Plasma needle:
non-thermal atmospheric plasmas in
dentistry

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Technische Universiteit Eindhoven, op gezag van de
Rector Magnificus, prof.dr.ir. C.J. van Duijn, voor een
commissie aangewezen door het College voor
Promoties in het openbaar te verdedigen
op dinsdag 17 oktober 2006 om 16.00 uur

door

Raymond Ernst Johannes Sladek

geboren te Culemborg
This research was sponsored by the Netherlands Organization for Scientific Research (NWO).

A catalogue record is available from the Library Eindhoven University of Technology
ISBN-10: 90-386-2858-7

Copyright © 2006 R.E.J. Sladek

All rights reserved. No part of this book may be reproduced, stored in a database or
retrieval system, or published, in any form or in any way, electronically, mechanically,
by print, photoprint, microfilm or any other means without prior written permission of
the author.

Printed by Printservice Technische Universiteit Eindhoven, Eindhoven, The Nether-
lands

Cover design by Oranje Vormgevers. Photo: Bart van Overbeeke Fotografie.
# Contents

1 General introduction  
   1.1 History and setting of the plasma needle project  
   1.2 Scope of the thesis  
   1.3 Outline of the thesis  

2 Plasmas in dentistry: an overview  
   2.1 Non-thermal atmospheric plasmas  
      2.1.1 What is a plasma?  
      2.1.2 Why plasma?  
      2.1.3 Safety issues of plasmas  
      2.1.4 The plasma needle  
   2.2 Dentistry  
      2.2.1 The mouth as a microbial habitat  
      2.2.2 Factors affecting the growth of micro-organisms in the mouth  
      2.2.3 Dental plaque  
      2.2.4 Dental caries (decay) and periodontal disease  
      2.2.5 Prevention and treatment of caries  
      2.2.6 Model systems  

3 Characterization of the plasma needle  
   3.1 Introduction  
   3.2 Experiment  
      3.2.1 The plasma set-up  
      3.2.2 The power measurement  
      3.2.3 The thermal probe  
   3.3 Results and discussion  
   3.4 Conclusions  
   3.5 Acknowledgments
4 The plasma needle in dentistry: a feasibility study 55
   4.1 Introduction .......................................................... 56
   4.2 Experimental set-up ............................................... 56
   4.3 Temperature measurements ...................................... 58
   4.4 Bacterial decontamination ....................................... 60
       4.4.1 Preparation of cells for exposure ......................... 60
       4.4.2 Sterilization rate (killing curves) ...................... 61
   4.5 Conclusion ............................................................. 62

5 Deactivation of *E. coli* by the plasma needle 65
   5.1 Introduction .......................................................... 66
   5.2 Experimental procedure ......................................... 67
       5.2.1 The plasma needle set-up .................................. 67
       5.2.2 Power measurements ........................................ 68
       5.2.3 Temperature measurements .................................. 69
       5.2.4 Bacterial sample preparation .............................. 69
       5.2.5 Sample treatment by plasma .............................. 71
       5.2.6 Sample treatment by heating .............................. 71
       5.2.7 Observations .................................................. 71
   5.3 Results ................................................................. 72
       5.3.1 Deactivation by plasma needle ............................ 72
       5.3.2 Deactivation by heat ........................................ 73
   5.4 Discussion ............................................................ 74
   5.5 Conclusions ........................................................... 77

6 Treatment of *S. mutans* biofilms 81
   6.1 Introduction .......................................................... 82
   6.2 Materials and methods .......................................... 82
       6.2.1 The atmospheric plasma .................................... 82
       6.2.2 Growth and plasma treatment of *S. mutans* biofilms . 84
       6.2.3 Treatment of *S. mutans* biofilms with chlorhexidine digluconate . 84
       6.2.4 Analysis of bactericidal effects of treatment on biofilm growth . 84
       6.2.5 Statistical analyses ......................................... 85
   6.3 Results ................................................................. 85
       6.3.1 Antimicrobial effects of plasma and chlorhexidine treatments on growth of *S. mutans* biofilms . 85
   6.4 Discussion ............................................................ 88
   6.5 Acknowledgments .................................................... 89
7 Treatment of microplate biofilm microcosms
  7.1 Introduction .................................................. 94
  7.2 Materials and methods ....................................... 96
  7.2.1 The plasma needle apparatus ................................ 96
  7.2.2 Growth conditions and experimental protocol .......... 97
  7.2.3 Application of the plasma and CHX ....................... 98
  7.2.4 Microbiota profiling of the microplate plaques using Checkerboard DNA-DNA (CKB) analysis ......................... 99
  7.2.5 Analysis of plaque ultra-structure using transmission electron microscopy .................................. 100
  7.2.6 Statistical analysis ....................................... 100
  7.3 Results ....................................................... 100
  7.3.1 Growth of the microplate plaques ....................... 100
  7.3.2 Microbiota composition of the microplate plaques ....... 105
  7.4 Discussion .................................................... 112
  7.5 Acknowledgments ............................................ 115

8 Wettability and growth of \textit{E. coli} and \textit{S. mutans} 119
  8.1 Introduction .................................................. 120
  8.2 Materials and methods ....................................... 121
     8.2.1 The plasma needle set-up ................................ 121
     8.2.2 Contact angle measurements ............................. 122
     8.2.3 \textit{Escherichia coli} .................................... 124
     8.2.4 \textit{Streptococcus mutans} ................................. 124
  8.3 Results ....................................................... 125
     8.3.1 Contact angle measurements ............................. 125
     8.3.2 Bacterial growth and adhesion .......................... 126
  8.4 Discussion .................................................... 127
  8.5 Conclusion .................................................... 130
  8.6 Acknowledgments ............................................ 131

9 General discussion ........................................... 133
  9.1 Introduction .................................................. 134
  9.2 The plasma needle design and operational parameters .... 134
  9.3 Inactivation mechanisms ...................................... 137
  9.4 Model systems ................................................ 138
  9.5 Penetration depth ............................................ 139
  9.6 Plasma needle versus ozone treatment ...................... 140
  9.7 Conclusion and future recommendations ..................... 141
Chapter 1

General introduction

1.1 History and setting of the plasma needle project

The plasma needle project was started in 2002 by dr.ir. Eva Stoffels-Adamowicz at the Eindhoven University of Technology (TU/e). The project was split in two parts; (1) medical/surgical applications, plasma needle treatment of cells and tissues [1], and (2) dental applications, plasma needle treatment of bacteria and biofilms. This thesis concerns with the latter part, the use of the plasma needle in dentistry.

This challenging, multidisciplinary research project required expertise from both dentistry and physics. The dental parts of this research project were performed at the Academisch Centrum Tandheelkunde Amsterdam (ACTA) and at the Dental Research Group of the Wellington School of Medicine and Health Sciences (University of Otago, New Zealand). The plasma physics related studies were conducted at the faculty of Applied Physics and Biomedical Engineering at the Eindhoven University of Technology.

