# The Application of Cold Atmospheric Gas Plasma (CAP) to Inactivate *Acanthamoeba* and other Ocular Pathogens

WAYNE HEASELGRAVE [1*], GILBERT SHAMA [2], PETER W. ANDREW [3], and MICHAEL G. KONG [4*]

[1] University of Wolverhampton, School of Biomedical Science, MA Building, Wulfruna Street, WV1 1LY. UK.

[2] University of Loughborough, Department of Chemical Engineering, Loughborough, Leicestershire, LE11 3TU. UK

[3] University of Leicester, Department of Infection, Immunity & Inflammation, Medical Sciences Building, University Road, Leicester, LE1 9HN. UK.

[4] Old Dominion University, Frank Reidy Center for Bioelectrics, 4211, Monarch Way Norfolk, VA 23529. USA

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[*]**Corresponding authors. Mailing address**:

University of Wolverhampton, School of Biomedical Science, MA Building, Wulfruna Street, WV1 1LY. UK. Tel: +44 (0)1902 322 709 Email: w.heaselgrave@wlv.ac.uk

Old Dominion University, Frank Reidy Center for Bioelectrics, 4211, Monarch Way Norfolk, VA 23529. USA. Tel: +1 757 683 7047; Email: mkong@odu.edu

## Abstract

***Objectives:*** Currently there are estimated to be approximately 3.7 million contact lens wearers in the UK and 39.2 million in North America. Contact lens wear is a major risk factor for developing an infection of the cornea known as keratitis due to poor lens hygiene practices. Whilst there is an international standard for testing disinfection methods against bacteria and fungi (ISO 14729), no such guidelines exist for the protozoan *Acanthamoeba* which causes a potentially blinding keratitis most commonly seen in contact lens wearers, and as a result many commercially available disinfecting solutions show incomplete disinfection after 6 and 24 hr exposure.

***Methods:*** Challenge test assays based on international standard (ISO 14729) were used to determine the antimicrobial activity of Cold Atmospheric Gas Plasma (CAP) against *Pseudomonas aeruginosa*, *Candida albicans* and trophozoites and cysts of *Acanthamoeba polyphaga* and *Acanthamoeba castellanii*.

***Results:*** The complete inactivation of *P. aeruginosa* and *C. albicans* was achieved in 0.5 min and 2 min respectively. Trophozoites of *A. polyphaga* and *A. castellanii* were completely inactivated in 1 min and 2 min respectively. Furthermore, for the highly resistant cyst stage of both species complete inactivation was achieved after 4 min exposure to CAP.

***Conclusions:*** This study has demonstrated that the CAP technology is highly effective against bacterial, fungal and protozoan pathogens. The further development of this technology has enormous potential as this approach is able to deliver the complete inactivation of ocular pathogens in minutes compared to commercial multipurpose disinfecting solutions which require a minimum 6 hrs.

## Importance

This study has demonstrated that the CAP technology is highly effective against bacterial, fungal and protozoan pathogens. The further development of this technology has enormous potential as this approach is able to deliver the complete inactivation of ocular pathogens in minutes compared to commercial multipurpose disinfecting solutions which require a minimum 6 hrs.

## Introduction

Currently there are estimated to be approximately 3.7 million contact lens wearers in the UK (1) and 39.2 million in North America (2). Contact lens use is a major risk factor for developing an infection of the cornea known as keratitis (3, 4). Keratitis associated with contact lenses has been attributed to poor contact lens hygiene practices, including failure to comply with the manufacturers' guidelines which includes the practice of 'topping off' existing solution with a small amount of fresh solution in the lens case instead of discarding and using fresh solution each day (4-6).

The exact numbers of contact lens wearers developing keratitis worldwide is not known, but a recent study from the USA reported that there are estimated to be 930,000 doctors' appointments and 58,000 emergency appointments annually for keratitis in contact lens wearers costing approximately $175 million in health care expenditure (7). The range of organisms causing keratitis is wide and includes the herpes simplex virus, bacteria including *Pseudomonas aeruginosa*, fungi including *Candida albicans,* and the protozoan *Acanthamoeba* amongst many others.

