Antibacterial Efficacy of Atmospheric Pressure Non-Thermal Plasmas
Prof. Brendan Gilmore, Queen’s University, Belfast, Ireland
The A. Denver Russell Memorial Teleclass Lecture for 2014

The Biofilm Theory

- First proposed in 1978 in a publication in Scientific American “How bacteria stick” (Bill Costerton, 1934 – 2012)
- Earlier observations by van Leeuwenhoek (1684), Henrici (1933) and Zobell (1943)
- Studying bacteria in natural ecosystems, such as mountain streams
- Engineered systems
- Medical Microbiology

Bad Bugs, No Drugs: No ESKAPE!
An Update from the Infectious Diseases Society of America, January 2009

"The Infectious Diseases Society of America (IDSA) continues to view with concern the lean pipeline for novel therapeutics to treat drug-resistant infections, especially those caused by gram-negative pathogens. Infections now occur that are resistant to all current antibacterial options."

ESKAPE = Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas, and Enterobacter

As Antibiotic Discovery Stagnates ... A Public Health Crisis Brews
Infectious Diseases Society of America, July 2004

"Infectious diseases physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future. There simply aren’t enough new drugs in the pharmaceutical pipeline to keep pace with drug-resistant bacteria/infections, so-called “superbugs.”"
Plasma – An Overview

- Plasma is the regarded as the fourth state of matter which is similar to the gaseous state but with certain degrees of ionisation and a higher energy content.
- Produced on laboratory scale by flowing gas through an electric field which drives the ionization, excitation and dissociation of gaseous molecules.
- This produces high densities of reactive oxygen and nitrogen species (RONS), charged particles (ions and electrons), radiation (from UV to IR), and electromagnetic fields.

Non-Thermal Plasma

- Thermal plasmas have for many years been used in sterilization of medical equipment, packaging, implants.
- Advantages include rapid bactericidal activity and access to narrow/confined recesses.
- Recently, atmospheric pressure, low temperature (‘non-thermal’ or ‘cold’) plasmas have been developed.
- Typically less than 40°C at point of application.
- Capable of delivering unique reactive dry chemistry at ambient temperatures to delicate surfaces – potentially viable tissues.
- This has given rise to the emerging field of ‘Plasma Medicine’.

Plasma – reactive species

- Variable, tunable according to input gas admixture.
- Base gas usually Helium or Argon.
- With varying % air/nitrogen/oxygen.
- Gives rise to:
  - Reactive Oxygen Species: ozone, atomic oxygen, single delta oxygen, peroxide, hydroxyl radicals.
  - Reactive Nitrogen Species: nitric oxide, nitrite, nitrate, peroxynitrite.
  - Neutral species.

Overview: Why APNTP?

- Provides highly reactive environment at ambient temperature and pressure.
- Tunability of plasma chemistry which makes it possible to optimise for different applications.
- Low capital and operational cost.
- Personnel and environment friendly.
- Utilisation of virtually non-toxic gases (He, Ar, O₂, N₂).
- Absence of harmful residues.
- Multiple conformations (power input, electrode configurations, plasma geometry).

Plasma Source Configuration

- Non-thermal, non-equilibrium dielectric barrier discharge (DBD)-type plasma jet designed and manufactured in-house.
- Quartz tube (6mm outer lumen diameter, 4mm lumen diameter).
- Two 2mm copper electrodes (2mm), separation distance 25mm.
- High voltage pulse source operating at variable repetition of 20 kHz (and 40kHz for rate of biofilm kill comparison).
- Voltage amplitude of 6 kV applied to downstream electrode, positioned 5mm from end of the plasma tube.
- Plasma operated with a gas mixture of 99-100% helium, 0-1% Oxygen, flow rate 2 SLM into ambient air.

Overview: Plasma Jet

- Schematic diagram (A) and photograph (B) of the plasma jet.

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Photographs of P. aeruginosa seeded agar plates (9 cm in diameter) showing inhibition zones as a result of APNTP jet exposure for (a) 0 s, (b) 120 s, and (c) 240 s. [Alkawareek et al. 2012a]

<table>
<thead>
<tr>
<th>Exposure time (sec)</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>B. cereus</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>25</td>
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<tr>
<td>120</td>
<td>40</td>
<td>41</td>
<td>42</td>
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</tr>
<tr>
<td>240</td>
<td>46</td>
<td>44</td>
<td>47</td>
<td>45</td>
</tr>
</tbody>
</table>

Measured inhibition zone diameters of four bacterial species following plasma exposure.


Survival curves for planktonic bacteria, suspended in PBS, of four bacterial species upon exposure to the 20 kHz atmospheric pressure plasma jet.

Comparison between fraction cell killed values obtained using XTT assay and plate count method for the bacterial species in planktonic mode of growth.

Survival curves for 48-hour biofilms, grown on Calgary Biofilm Device, of four bacterial species upon exposure to the 20 kHz atmospheric pressure plasma jet.