1.2 Scope of the thesis

Plasma is defined as the fourth state of matter. The other states of matter are solid, liquid and gas. The fourth state, plasma, is an ionized gas. Most of the matter in the universe is a plasma. For example, huge objects like stars are plasmas. But there are also man-made plasmas, daily used on our planet. Plasmas are used in industrial and in medical applications [2–6]. In industry plasmas are used in numerous technologies: plasma TV displays, lighting systems and power systems. A relatively new area is the use of plasmas in biomedical and dental applications. In this work we focus on the application of plasmas in dentistry.

Plasmas contain free moving electrons, ions and radicals. Energy is needed to make and sustain a plasma. In our case we coupled this energy in our plasma by radio-
General introduction

frequency (RF) power. Plasma temperatures range from relatively cold to hot (like the central core of a star). The plasma generated by the plasma needle is small-sized and non-thermal. The electron temperature is much higher than the temperature of the neutral particles and ions, which in our plasma is near room temperature. The important characteristic of plasmas is that they are chemically active. Inside the plasma, reactive species like radicals are generated that can be used in different applications [7–10].

The scope of this thesis is the investigation of a novel application in dentistry, the plasma needle. The plasma needle is used to generate a non-thermal atmospheric microplasma for the treatment of dental cavities.

In dentistry dental biofilms, commonly referred to as dental plaque, are widely studied [11–13]. Dental biofilms consist of bacteria that adhere to the tooth surface in bacterial communities. Certain species in a plaque can participate in dental caries (decay) or periodontal disease. For example, certain species in plaque can produce acids that irreversibly solubilize the tooth mineral. The net loss of the tooth mineral results in the formation of a dental cavity.

Non-thermal atmospheric plasmas are very efficient in the deactivation of bacteria and therefore widely used [14, 15]. The aim of this project is to investigate the effectiveness of plasma against the caries bacteria. A promising application of the plasma needle in dentistry is disinfection of dental cavities, to stop caries without removing too much of the healthy tissue. To apply the plasma in a dental cavity, the plasma needle should be easy to handle and operate. The plasma needle treatment needs to be safe and efficient (no bulk destruction of the tissue and efficient deactivation of bacteria).

Plasma needle treatment in dentistry is not limited to plain caries. In the future the plasma needle can also be used for the treatment of periodontal pockets or in root canal treatments. The idea is basically the same: deactivation of bacteria in a biofilm.

The plasma needle project started with characterization and development of the plasma needle. Our first plasma needle set-up consisted of a closed system, a needle confined in a box. This closed system could not be used in dental practice, so an open system was developed. The advantage of this open system is that it can be used in oral cavities.

1.3 Outline of the thesis

In Chapter 2 an overview is given of non-thermal atmospheric plasmas and their role in dentistry. The chapter begins with an overview of plasmas, their applications and safety issues. The plasma needle is introduced and the properties of the plasma needle are described. In the second part of the chapter we describe the mouth as a microbial habitat. Dental plaque is described and an overview of dental caries (decay) and pe-
1.3. Outline of the thesis

Dental and periodontal disease is given. There are several prevention and treatment techniques for dental caries. Some of these techniques, like antimicrobial agents, can be tested against bacteria in planktonic state or in dental biofilm (plaque) model systems. An overview of the model systems is given.

As mentioned in Section 1.2, the project started with the characterization and development of the plasma needle. In Chapter 3 the plasma needle is characterized in terms of dissipated and emitted power per unit surface.

In Chapter 4 a feasibility study on the use of the plasma needle in dentistry is described. The temperature in the tooth and the efficiency of killing Escherichia coli during plasma treatment are described.

Chapter 5 is about the plasma needle treatment of Escherichia coli. In this chapter a parameter study on the deactivation of E. coli is presented.

After this parameter study, the plasma needle was tested for its bactericidal activity against biofilm cultures of a key cariogenic bacterium Streptococcus mutans grown under different sucrose concentrations. These single-species biofilms were treated by the plasma needle and a 0.2 % chlorhexidine digluconate (CHX) rinse as a positive antimicrobial reference. These S. mutans biofilm experiments are described in Chapter 6.

Chapter 7 is devoted to the treatment of multi-species biofilms, the microplate biofilm microcosms. The effects of the plasma needle and CHX on growth and species composition of the MBMs are presented.

In addition to the microbiological experiments, the surface modification of various materials by the plasma needle was investigated. The modification of the materials is given in Chapter 8. Furthermore, the effects of this modification on growth of two bacterial species, E. coli and S. mutans, are described.

The thesis ends with general discussions and conclusions in Chapter 9. In this chapter the general findings, conclusions and future recommendations are discussed and summarized.

A list of publications related to this work can be found in the Publications related to this work chapter.
References


Chapter 2

Plasmas in dentistry: an overview

Abstract– In this chapter an overview is given of non-thermal atmospheric plasmas and their role in dentistry. The chapter begins with an overview of plasmas and their applications and safety issues. The plasma needle is introduced and the properties of the plasma needle are described. In the second part of the chapter we describe the mouth as a microbial habitat. Dental plaque is described and an overview of dental caries (decay) and periodontal disease is given. There are several prevention and treatment techniques for dental caries. Some of these techniques, like antimicrobial agents, can be tested against bacteria in planktonic state or in dental biofilm (plaque) model systems. An overview of the model systems is given.

Large parts of this chapter were published as:

2.1 Non-thermal atmospheric plasmas

2.1.1 What is a plasma?

Blood is composed of cells and plasma. When blood is cleared of its cells the plasma remains. This blood plasma was named by Johannes Purkinje (1787-1869) after the Greek word, which means "moldable substance". In 1927 Irving Langmuir first used this term to describe an ionized gas, perhaps because it reminded him of blood plasma.

A plasma is defined as the fourth state of matter. It was first identified by Sir William Crookes in 1879. Plasma is an ionized gas containing free moving charge carriers: electrons and ions [1–4]. Over 99% of the visible universe is made up of plasma. For example, the matter in stars or nebulae is plasma. There are also man-made plasmas on our planet, daily used in industrial and medical applications [5–9]. In industry plasmas are used in numerous applications: plasma TV displays, surface processing, lighting systems or in power systems.

An important characteristic of plasmas is that they are chemically active. Inside the plasma reactive species like active radicals or excited molecules are generated that can be used in surface processing. In the last decades non-thermal plasmas have made a fast career in surface processing technology. At present, virtually any surface treatment can be performed in a plasma reactor: etching (fabrication of semiconductor elements); deposition of amorphous silicon layers for solar cells; deposition of various thin coatings: hard/protective layers (diamond), nano-structured composite films; cleaning/ashing; tailoring of surface properties: wettability, surface energy, adhesion. The non-destructive character of the plasma, and its versatility in interactions with (non-organic) surfaces have inspired a completely new application: plasma-surface treatment in medical care.