Bacterial keratitis is frequently caused by the Gram negative bacillus *P. aeruginosa,* and in a recent study conducted in India this organism was found to be the causative agent in 29% of patients presenting with bacterial keratitis (8). A recent Danish study (9) implicated *C. albicans* in 52% of patients presenting with fungal keratitis.

Although bacteria and fungi are considered the main etiological agent for keratitis the free living protozoan *Acanthamoeba* can be an opportunistic pathogen of humans causing a potentially blinding corneal infection known as Acanthamoeba Keratitis (AK) (10, 11). *Acanthamoeba* has a virtually ubiquitous distribution in nature and can be found in most soil and aquatic environments including gardens, household dust, heating systems, cooling towers, lakes, rivers, and tap water (12-17). *Acanthamoeba* is a dimorphic organism that has two distinct life cycle stages, the motile feeding trophozoite stage and the dormant and highly resistant cyst stage (18). *Acanthamoeba* transforms into the cyst stage in response to adverse environmental conditions including nutrient depravation, exposure to various chemical agents and changes in osmolarity (19-21). The cyst stage is highly resistant to chemical disinfection and desiccation, and remains viable and pathogenic even after decades in storage (22-24). There is currently no licensed drug to treat AK, but unlicensed treatment does exist and consists of the application of a biguanide disinfectant usually PHMB or chlorhexidine and a diamidine directly to the eye. However, treatment times are long and treatment failure is common (25).

The transformation of trophozoites into highly resistant cysts in response to adversity poses greater challenges to disinfection by MPS solutions. This is due to the fact that great variability in the disinfecting efficacy against *Acanthamoeba* spp has been reported between different commercially available MPS's, with many solutions delivering less than a 0.5 log reduction in viability of the cyst stage (26-28). This lack of efficacy might be attributed to the current ISO

guidelines, which outline the microbiological requirements and test methods for products and regimens for hygienic management of contact lenses, but do not include efficacy testing standards against *Acanthamoeba* spp (29). However, a standardised method has recently been proposed for assessing biocidal efficacy of contact lens care solutions against cysts and trophozoites of *Acanthamoeba* (26). Despite this many of the commercially available solutions lack any activity against *Acanthamoeba* cysts even after 6 hours of exposure which is the recommended disinfection time for most lens solutions.

Cold atmospheric plasmas (CAP) are gas discharges at ambient pressure and at temperatures close to those of ambient conditions (30, 31). Treatment is usually defined in terms of time of exposure and electrical power used to sustain the gas discharge. Their biological effects are attributed to the generation of a wide range of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Gas plasmas have been shown to be capable of inactivating a wide range of micro-organisms including viruses, bacteria and fungi (30, 31). However, protozoans have received little attention in this regard, and what studies have been conducted have focussed on inactivation of the water-borne pathogen *Cryptosporidium* (32, 33). Therefore we report here on the capability of a cold gas plasma against *Acanthamoeba polyphaga* and *Acanthamoeba castellanii* in addition to the bacterial and fungal ocular pathogens, *Pseudomonas aeruginosa* and *Candida albicans* in order to establish whether its use could ultimately constitute the basis of a method for treating contact lenses.

## Materials and Methods

**Chemicals.** All chemicals were obtained from Sigma Chemical Company Ltd (Poole, UK) unless otherwise stated.

**Test organisms and culture.** *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231), were obtained from the American Type Culture Collection (LGC Standards, Teddington UK). *P. aeruginosa* was cultured on Tryptone Soy Agar and *C. albicans* on Sabouraud Dextrose agar (Oxoid, Basingstoke, UK) for 24 hrs at 32°C to allow for colony formation. After incubation, suspensions of bacteria and fungi were prepared in ¼ strength Ringer's solution to a concentration of $1 \times 10^7$ - $1 \times 10^8$ cfu / mL.

*Acanthamoeba polyphaga* (Puschkarew) Volkonsky (1931) (CCAP 1501/3G) was obtained from the Culture Collection of Algae and Protozoa (CCAP, Oban, UK). This is a clinical isolate obtained from a patient undergoing a keratoplasty in the USA in 1974. *Acanthamoeba castellanii* (Douglas) Page (ATCC 50370) was obtained from the American Type Culture Collection (LGC Standards, Teddington, UK). This is a clinical isolate obtained from a keratitis patient in New York, USA in 1978. Trophozoites of both species were adapted to growth in tissue culture flasks (Nunc Life Technologies, Paisley, U.K.) at 32°C in a semi-defined axenic culture medium as described previously (34). Cysts were prepared from late log phase cultures using Neff's chemically defined encystment medium (35). Cysts were counted using a Modified Fuchs Rosenthal haemocytometer, adjusted to $5 \times 10^6$/mL and stored at 4°C for use within 2 weeks.