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XTT Assay vs. Plate Count Method
(P. aeruginosa Biofilm)

Comparison between % cell reduction values obtained using XTT assay and plate count method for P. aeruginosa biofilm

Effect of Frequency Variation

Log survival curves of P. aeruginosa biofilm cells upon exposure to a 20 kHz and a 40 kHz plasma jet.


LIVE/DEAD Stain and Confocal Microscopy

3D rendered confocal laser scanning micrographs of P. aeruginosa biofilms exposed to the plasma jet for 0s (a and d), 60s (b and e), and 240s (c and f). Green color indicates surviving cells whereas red color indicates dead cells. Magnification power is 200x (a-c) and 600x (d-f).


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Cellular Targets & Mechanism

Effect on Plasmid DNA: Addition of AA

Effect on Plasmid DNA: Rate of SC Damage

Effect on Plasmid DNA

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Effect on Plasmid DNA

Influence of % O₂ on Inactivation Rate

Effect on Proteinase K Activity: Michaelis–Menten Plot

Lipid Peroxidation - TBARS Assay

Malondialdehyde (MDA) is a product of the peroxidation of polyunsaturated fatty acids, usually caused by ROS.

MDA reacts with two equivalents of thiobarbituric (TBA) acid to give a fluorescent red derivative that can be assayed colorimetrically or fluorometrically.

MDA is a ROS itself and can form covalent adducts with proteins and purine deoxynucleosides (A & G) in DNA.
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Lipid Peroxidation: MDA Concentration

Leakage of Intracellular Components: Extracellular [ATP]

Cellular Targets of Non Thermal Plasmas

E. coli + Catalase

M. luteus + Catalase

H$_2$O$_2$ Production in Plasma-Exposed PBS

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P. aeruginosa Biofilm Plasma Tolerance

Cold Plasma – Biofilm Tolerance

Plasma – Biofilm Tolerance

Persister Cell Susceptibility

Tolerance mediated by extracellular biofilm components, such as polysaccharide, alginate, eDNA

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Effect of Different Alginate % survival of P. aeruginosa

Effect of eDNA on P. aeruginosa survival

Combined effect of DNA and Alginate on P. aeruginosa survival

Combined effect of Alginate and eDNA on log<sub>10</sub> reduction of P. aeruginosa viability

Exposure Time (minutes) | log<sub>10</sub> Reduction in PA01 Viability
---|---
0 mcg/ml DNA & 0 % Alginate | 0 mcg/ml DNA & 0.5 % Alginate | 5 mcg/ml DNA & 0.5 % Alginate | 50 mcg/ml DNA & 0.5 % Alginate
0.25 | 0.29±0.09 | 0.08±0.06 | 0.07±0.08 | 0.03±0.03
0.5 | 0.42±0.02 | 0.15±0.08 | 0.09±0.07 | 0.05±0.05
1 | 0.85±0.1 | 0.73±0.17 | 0.67±0.17 | 0.48±0.06
2 | 1.49±0.1 | 0.83±0.17 | 0.74±0.09 | 0.60±0.12

MS2 Phage Inactivation

The Daily Telegraph
Norovirus closes hundreds of wards

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Summary
- Rapid bactericidal effect, more than one target, more than reactive one species involved
- >4 log reduction in 48 hr P. aeruginosa biofilm in 4 minutes
- Biphasic biofilm kill curve may indicate a ‘shielding effect’ from surface layers of biofilm or
- Sequestration of active species by cellular component of sacrificial outer layer of biofilm
- Plasma interaction with liquid has implications for planktonic kill rate – rate of propagation of reactive species
- Multiple cellular targets (interactions with lipid membrane, protein, DNA)
- Effect of biofilm subpopulations (persist) must be considered in chronic or longterm infections
- Biofilm components are critical mediators of bacterial biofilm tolerance to non-thermal plasma treatment

Future Directions (QUB Plasma Medicine Group)
- Invest Northern Ireland funded Proof of Concept Grant
- Development of a portable system based on the device described for hospital control of biofilms, planktonic bacteria and viral pathogens
- WARD Testing – Infection control (2014)
- Safety and Biocompatibility testing
- Phase I safety trials
- Trials in animals (and eventually patients) topical wounds

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Timo Gans

Coming Soon
April 7
CHLORHEXIDINE PATIENT BATHING AS A MEANS TO PREVENT HEALTHCARE ASSOCIATED INFECTIONS
Prof. Mark Rapo, University of Nebraska Medical Center

April 24 (Free Teleclass)
ARE WE TOO CLEAN FOR OUR OWN GOOD? THE HYGIENE HYPOTHESIS AND ITS IMPLICATIONS FOR HYGIENE, LIFESTYLE, AND PUBLIC HEALTH
Dr. Sally Bloomfield, London School of Hygiene and Tropical Medicine

May 5 (Free – WHO Teleclass – Europe)
SPECIAL LECTURE FOR 5 MAY, 2014
Prof. Didier Pittet, World Health Organization

May 8
VENTILATOR-ASSOCIATED EVENTS: A PATIENT SAFETY OPPORTUNITY
Dr. Michael Kompier, Harvard Medical School

Thanks to Teleclass Education
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