There are several methods to generate non-thermal plasmas. Energy is needed to produce and sustain a plasma. This can be done in several ways: by thermal, electrical, or light energy. Usually, a discharge in gas is induced electrically. In this case, only charged species (electrons and ions) can gain energy from the electric field. When these particles are in minority, heating of neutral molecules will be limited. Thus, diffuse plasmas where the fraction of ionized species is below 0.1 %, are usually non-thermal. This situation is readily achieved under reduced pressures, in the range of 10 to 1000 Pa. The effect of low pressure is two-fold: in a rarefied gas ionization events are scarce, which keeps the charge density low. Moreover, the frequency of elastic collisions between electrons and molecules is low, so electrons do not have much chance to convey their energy to the gas.

Low-pressure plasmas are of great value in fundamental research as well as in technology, but they have many serious drawbacks. These plasmas must be confined in
massive vacuum reactors, their operation is costly, and the access for observation or sample treatment is limited. One of the recent trends focuses on developing new plasma sources, which operate at atmospheric pressure, but retain the properties of low-pressure media. Non-thermal atmospheric plasmas can be obtained using the following principles:

**Transient plasmas**

The frequency of energy transfer in collisions between electrons and gas is given by:

\[ \nu [s^{-1}] = \left( \frac{m_e}{m_a} \right) 2 n_a \sigma_{ea} v_e \]  

where \( m_e / m_a \) is electron to atom (molecule) mass ratio, \( \sigma_{ea} \) is their mutual collision cross-section, \( n_a \) is the atom density and \( v_e \) is the electron velocity. In atmospheric plasmas \( \nu \) is about \( 10^8 \) collisions/s; for efficient gas heating at least 100-1000 collisions are necessary. Thus, if the plasma duration is shorter than \( 10^{-6} \)–\( 10^{-5} \) s, gas heating is limited.

**Micro-plasmas**

Small-sized atmospheric plasmas are usually non-thermal. This is simply a consequence of their low volume to surface ratio. Energy transfer from electrons to gas atoms/molecules occurs in the volume, and the resulting heat is lost by conduction through the plasma boundary surface. Suppose that the temperature in the plasma should not be higher than \( \Delta T \) degrees above the ambient temperature, what is the maximum size of the plasma glow? A simple balance between electron-impact heating and thermal losses can be made for a spherical glow with a radius \( L \):

\[ \frac{m_e}{m_a} v_{ea} n_e k_B T_e \frac{4}{3} \pi L^3 = \frac{\kappa}{L} \frac{\Delta T}{L} 4 \pi L^2 \]  

where \( m_{e,a} \) is the electron/atomic mass, \( v_{ea} \) is the electron-atom collision frequency and \( \kappa \) is the thermal conductivity of the gas. This allows to estimate a typical plasma size:

\[ L = \sqrt{\frac{m_a}{m_e} \frac{3\kappa \Delta T}{v_{ea} n_e k_B T_e}} \]  

Dependent on the plasma conditions, the typical length scales of non-thermal plasmas with \( \Delta T < 10^\circ C \) are of the order of 1 mm.
Dielectric barrier discharges

The dielectric barrier discharge (DBD) consists of two flat metal electrodes that are covered with dielectric material (at least one of the electrodes is covered) [10]. The two electrodes are aligned parallel and separated by a variable gap. An alternating voltage is applied to the electrodes and this induces a voltage between the two dielectric layers. The applied voltages are in kV range at frequencies ranging from Hz to a few kHz. When the breakdown voltage is reached a discharge starts. Ionization occurs and an electrical current starts flowing in the gas. This discharge current is limited by the dielectric layer: the layer is charged by incoming ions or electrons and this surface charge reduces the electric field and therefore limits the transport of charge to the electrodes. In this process the glow discharge does not transit into an arc. The DBDs have a large surface-to-volume ratio, because the electrodes are large and the distance between them (the gap) is small. This promotes heat diffusion losses and maintains a low gas temperature.

2.1.2 Why plasma?

Non-thermal atmospheric pressure plasmas are very effective in killing bacteria. This makes these plasmas very useful for various biological and medical applications, such as: sterilization of medical instruments, decontamination in biological warfare [11] and air filters in hospitals.

In this section we describe the roles of heat, ultraviolet radiation, charged particles and active species in the inactivation of bacteria. Studies showed that the chemically active species, such as free radicals, play the most important roles in the inactivation.

The inactivation factors are [6]:

- **Heat**
  Heat can inactivate bacteria. Conventional sterilization methods are based on heat. There are two types of sterilization, by moist or by dry heat. Moist heat requires a temperature of 121 °C at a pressure of 15 psi (=1.034 bar). Dry heat requires a temperature of 170 °C for 1 hour.

- **Ultraviolet radiation**
  Ultraviolet radiation causes dimerization of thymine bases in the DNA strands. This DNA damage inhibits the replication of bacteria. The wavelengths in the range 220 – 280 nm at the irradiation intensities of several mW/cm² are known
to have the optimal inactivation effect. However, studies showed that UV is not an important decontaminating factor in treatments with low temperature atmospheric air plasmas [11, 12].

- **Charged particles**
  Charged particles from the plasma cause cell membrane charging, which may rupture the outer membranes of bacteria [13, 14]. This is because the electrostatic force caused by the build-up of charge on the membrane can overcome its tensile strength and cause rupture. Mendis et al. [13] claimed that such rupture is more likely to occur for gram-negatives, because they have an irregular surface. Electrostatic rupture can occur only in case of direct exposure to the plasma glow; it does not play a role in remote/afterglow treatment.

- **Reactive species**
  The reactive species in non-thermal atmospheric air plasmas are generated through electron impact excitation and dissociation. In these air plasmas we can find nitrogen– and oxygen–based species such as atomic oxygen, ozone, NO, NO\(_2\), and OH\(_\cdot\). The nitrogen– and oxygen–based species are expected to play a crucial role in the inactivation, because these species have direct chemical interactions with the membranes of bacteria. The active species are short living radicals in gas phase that can dissolve in liquid [15]. After recombination/reaction the radicals are destroyed, so that no radicals remain after plasma exposure.

  The membranes are made of lipid bilayers. They form a protective barrier where compounds are transported in and outside the cell. Important components of the membrane are the unsaturated fatty acids and the proteins; both are involved in transport processes across the membrane. The unsaturated fatty acids are susceptible to attacks of OH\(_\cdot\). Hydroxyl radicals can therefore destroy membrane lipids and thereby deactivate the bacteria. The same is true for the proteins: these are also susceptible to the attack of radicals when exposed to plasma.

### 2.1.3 Safety issues of plasmas

What is a “bio-compatible” plasma? It depends very much on what one expects from plasma treatment. But generally, everyone agrees that damage to the living organism should be avoided or at least minimized, and that refined/selective modifications are preferred to non-specific effects. This imposes quite a number of restrictions on the
plasma source, in particular on its thermal and electrical properties and its chemical activity (toxicity). Before going into details about the possibilities and consequences of in vivo plasma treatment, some facts concerning basic features of atmospheric plasmas will be presented, and the necessary safety requirements will be discussed.