**The CAP generating apparatus.**

The CAP system used in the present study was an ambient air plasma confined to the surface of a sheet electrode of metallic mesh used as the ground electrode (36). A solid polymer plate was

147 used to separate the ground electrode from the powered sheet electrode, and the electrode unit
148 was powered at an electrical power density of 50mW/cm$^2$ and at 20kHz. With the ground
149 electrode facing the microbial sample (described below), the electrode unit was suspended above
150 the sample as shown in Fig 1. Dominant ROS and RNS impinging on the sample included $O_3$,
151 excited $O_2$, $HO_2$, $H_2O_2$, $N_2O/HNO_2$, $NO_3/HNO_3$, $HNO_2$, $N_2O$ and $N_2O_5$ (37)
152
153 **Acanthamoeba CAP assays.** Trophozoites from a late log phase suspension or cysts were
154 counted using a Modified Fuchs Rosenthal haemocytometer, and adjusted to 5 x $10^4$ cells in 20
155 ml of ¼ strength Ringer's solution. The suspension containing the cysts or trophozoites was then
156 poured into a filtration cup (Sartorius, Epsom, UK) that was attached to a manifold unit
157 (Sartorius, Epsom, UK). The suspension was passed through a cellulose acetate filter with a 0.2
158 µm pore size (Pall, Portsmouth, UK). The purpose of the filtration step was to allow the
159 organisms to settle onto the surface of the membrane and to be evenly distributed. The suction
160 pressure was provided using a hand pump and was kept below 10 cm Hg to prevent excess
161 suction stress and to prevent deformation and damage to the microorganisms. Once the filtration
162 was complete the cellulose acetate filter was removed aseptically from the suction cup using
163 forceps and placed in the middle of the surface of an agar plate containing 2.5% (w/v) Non-
164 Nutrient Agar (NNA) No. 1 (Lab M, Bury UK). The purpose of the agar was both to provide
165 some moisture to prevent excess drying of the filter and to provide structural support to stop the
166 filter curling. Furthermore, the CAP generating electrode had been fashioned onto a Petri dish
167 lid and so fitted closely to the agar plates. The filters on the agar surface containing the
168 microorganisms were exposed to the CAP generating electrode for 0, 0.25, 0.5, 1, 2, 4 and 8
169 min. Each of the timepoints was performed using a separate filter in triplicate. The CAP
170 apparatus was used in cycles of 30 sec on and then 30 sec off to prevent overheating but the

exposure time only relates to the time when the electrode was on. For example 1 minute exposure was achieved by 30 secs on, followed by 30 secs off followed by a further 30 secs on.

After exposure to the CAP electrode for the specified time the filter was removed from the surface of the agar and placed in a 50 ml centrifuge tube containing 10 ml of ¼ strength Ringer's solution, before being swabbed with a sterile cotton tipped applicator (Puritan, Guilford, USA) to remove any adherent cells into the ¼ strength Ringer's solution. The viability of the organisms was then determined using the previously published Spearman-Karber method with minor modifications (34). Briefly, this involved transferring 200 µl aliquots of the *Acanthamoeba* suspension to the wells of a 96 well microtitre plate in quadruplet before performing three 10-fold serial dilutions of each. From the original concentration of cells the serially diluted wells ($10^0$, $10^1$, $10^2$ and $10^3$) would have contained approximately 1000, 100, 10 and 1 cells respectively. The plates were examined microscopically for the presence of excysted trophozoites and/or trophozoite replication. The reduction in viable cysts or trophozoites was plotted as the change in log viability for each time point compared to zero time viability. All experiments were performed in triplicate.

