exposure time.

With the used plasma device, we showed that the viral inactivation was greater than three log (after 240 s), demonstrating that CAP is much more effective than UV alone.

More recently the potential of inactivation of different viruses, among them adenovirus in solution, by ozone (O_3) was determined [28]. Amongst others (as stated above) CAPs also contain ozone. The plasma device used for the viral inactivation experiments in this study generates at maximum a concentration of a few tens of ppm (in a closed volume; see section 2). Murray *et al* [28] reported that a mixed gas of O_2 and ozone with a ozone concentration of 1200 ppm led to a five log reduction of adenovirus after 60 min of treatment. From the viewpoint of ozone, the concentration mentioned in [28] is a factor 100 higher than in our experiments. In addition Murray *et al* used a more than ten fold longer treatment time.

This implies that the inactivation of adenovirus by our plasma device cannot be due to ozone production alone. This suggests a synergetic effect of all possible species produced in the non-equilibrium plasma chemistry–electrons, charged and uncharged particles, excited atoms and molecules, reactive species and UV light (there are some 600 chemical reactions in air, involving oxygen, nitrogen and water vapour [29–32]).

At this point it is worth mentioning that some of these produced species are identical to the reactive agents, which our own immune system produces [30, 33]—OH, NO, O_2^*, H_2O_2, NO_2, NO_3, etc—and may therefore help to inactivate viruses as well. Redox molecules such as NO and ROS are key mediators in the immune system and are produced endogenously: They are not only involved in pathogen and tumour eradication, but also in the modulation of immune responses such as inflammation and immunosuppression during wound healing and tissue-regeneration [31, 34]. NO with its intermediates (·NO radicals, NO^-, NO^+) and adducts (NO_2, NO_2^-, NO_3^-, N_2O_3, N_2O_4, $ONOO^-$, etc) and ROS (O_2^-, H_2O_2, etc) have diverse functions in the immune system—ranging from antimicrobial, antiviral, immuno modulatory (stimulatory or suppressive), cytotoxic to cytoprotective [32]. NO is endogenously produced by different isoforms of nitric oxide synthase (NOS) and ROS by NADPH oxidase (NOX) e.g. in macrophages and natural killer cells that are key cells in the innate immune system—the rapid, not pathogen-specific defence system of mammals. The direct antimicrobial activity of NO is thought to be via DNA mutation, inhibition of DNA repair and synthesis, inhibition of protein synthesis, modulation of protein activity and membrane lipid peroxidation [35]. Additionally, they initiate and modulate signalling pathways that allow for full expression of adaptive immune response.

For a variety of different RNA and DNA viruses, inhibition of viral replication has been demonstrated [36]. Recently Cao *et al* [37] were able to demonstrate that NO indeed inhibits adenoviral replication via E1A protein. Although the precise mechanism of the direct inactivation of adenovirus has not been elucidated, other viruses are eradicated by NO-mediated nitrosilation of cysteine residues critical for viral infection, transcription, integration or maturation [34].

In our inactivation experiments with CAP we first compared plasma-treated adenovirus coding for the fusion protein of eGFP and firefly luciferase with control treated virus with respect to infectivity and gene expression by infecting a cell line (CMS-5) that does not support viral replication. We observed a two to three log inactivation (compared with the controls) after treatment of the vectors in solution for 4 min. When adenovirus was pre-diluted prior to the treatment with CAP a less pronounced effect of viral inactivation was achieved at lower virus doses. These results might be interpreted by an increased likelihood of adenovirus particles encountering reactive N/O species in solution, when present at higher virus doses, although other mechanisms cannot be ruled out. Likewise the inactivation of the recombinant adenovirus was observed, when cells that support the adenoviral replication (HEK 293) were infected with virus treated with CAP: as already reported at the highest virus dose tested all cells survived (100%), whereas the untreated controls resulted in 60% cell killing after 48 h. Furthermore the reduction in infectious titer was analysed using an end-point dilution assay yielding an up to six orders of magnitude inactivation of virus.

These data indicate that both the infectivity of the used adenovirus as well as the replication are very effectively inhibited by CAP.

In order to gain insights into the mechanisms of plasma inactivation, suspension experiments with *E. coli* bacteria were performed. 'Dry' bacteria on agar can be inactivated with CAPs in approximately 10 s to log 4–5 (see, e.g., figure 1). By treating a bacteria suspension with 240 s with CAP a reduction comparable to the viral inactivation rates was achieved which was less efficient when compared with the bacteria experiments on agar [38]. A full discussion on this is given in a forthcoming publication by Boxhammer *et al*. The explanation for this probably resides in three processes,

(1) In air the reactive molecules move faster, hence 'impacts' are more frequent and particle fluxes are higher than in solution (for the same concentration).
(2) The air chemistry is different, more short-lived and excited species may be able to interact with the specimen.
(3) Electromechanical and localized thermal effects (e.g. through molecular de-excitation or surface recombination) in air may lead to more efficient and frequent 'breaching' of the bacteria membrane or the viral protein capsid.

In solution, we have seen that the used plasma device produces only minor increases in temperature, and a minor drop in pH. Also, the UVC production is quite low, so that we may safely assume that the observed adenovirus inactivation is most likely due to reactive nitrogen and oxygen species produced by CAP in the fluid. These species can act on the one hand on the protein level leading to protein peroxidation and destruction of the capsid or they can damage DNA leading to reduced gene expression and elimination of virus DNA replication or both.

The inactivation of viruses by CAP using bacteriophages such as lambda or Phi X174 as surrogate for viruses have been examined and published by a number of authors [39–41]. For the inactivation of bacteriophage lambda by CAP Yasuda *et al* [40] suggest a mechanism that mainly affects the viral coat protein and to a lesser extent causes damage of the bacteriophage DNA. They also show experimental evidence that only at long CAP exposures is degradation of proteins (or DNA) detectable. However, GFP and lambda coated protein can be inactivated quickly, suggesting chemical modification such as oxidation or reduction [40].

Since adenoviruses consist of DNA, packaged tightly in the capsid—a protein coat—the main mechanism of inactivation by CAP might be similar and on the level of the capsid proteins. The fact that the production of functional eGFP and luciferase after infection of cells with plasma-treated adenovirus is still possible, although to a lesser extent might indicate that the destruction of DNA is not the major mode of virus inactivation.

However, more research to substantiate this hypothesis is required.

6. Conclusions

Adenovirus in solution was exposed to cold atmospheric plasma (CAP) from a remote (not embedded) source. The plasma was generated by micro-discharges in air and produced many chemical products, of which significant fractions are identical to the ROS/RNS species produced by our own immune system in response to microbial attack. These species may dissolve in the embedding fluid of the viruses and inactivate the adenoviruses. Tests were conducted to determine the inactivation efficiency as well as the inhibition of replication due to the plasma treatment. Results are that a plasma treatment time of 240 s is sufficient to inhibit replication and (using an end-point-dilution assay) that an up to six log reduction in adenovirus is achieved. This is not due to changes in the pH value of the medium, nor is it due to heating. The conclusion is that the dissolved ROS/RNS are likely to be responsible, making CAP a potentially very effective agent for control of viral infections.

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