Plasmas are often very complex mixtures; in fact, they owe their specific properties to the synergy of various components: charged particles (electrons, positive and negative ions), metastables, ro-vibrationally excited molecules, active radicals and (UV) photons. Non-equilibrium plasmas are particularly complicated, as their composition and properties cannot be easily predicted from equilibrium-based models (Boltzmann and Saha equilibrium, Maxwell-Boltzmann energy distributions, reaction balances based on single temperature). On the other hand, non-equilibrium features make such plasmas extremely valuable for refined surface treatment. It is a great challenge for the experimentalist to characterize the plasma and to tailor its properties to achieve desired surface modification.

In the next sections the safety requirements are briefly discussed.

Thermal properties

Non-equilibrium plasmas are often referred to as “non-thermal”. In such plasmas electron temperatures can be 100 to 1000 times higher than in neutral gas temperature. In this way light and energetic particles (electrons) create a chemically reactive medium, while the gas is sustained at room or slightly elevated temperatures and thermal damage to the surroundings is avoided.

Thermal damage was already of much concern in processing heat-sensitive (non-living) surfaces, like plastics and fabrics. For living tissues, it becomes even more crucial. Temperature elevation above 43 °C causes damage to cells and tissues in most cases. The extent of damage depends on the sort of cell/tissue and duration of the exposure to heat. Thermal effects were elaborately studied in relation to laser surgery [16]. Figure 2.1 shows a curve, indicating which temperatures and exposure times lead to irreversible tissue damage. This curve can be used as a rough orientation, but one has to keep in mind that some tissues are much more sensitive to heat than one could deduce from Figure 2.1. For example, a temperature increase of only 2.2 °C in the dental pulp causes partial pulp necrosis [17]. Teeth suffer pulpal necrosis in 15 % of cases when heated to more than 5.5 °C, and in 60 % of cases when heated by more than 11 °C. Conversely, tissues like skin can withstand temperature elevation to 60 degrees or more without substantial damage [18].

Dependent on the desired effect of plasma treatment, rigorous control of gas temperature in the plasma, and surface temperature of the exposed tissue, may be necessary. In non-specific treatment, like burning and coagulation of wounds, heating of the tissue
2.1. Non-thermal atmospheric plasmas

Figure 2.1: A curve indicating the maximum temperature that tissue can tolerate for a given period of time (after Niemz 1996) [16].

is desired as an important part of the therapy. For this purpose hot plasmas may be used, and the temperature is not so critical as long as there is no carbonization or deep damage. In other applications, like specific treatment without tissue devitalization, temperature is an essential issue. The tissue may be warmed up to at most a few degrees above the ambient temperature, and treatment time must be limited to several minutes.

There are many techniques to determine plasma and surface temperatures. In most plasma physical works, spectroscopic measurement of relative intensities of rotation bands (rotation temperature) is a popular method to determine the gas temperature. Unfortunately, the accuracy is somewhat limited: at low temperatures (close to room temperature) the intensity of higher rotational bands is very low, and the error bar is in the best case on the order of ten degrees. This may not be so critical in plasma physics, but in medicine every degree above body temperature may become a problem. In biology (biophysics) and material technology thermocouples are often used. In the present context, thermocouples are more useful as they provide direct information about the thermal flux towards the exposed surface. In the table below (Table 2.1) a few typical non-thermal plasma sources and the corresponding gas temperatures are listed. Most of these results have been obtained using spectroscopic methods: optical emission and CARS [19]. Moon and Choe [20] have calibrated optical emission spectroscopy against thermocouples. Stoffels et al. [5, 21] has also used both methods.
Table 2.1: Gas temperature of non-thermal plasma sources

<table>
<thead>
<tr>
<th>Plasma source</th>
<th>Type</th>
<th>Gas</th>
<th>T(K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atmospheric Pressure Plasma Jet (APPJ) [22]</td>
<td>RF capacitively coupled</td>
<td>Helium, argon</td>
<td>400</td>
</tr>
<tr>
<td>Atmospheric glow [23]</td>
<td>AC/DC glow above water</td>
<td>Air</td>
<td>800–1500</td>
</tr>
<tr>
<td>Cold arc-plasma jet [24]</td>
<td>AC 10–40 kHz</td>
<td>Air, N₂, O₂</td>
<td>520</td>
</tr>
<tr>
<td>Microwave torch [20]</td>
<td>2.45 GHz</td>
<td>Argon + O₂</td>
<td>2200</td>
</tr>
<tr>
<td>AC plasma [20]</td>
<td>AC</td>
<td>Helium + O₂</td>
<td>800–900</td>
</tr>
<tr>
<td>DBD [19]</td>
<td>Dielectric barrier</td>
<td>N₂ + O₂ + NO</td>
<td>300</td>
</tr>
<tr>
<td>Pulsed DBD [25]</td>
<td>Dielectric barrier</td>
<td>Argon + H₂O</td>
<td>350–450</td>
</tr>
<tr>
<td>Atmospheric glow [26]</td>
<td>DC glow with micro-hollow cathode electrode</td>
<td>Air</td>
<td>2000</td>
</tr>
<tr>
<td>Plasma needle [21]</td>
<td>RF micro-plasma</td>
<td>Helium (+H₂O)</td>
<td>300</td>
</tr>
</tbody>
</table>

The influence of electricity

Gas discharge and electricity are inevitably coupled. At atmospheric pressure the breakdown voltages may be quite high - from several hundreds of Volts to even 10 kV, depending on the type of discharge (DC, RF, microwave), electrode gap and gas composition. Such high electric fields are surely a matter of concern for the health of the patient: they may interact with the nervous system, disturb the heartbeat, and cause damage to the individual cells.

Electricity has always fascinated mankind. Lightning is probably its most awesome and frightening manifestation, but most of the commonly encountered phenomena – sparks in dry air, shocks experienced after touching some surfaces, charging of hair and clothes – are not so destructive by far. Yet, the electric voltages that induce such phenomena may be considerably high; for example, sparks are generated when the local field strength is about $3 \cdot 10^6$ V/m. The reason for their harmlessness lies in very low electric currents and consequently low power dissipation. Thus, one can tentatively state that damage is related to (excessive) electric power rather than voltage or field strength.
Electric methods are not unfamiliar in medicine. Electrosurgery may be the most prominent example: substantial electric power is dissipated in a living tissue, with controlled coagulation/cutting as a result. However, not only electrosurgical devices, but most of the advanced medical equipment involves electricity. With the introduction of new techniques, a thorough study of the effects of electric fields and EM radiation on living beings was undertaken. In particular, much attention was given to alternate (high-frequency) currents passing through the body [27–29]. A very important issue, revealed by these studies is the decreased sensitivity of nerves and muscles to high-frequency currents. The threshold current that causes irritation is only 1 mA at 10 Hz, but it increases dramatically with increasing frequency – at 100 kHz it is as high as 0.1 A. This result is of major importance for electrosurgery and plasma treatment: it implies that for medical applications high-frequency sources should be employed. The ”safety limit” lies around 100 kHz, but to be “on the safe side”, most of the equipment operates at 300 kHz or higher.