**Bacterial and fungal assays.** The bacteria and fungi were tested according to the internationally recognised ISO 14729 method used for the evaluation of contact lens solutions against ocular pathogens (29) with modifications. Briefly, the organisms were cultured as stipulated in the ISO guidelines and viable bacteria and fungi at a concentration of $1 \times 10^7$ were re-suspended in 10 mL of ¼ strength Ringer's. As for the *Acanthamoeba* assays, the suspensions were placed into the filter unit and suction was applied to allow the organisms to settle onto the surface of the

filter paper. The filter paper was then transferred to the surface of an agar plate for support and then exposed to the plasma.

After exposure to the plasma the filters were removed from the agar and placed in a tube containing and 10 ml ¼ strength Ringer's solution. The surface of the cellulose acetate filter was swabbed aseptically to remove adherent organisms and then vortexed to bring about the detachment of cells. To determine viability, the suspensions were serially diluted and 50 µL of suspension for each dilution was cultured on the appropriate agar medium using the method of Miles & Misra. (38) The reduction in viable cells was plotted as change in log viability for each time point compared to zero time viability, as described previously (39).

***Acanthamoeba* CAP contact lens assay.** For the contact lens assay a Polymacon contact lens was placed convex side up on the surface of an NNA agar plate for support. The suspension of *A. castellanii* cysts was adjusted to 5 x $10^4$ cells in 100 µl of ¼ strength Ringer's solution. This suspension was then applied to the convex side of a Polymacon contact lens before exposure to plasma. The viability was then determined using the same method described for the *Acanthamoeba* CAP assay above.

**Data analysis.** Statistical analysis was performed using one-way analysis of variance (ANOVA) of data from triplicate experiments on the InStat statistical software package (GraphPad, La Jolia, USA).

## Results

### Bacterial and fungal assays.

The effect of CAP against *Pseudomonas aeruginosa* and *Candida albicans* is shown in Table 1. CAP caused the complete inactivation (≥7 log reduction in viability) of *P. aeruginosa* within 0.5 min and with *C. albicans* complete inactivation (6.85 log reduction in viability) was achieved after 2 min exposure. Under control conditions in the absence of the plasma ≤0.06 log reduction in viability was observed for both organisms (Table 1). The log reductions observed with the CAP at complete inactivation for both organisms compared to the controls at the same time point was highly statistically significant (p=<0.001).

### *Acanthamoeba* trophozoite assays.

The effect of CAP against trophozoites of *A. polyphaga* (CCAP 1501/3g) and *A. castellanii* (ATCC 50370) is shown in Table 2. CAP resulted in the complete inactivation (≥3.5 log reduction in viability) of *A. polyphaga* within 1 min and with *A. castellanii* this was achieved after 2 min exposure. In control experiments in the absence of the plasma ≤0.08 log reduction in

viability was observed for both strains of *Acanthamoeba* (Table 2). The log reduction observed with the CAP at complete inactivation for both organisms compared to the controls at the same time point was highly statistically significant (p=<0.001).

***Acanthamoeba* cyst assays.**

The effect of CAP against cysts of *A. polyphaga* (CCAP 1501/3g) and *A. castellanii* (ATCC 50370) is shown in Table 3 and Figure 2. CAP produced complete inactivation (≥3.5 log reduction in viability) of *A. polyphaga* within 4 min and with *A. castellanii* this was achieved after 2 min exposure. For control experiments ≤0.08 log reduction in viability was observed for both strains of *Acanthamoeba* (Table 3). The log reductions observed with the CAP at complete inactivation for both organisms compared to the controls at the same time-point was highly statistically significant (p=<0.001). The inactivation data from Table 3 for *A. castellanii* cysts has been plotted in Figure 2 to reveal the form of the inactivation curve: a short 'shoulder' occurs at low treatment times and is followed by a phase of rapid decline leading to complete inactivation.

***Acanthamoeba* contact lens assay.**

The effect of CAP against cysts of *A. castellanii* (ATCC 50370) on the surface of a Polymacon contact lens is shown in Table 4. CAP produced the complete inactivation (≥3.5 log reduction in viability) of *A. castellanii* cysts after 12 min exposure. The log reduction observed with the CAP at complete inactivation compared to the controls at the same time point was highly statistically significant (p=<0.001).