Presently, much attention is given to interactions of electric fields with the cell membrane. Cell death due to membrane rupture is one of the hazards related to electric fields – electroporation is a well-known phenomenon, and it may become an undesired side effect during plasma treatment. The rupture occurs when the potential difference across the cell membrane is 1 V [29]. Since the membrane has a thickness of several nm, external electric fields, which causes this damage, must be on the order of $10^8$ V/m. In man-made plasmas electric fields are typically much lower: in extreme cases (plasma sheath at the powered electrode) they are about $10^5$ - $10^6$ V/m. During plasma treatment, a sheath is formed around the exposed (grounded) object, but the electric field in this sheath is lower than $10^5$ V/m. Thus, plasma-induced electric fields should not inflict too much damage. In some cases, the action of the electric field may even become beneficial. Field-induced reversible permeabilization of the membrane is an interesting effect with many potential applications.

The effects of transient electric fields on living cells was elaborately studied by Schoenbach et al. [30] and Beebe et al. [31] (see also [32, 33]). The duration of the electric pulse was from 60 ns to 100 µs, the electric field strength was 3–15 · $10^6$ V/m. The authors found conditions in which membrane electroporation is reversible. Furthermore, they reported interesting modifications to the cell interior. Short electric field pulses are capable of inducing programmed cell death (apoptosis) in cancer cells, so they may be used therapeutically, e.g. to suppress tumor growth.

**Toxicity**

Plasma is a rich source of radicals and other active species. Free radicals have earned a bad name in biology and medicine, because of their capability of causing severe cell
damage. Especially the ROS (reactive oxygen species) are well known as evildoers. The ROS family comprises radicals like O, OH and HO$_2$, peroxide anions O$_2^-$ and HO$_2^-$, ozone and hydrogen peroxide. These species are easily created in ambient air and water (e.g. due to radiation), and they live long enough to reach the cell and attack the organic matter. When the ROS level in body fluids becomes too high, various types of damage occur, known under a common name of oxidative stress. It is believed that oxidative stress bears at least partial responsibility for diseases like arteriosclerosis, cancer and respiratory problems. Moreover, high concentrations of oxygen radicals accelerate ageing of cells and tissues. On the cellular level, several effects leading to cell injury have been identified:

- lipid peroxidation - the oxidation of unsaturated lipids in the cell membrane (damage to the membrane).
- DNA damage - oxidation of DNA bases, leading to breakage of the DNA strand.
- protein oxidation - generally not so harmful, because damaged proteins are efficiently replaced. However, it can temporarily decrease the enzyme activity.

On the other hand, free radicals have various important functions in the body. Small amounts of them are produced by the organism itself. For example, macrophages generate ROS to destroy the invading bacteria, and endothelial cells (inner artery wall) produce nitric oxide (NO) to regulate the artery dilation. It is not completely clear what radical concentrations are indispensable for the proper functioning of the body, and which are dangerous. There must always be a compromise between benefit and damage, but the numbers can vary from individual to individual. Radical production by the body during physical exercise can increase the ROS concentration in blood plasma even up to 0.1 mM [34], however, physical activity is generally considered wholesome.

Radiation

UV irradiation is a well-known factor that induces cell injury [35]. Three spectral regions can be distinguished:

- UV-A (315-380 nm) - the least harmful; causes tanning but may also accelerate skin aging.
- UV-B (280-315 nm) - absorbed by the DNA, causes DNA damage; carcinogenic.
- UV-C (100-280 nm) - more aggressive than UV-B, damages DNA, proteins and cellular lipids. However, radiation below 150 nm is blocked by water (absorption coefficient $10^3 - 10^4$ cm$^{-1}$)
It is known that UV radiation in the B and C range can efficiently inactivate bacteria [36]. UV (in all ranges) is also an important factor in bacterial sterilization using low-pressure plasmas [37]. However, non-thermal plasmas under atmospheric pressures are generally poor sources of UV [14]: the irradiated power in the 200-300 nm region is on the order of 1 mW/cm$^2$. Sosnin et al. [38] investigated the effects of UV radiation on bacteria (E. Coli) and mammalian cells (CHO-K1 fibroblast type cells). They used narrow band lamps with about 80% of the irradiated power in one transition: 282 nm for a XeBr lamp, 222 nm for a KrCl lamp and 206 nm for an I lamp. The lethal UV doses for bacteria (reduction to $10^{-4}$ of the original population) were about 50 mW/cm$^2$ for 222 nm and 150 mW/cm$^2$ for 282 nm. The lethal doses for fibroblasts were substantially higher – irradiation with 0.5 W/cm$^2$ did not result in direct cell damage, necrosis (cell death) started from 0.7 (at 206 nm) and 1 W/cm$^2$ (at 282 nm). These results show that living cells are much more resistant to UV than bacteria. This is a potentially interesting effect: moderate doses of UV light can inactivate bacteria in an infected wound, while the cells responsible for tissue repair (fibroblasts) remain unharmed. It is known that UV exposure can accelerate wound healing [39], probably due to disinfecting and thus reducing the inflammation. A more detailed investigation is needed to decide whether UV treatment can become a competitive technique for wound sterilization. As regards atmospheric plasmas, it seems unlikely that their weak UV radiation can have much effect on cells and bacteria. The specific cell responses are probably induced by other factors, like plasma chemistry.

### 2.1.4 The plasma needle

The plasma needle [40] fulfills the requirements of being small, precise in operation, flexible and absolutely non-thermal. This small size distinguishes the plasma needle from larger atmospheric plasmas intended to treat large areas. The small size is desired for dental or medical use, where high precision is required. The plasma needle is a typical atmospheric capacitively coupled radio-frequency (13.56 MHz) micro-discharge created at the tip of a sharp needle. The experimental scheme, including a photograph of the flexible hand-held plasma needle is shown in Figure 2.2.

Like most atmospheric discharges, the needle operates most readily in helium: the breakdown voltage needed for ignition is low, only 200 V peak-to-peak. In fact, using helium as a carrier gas has some more advantages: the thermal conductivity (144 W/m/K) is very high, and consequently the plasma temperature can be low. Moreover, helium is light and inert - possible tissue damage due to ion bombardment and toxic chemicals can thus be excluded. The therapeutic working of the plasma depends on the additives. Small doses of active species may be beneficial, while large doses inflict damage [15]. In case of plasma needle, the amount of active species is easy to regulate. The
The plasma needle consists of a tungsten wire (0.3 mm diameter) with a sharp tip at the end, confined in a Perspex tube (4 mm inner diameter). The Perspex tube is filled with helium via the gas inlet. A radio-frequency (RF) voltage is applied to the needle at 13.56 MHz. The signal is produced by a waveform generator and amplified by a RF amplifier. The dissipated power is monitored using a probe connected via a dual-directional coupler to a power meter. To optimize the amount of power deposited in the plasma, a matching network is introduced between the power generator and the plasma needle [41].

The right dose can be administered by adjusting plasma power, distance to the substrate, treatment time and gas composition. Light emitted by the plasma was monitored to determine the species composition of the plasma (Figure 2.3). The species found in our plasma are: He, N₂, N₂⁺, OH⁻ and O⁻. It should be noted that ozone is difficult to detect by spectroscopy (only weak emission bands have been reported in literature) [15].

The usage of helium as carrier gas does not significantly reduce the flexibility of the needle. The supplied flows are low (0.5–2 l/min) and the plasma tolerates substantial admixtures of other gases. The glow can be applied directly to the substrate. In Figure
2.1. Non-thermal atmospheric plasmas

2.4b one can see how the plasma interacts with human skin: it spreads over the surface without causing any damage or discomfort. So far, helium plasmas with about 1% of air have been used [15].

![Plasma Images](image)

Figure 2.4: Two modes of plasma operation: a) plasma is concentrated at the needle tip, grounded electrode is remote (at least 3 mm away); b) plasma is in contact with the treated object (dry skin, thus poorly grounded).

The visual appearance (size, shape, brightness) and the electrical and thermal properties of the plasma are strongly dependent on the configuration. The crucial parameters that determine the plasma performance are: electrode gap (distance from the needle to the grounded object), needle thickness and the gas composition. One can generalize that thin and sharp needle electrodes produce small glows, which are stable and easy to ignite at relatively low voltages. This is not surprising, because plasma ignition requires a certain electric field strength, and for a sharp needle, this critical field can be realized at a lower input voltage. Small glows (< 1 mm in diameter) consume very little power and maintain a low gas temperature [40]. A small glow size offers better precision of treatment, the latter being of particular importance in dentistry. However, it was also observed that small, low power glows cannot be operated in certain gas mixtures, e.g. in presence of electronegative gases (air). Plasma needle will ignite in air, but it requires a much higher voltage (> 600 V) and higher power input (several Watts).
An increase in power input does not generally lead to drastic changes in essential plasma parameters like electron temperature and density. Kieft et al. [41] characterized the electrical features of the plasma needle. Furthermore, they investigated other plasma properties like glow size and charge density. It must be noted that electron density measurement in small-size, low-density plasmas is generally a difficult task. Most available methods have a too low spatial resolution (interferometry), are too intrusive (probes), are sensitive to stray light (Thomson scattering), or are insensitive to low charge densities (Stark broadening). Thus, in case of the plasma needle an indirect method was applied. Plasma impedance ($Z = Z_r + iZ_i$) was determined from the voltage-power characteristics supported by a matching network model, and the corresponding conductivity ($\sigma$) was used to estimate the average electron density ($n_e$):

$$\sigma = \frac{n_e e^2}{m_e v_{ea}}$$  \hspace{1cm} (2.3)

where $e$, $m_e$ and $v_{ea}$ are electron charge, mass and collision frequency with neutral gas (relevant for plasmas with low charge density). For the collision frequency, a typical electron temperature of 2 eV was taken, the estimated frequency was $1.6 \cdot 10^{12}$ Hz. The conductivity was obtained from the resistance and typical length scale of the plasma (plasma diameter, determined by visual observation): $\sigma = (Z_r L)^{-1}$. When the power increases, the plasma expands in volume, while the electron density does not change significantly. The magnitude of $n_e = 10^{17}$ m$^{-3}$ is in very good agreement with theoretical predictions from a fluid code [42]. Optical emission spectroscopy allowed determination of the electron excitation temperature [40], which also appeared to be approximately constant with power. Electron density and (excitation) temperature are important parameters that condition the plasma activity (excitation, ionization and formation of active radicals). Therefore, one can conclude that atmospheric plasmas operated at higher power have about the same activity and efficiency as the low-power ones. However, an upcoming drawback of increased power is the elevated gas temperature – when the plasma glow expands, cooling by thermal diffusion becomes less efficient and the temperature can reach even a few hundred degrees [40]. Gas temperature of the plasma is one of the most important issues in treatment of heat-sensitive (biological) objects. It was extensively studied using various experimental techniques: optical emission spectroscopy [40], thermocouples (see Chapter 3 and 4) [43], liquid crystal temperature strips (see Chapter 5) and mass spectrometry [44]. The latter is based on determination of neutral gas density ($n$) from Residual Gas Analyzer (RGA) signals; then, the law $p = nk_BT$ is applied assuming constant (atmospheric) pressure. The methods for temperature determination are quite different, and they provide different but often complementary information. For example, optical emission is a typical
gas-phase technique. The data it provides originate from the hottest part of the plasma: the active zone, which yields the highest emission intensity. Therefore it is not surprising that a temperature as high as a few hundreds of degrees is observed. In contrast, mass spectrometry provides downstream information, as it records the density of gas flowing into the mass spectrometer. The corresponding temperatures are lower. Nevertheless, the trends obtained by various methods are quite consistent. It is evident that gas heating occurs only at high power input; this is also coincident with an increase in the plasma glow size. The thermocouple and the temperature strip are not gas-phase methods. In fact, they are more relevant from the point of view of biomedical applications, because they provide the information about the heat that the treated object will suffer. It follows that heating of the sample by plasma is usually tolerable. This is because of a large heat capacity of samples: one can treat a living tissue for several seconds or even minutes without inducing thermal damage. Furthermore, convective cooling is of importance in sustaining a low plasma temperature. In most experiments a gas flow of 2 l/min was used, corresponding with a flow speed of 1.4 m s\(^{-1}\).

A general feature of the plasma needle is the existence of two operation modes: the unipolar one, observed when the needle is separated by several millimeters from the ground, and the bipolar one, where plasma is sustained between the needle tip and the ground (Figure 2.4).

The transition between the modes is abrupt: when the needle-to-surface gap is brought close to the critical distance (typically 3 mm for 100-300 mW power), the glow suddenly “leaps” over, expands and increases in brightness. As expected, the bipolar mode is much more effective for treatment of biological and other samples. Several experiments were conducted to assess plasma activity in both modes. The results can be summarized as follows:

- Density of active oxygen species, diffusing from the plasma into the liquid medium, decreased drastically with increasing distance from liquid interface (by a factor of 10 from 1 mm to 3 mm). These measurements were performed using a fluorescent label probe (CM-H\(_2\)DCFDA) that allows detection of free oxygen radicals in the fluid with sensitivity in the sub-micromolar range [45].