## Discussion

This study reveals for the first time that CAP can bring about the complete inactivation of all commonly encountered contact lens pathogens tested including highly resistant cysts of *Acanthamoeba*. The predominant mechanism by which CAP brings about microbial inactivation is through the generation of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) including $O_3$, excited $O_2$, $HO_2$, $H_2O_2$, $N_2O/HNO_2$, $NO_3/HNO_3$, $HNO_2$, $N_2O$ and $N_2O_5$ (37). The ocular pathogens investigated here included the protozoans *A. polyphaga* and *A. castellanii*, the bacterium *P. aeruginosa* and the yeast *C. albicans*. Treatment by the methods described here resulted in the complete inactivation of *P. aeruginosa* and *C albicans* in 0.5 min and 2 min respectively. With trophozoites of *A. polyphaga* and *A. castellanii* we were able to achieve complete inactivation in 1 min and 2 min respectively. Furthermore, for the highly resistant cyst stage of both species, complete inactivation was achieved after 4 min exposure to CAP.

CAP technology offers a significant performance advantage over current chemical disinfection methods as supported by the finding in this study that complete disinfection of the test organisms was achieved in less than 4 min. In a recent study in which a number of commercial multipurpose solutions (MPS) were tested against *P. aeruginosa* and *Fusarium solani,* a fungus included in the ISO testing guidelines (29), a greater than 5 log reduction was obtained for both organisms with the commercial products ReNu MultiPlus® (Bausch & Lomb, Rochester, USA) and OptiFree Express® (Alcon, Ft Worth, USA) that was achieved after the minimum disinfections times for these products of 4 and 6 hrs respectively (40). In another recent study in which the efficacy of ReNu MultiPlus® (Bausch & Lomb, Rochester, USA) and OptiFree RepleniSH® (Alcon, Ft Worth, USA) against *A. polyphaga* and *A. castellanii* were investigated, less than 1 log reduction with cyst preparations even after 6 and 24 hr exposure to the solutions was achieved (26).

A number of disinfection methods for contact lenses that do not rely on the use of chemical compounds have been proposed in recent years. These include microwave treatment, sonication and UV light treatment. Low power microwave treatment was found to inactivate *P. aeruginosa*, *Staphylococcus aureus* and *S. epidermidis* after 15 mins (41). Microwave treatment was also shown to be effective in inactivating *Acanthamoeba* present on contact lens cases (42).

Although sonication has been proposed as a disinfection strategy, it has attracted relatively little interest (43). With reference to UV treatment, in a recent study from Johnson and Johnson Vision Care (Jacksonville, USA) reductions in viability of *Acanthamoeba* cysts were obtained that are comparable to those reported here (44). However, the times required to achieve a 3.8 to 4 log reduction in viability of the cysts using UV ranged from 18 to 21 min.

The possible effects of CAP treatment on the material of the contact lenses themselves lay outside the scope of the present study but constitutes an important consideration that warrants future investigation. However, CAP may hold other benefits to that of purely achieving disinfection. Lipids and other deposits from the eye are known to form on contact lenses and can impair cleanability as well as causing discomfort to wearers (45). It has been demonstrated that CAP proved superior to ultrasonic cleaning in removing stearic acid from polymer films (46). Moreover it was reported in the same study that CAP treatment improved the wettability of the polymer – a factor that has been shown to be important in contact lens care (45).

In the present study we restricted investigation to the direct exposure of the contact lenses to the plasma discharge but this is not the only means by treatment might be effected. In particular, it has been shown that certain plasma species survive in water sufficiently long enough to constitute an alternative way of achieving disinfection through contact with the plasma treated water (47). In a completely different departure to the potential that CAPs possess for the disinfection of contact lenses, it was recently demonstrated that the application of CAP to the surface of the eye is able to bring about the rapid disinfection of bacteria and fungi from the ocular surface without causing damage to the corneal epithelium and stroma (48).

Further work would need to be undertaken to establish the precise configuration which a personalised CAP-based device for treating contact lenses would take. However, the availability of battery-powered CAP devices is at least testament to the concept being feasible (49).

In conclusion, this study has demonstrated that the CAP technology is highly effective against the bacterial, fungal and protozoan pathogens tested. The further development of this technology has considerable potential as this approach is able to deliver the complete inactivation of ocular

pathogens in minutes compared to commercial multipurpose disinfecting solutions which show variable and often incomplete activity after 6 and 24 hrs.


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## Transparency Declaration

Nothing to declare

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