- The energy flux from the plasma, due to deposition of energetic particles, was almost constant as long as the plasma was in contact with the collecting probe, and decreased drastically when the glow was detached from the device’s surface. Experiments showed that in the bipolar mode, practically all input power in the plasma is deposited on the surface. This measurement made use of a calibrated thermal probe (a small metal disc attached to a thermocouple); energy flux from the plasma was calculated from the dynamic temperature changes of the disc. (see Chapter 3)
In mass spectrometric measurements, no ions and radicals were detected when the glow was not in contact with the orifice of the mass spectrometer. The experiment was performed with a new molecular beam mass spectrometer, developed by HIDEN Analytical (Warrington, UK), especially for the analysis of atmospheric media [44].

Direct tests on living cells showed no instantaneous effect when the plasma was not in contact with the sample surface [15]. This also served to certify that only gas flow and/or only RF radiation have little or no influence on cells. Of course, prolonged exposure of samples to flowing helium could cause desiccation, but this effect is of a different nature than plasma-induced cell reactions.

However, the rest amount of active radical species, present in the remote plasma (afterglow) region, was sufficient to inactivate bacteria. Studies on bactericidal properties of the plasma showed that bacteria that were approximately 1 cm away from the needle tip were still inactivated (see Chapter 5).

The above-mentioned studies showed that the length of the electrode gap, in combination with the applied plasma power, is crucial for the result of plasma treatment. This is the main motivation to construct a fully automated plasma device with a distance sensor and a feedback mechanism for the adjustment of the position [41].
2.2 Dentistry

2.2.1 The mouth as a microbial habitat

The mouth or oral cavity has an oral microflora with a characteristic bacterial composition. The study of the oral microflora goes back almost 400 years. Antoni van Leeuwenhoek (1632–1723)(Figure 2.5), a Dutch tradesman and scientist from Delft, discovered the first microbial species in the mouth and recorded the diversity of them. Today it is known that there are over 700 bacterial species present in the oral cavity [46].

![Figure 2.5: Antoni van Leeuwenhoek (1632–1723), (US National Library of Medicine).](image)

The oral microflora usually lives in harmony with the human body. The oral microflora has not merely a passive relationship with the host, it contributes to the defense system of the body (e.g. colonization resistance) [47]. The relationship with the host can be altered in two ways: 1) imbalance in the composition of the microflora (e.g. following frequent sugar intake), 2) presence of bacteria at sites normally not accessible to them (e.g. when bacteria enter bloodstream after tooth extraction). These alterations or imbalances can result in disease. The two most common diseases are caries and periodontal disease.
"Poor oral health causes harm directly. Three out of 10 people over 65 have lost all their teeth. In the USA, half of all adults have either gum disease or tooth decay; Americans spend more than 60 billion dollar a year to treat tooth decay alone (Pennisi, 2005) [46]."

There are several distinct surfaces present in the mouth: teeth, mucosal surfaces and gingival crevices. Each surface provides a different habitat because of its unique physical and biological properties.

**Teeth**

Teeth are the only hard non-shedding surfaces in the body. The tooth is composed of four tissues: enamel, dentine, cementum and pulp (Figure 2.6).

![Figure 2.6: Cross section showing parts of tooth, (Wikipedia, created by Sam Fentress).](image)

Enamel is the hardest and most mineralized structure in the human body. Normally it is the only part of the tooth exposed to the environment [47, 48]. Enamel is primarily composed of minerals (96%), water and organic material. The primary mineral of enamel is hydroxyapatite, $\text{Ca}_{10} \text{(PO}_4)_6 \text{(OH)}_2$. Enamel is hard but also very brittle [49].
The enamel is supported by its underlying dentine. Dentine is a protective layer that supports the crown and protects the pulp. It is a mineralized connective tissue with bundles of collagen filaments surrounded by mineral crystals. Dentine contains dentinal tubules which are micro-canals radiating outwards. The dentine is porous and made up of inorganic material (70%), organic material (20%), and water (10%) [49]. The dentine is softer than enamel. Because of that the dentin decays faster than the enamel. This rapid decay leads to severe cavities if not treated properly.

Cementum is a substance found in the root covering the dentine. It is softer than either enamel or dentin. The composition of cementum is almost similar to bone. The difference is that cementum lacks vascularization. The cementum is made up of inorganic material (45%) (mainly hydroxyapatite), organic material (33%) (mainly collagen) and water (22%) [49].

The pulp contains blood vessels and nerves. The blood and nerve cells are supplied from the tissues of the jaw via the root. The nerves detect pain and the blood vessels nourish the dentine [47].

Mucosal surfaces

The mucosal surfaces in the mouth are the lips, cheek, palate and tongue. Each surface contributes to the diversity of the micro-organisms found in the mouth. The oral microbes have their favorite sites. For example, on the tongue micro-organisms are living in groups (e.g. the tongue acts as a reservoir for anaerobes).

The mucosal surfaces and teeth are kept moist and lubricated by the flow of saliva. Saliva is a fluid containing ions and proteins. The function of saliva is to moisture food, so it can be swallowed easily. Saliva protects teeth from dental decay by plaque by its pH buffering property (mainly by bicarbonate). The pH value of saliva is between 6.75 and 7.25 [47]. The buffering property reduce the cariogenic effect of acids formed by bacterial fermentation of dietary carbohydrates. The acids demineralize the tooth structure. Saliva contains also calcium and phosphate to help remineralize the tooth structure and to make teeth less susceptible to decay. Finally, saliva contains immunoglobulins which help counteract infections of the mouth.

Gingival crevices and periodontal pockets

The gingival crevice is the protected habitat formed where teeth rise out of the gum. In periodontal disease (see Section 2.2.4) the gingival crevice will develop into a periodontal pocket. This periodontal pocket, caused by inflammation, results in an increase of the flow of gingival crevicular fluid (GCF).

The gingival crevice is bathed by the gingival crevicular fluid (GCF) (Figure 2.7).
This serum-like fluid contains nutrients and components of the host defense. The GCF regulates the microflora of the gingival crevice in health and disease, for example by immunoglobulins, leucocytes and enzymes which are present in the fluid.

![Diagram illustrating the periodontal pocket and the flow of gingival crevicular fluid (GCF).](image-url)

2.2.2 Factors affecting the growth of micro-organisms in the mouth

There are several important factors affecting the growth of micro-organisms in the mouth [47]:

- **Temperature**
  The temperature in the mouth is kept constant at 35-36 °C. This is suitable for growth and metabolism of a wide range of bacteria.

- **pH**
  The pH in the mouth is regulated by the flow of saliva. Many bacteria grow at a neutral pH. However, the population or bacterial composition within a habitat can shift as a result of fluctuations in the pH. Frequent exposures to acids, for example after consumption of sugars, will result in growth or colonization of acid-tolerant species (e.g. *S. mutans*).

- **Nutrients**
  There are two types of nutrients: endogenous and exogenous. The latter are supplied by the diet. The best example is the intake of carbohydrates. These dietary
2.2. Dentistry

carbohydrates can be broken down to acids. The endogenous (provided by the host) nutrients are supplied by the saliva. Saliva contains amino acids, peptides, proteins, glycoprotein and vitamins.

- Redox anaerobiosis/potential

Anaerobiosis is the separation of micro-organisms into aerobes and anaerobes. It is based on the ability to grow in oxygen. Aerobes need oxygen to survive, while anaerobes do not. However, in reality there is a wide range of oxygen tolerance among the organisms.

Oxygen results in the oxidation of the environment. The degree of oxidation-reduction is given by the redox-potential, Eh. Anaerobes require a reduced environment for their metabolism. Therefore the growth of anaerobes at a specific site in the mouth is related to the redox-potential at that site.

2.2.3 Dental plaque

Dental plaque is a complex oral biofilm made up of hundreds of microbial species organized in communities embedded in a matrix of polymers of bacterial and salivary origin [50, 51]. Plaque is found at several sites in the mouth. Most of the plaque is found on teeth. It is mainly accumulated on the protected sites of the teeth, for example in fissures (fissure plaque) or the gingival crevice (gingival crevice plaque). It is natural to have plaque on the teeth. Plaque is an important part of the host defense, because it hinders the intrusion of pathogenic bacteria.

The plaque or dental biofilm results from the colonization of micro-organisms on the tooth surface. First colonization of plaque on the tooth starts with the formation of the pellicle. This pellicle is a conditioning film made up of molecules secreted by the host and bacteria; the molecules are adhering to the tooth surface. Then secondary colonization starts through interbacterial adhesion [52]. This process of adhesion is called co-aggregation or co-adhesion. This stage of interbacterial adhesion results in the diversity of the plaque. The increasing diversity of the plaque will finally result in the formation of a stable climax community. The attachment, growth, removal and re-attachment of the bacteria in the plaque is a dynamic process that continuously takes place, also at the climax community.

Vertical gradients of the physical and chemical parameters exist over short distances in plaque (e.g. nutrients, O₂, pH, redox potential) (Figure 2.8). These gradients influence the growth and survival of the bacteria in plaque.
Topography and vitality

Topography and vitality of undisturbed dental plaque was studied by Netuschil et al. [53] and Auschill et al. [54]. They used vital fluorescence techniques combined with Confocal Laser Scanning Microscopy (CLSM) to analyze the spatial structure of plaque. Plaques were grown on enamel slaps in the oral cavity and analyzed ex vivo.

The dental plaque samples showed an uneven spatial distribution of vital and dead bacteria [54]. According to the observations of Auschill et al. the percentage of vital organisms was lower adjacent to the enamel surface, increased towards the central part and decreased again towards the outer layers. The observations showed that the plaque samples contained voids. Also channels have been seen in other studies [47]. This confirms that dental plaque has a more open structure than previously thought.

Auschill et al. found that the vitality of the bacteria was dependent on the biofilm age. Several studies showed that young and sparse dental plaque consisted of more dead material than living micro-organisms [55, 56].

Dead material is a major plaque component during the initial stages of plaque formation [57]. Experiments of Netuschil et al. showed that living bacteria were located on top of dead material. Dead cellular material may supplement the living bacteria with all the material needed for their growth [58, 59] and protect the living bacteria against bactericidal factors.
Composition

Each site in plaque can have a different bacterial composition as a result of the vertical and also horizontal gradients. This diversity results in a heterogenous plaque consisting of many micro-habitats. Each micro-habitat can support a complete different microbial community.

The microbial population shifts in developing dental plaque [60] and is dependent on the age of the biofilm. The plaque can accumulate beyond normal or healthy levels. Too much plaque will lead to shifts in the composition and this can initiate disease.

2.2.4 Dental caries (decay) and periodontal disease

Dental caries is the localized destruction of tooth tissue by bacterial fermentation of dietary carbohydrates (sugars) [47]. The acids produced by the fermentation of these dietary carbohydrates demineralize the enamel. During this demineralization calcium and phosphate ions are transported into the surrounding environment and a lesion or cavity will form. Caries begins with small demineralization areas under the enamel. The demineralization can progress through the dentine to the pulp. A lesion or cavity that approaches the pulp (Figure 2.6) can cause intense pain.

"The dissolution of enamel, particularly in acidic solutions, is a chemical reaction which appears to follow a simple rate law, but the physico-chemical basis of the process is extremely complicated and only partially understood …(Patel et al., 1987) [61]."

The dissolution/demineralization of the enamel, that is primarily composed of hydroxyapatite (see Section 2.2.1), is given by the following net reaction:

\[ \text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2 + 8\text{H}^+ \rightleftharpoons 10\text{Ca}^{2+} + 6\text{HPO}_4^{2-} + 2\text{H}_2\text{O} \]

After a meal it is normal to lose some tooth mineral as a result of the bacterial fermentation of carbohydrates into acids. Tooth mineral is replenished by the saliva that contains calcium and phosphate. However, when there is frequent intake of fermentable food, the low pH is sustained and a net loss of mineral occurs. This sustaining acid environment favors the growth and selection of aciduric organisms, such as \textit{S. mutans} and various lactobacilli.

The acids are produced by bacteria adhering to the tooth surface in dental plaque. However, only a limited number of bacterial species in the plaque participates in caries or periodontal disease, for example \textit{Streptococcus mutans} bacteria and various lactobacilli. The \textit{S. mutans} pathogen is one of the major causes of caries because it can store
polysaccharide and is able to continue to secrete acid long after a meal has been taken. Various lactobacilli have an important role in the progression of the lesion.

Periodontal disease is also a common disease that is related to dental plaque. In short, periodontal disease is the detachment of the gum from the teeth as a result of inflammation. This inflammation can be a response to dental plaque. The bacteria in plaque produce compounds (e.g. antigens, endotoxins, etc.) that can elicit an inflammatory response. These inflammation responses can be divided into two groups: gingivitis or periodontitis. Eventually periodontal disease can result in the loss of periodontal tissue, formation of a periodontal pocket or even loosening and loss of the tooth. [47]

There are several methods to prevent and treat dental decay or periodontal disease. In the next section we will describe several methods to prevent and treat dental caries.

### 2.2.5 Prevention and treatment of caries

Mechanical removal of dental plaque by efficient oral hygiene (e.g. daily toothbrushing) can almost completely prevent caries (or periodontal disease) [47]. Other approaches to prevent caries are fissure sealants, antimicrobial agents (e.g. mouthwash), fluoride, and sugar substitutes (e.g. xylitol). Cavities will form if the caries prevention is not sufficient.

Preparation or reparation of cavities prior to filling is done by removing necrotic, infected and demineralized tissue by means of mechanical drilling, laser techniques or ozone treatment [62–73]. During mechanical drilling and laser treatment heating takes place and this is usually painful. Moreover, vibrations are induced [63] and this can also cause pain sensation in the patient. Moreover, these methods are often destructive: an excess of healthy tissue must be removed to ensure that the cavity is free of bacteria. In addition, the remaining tooth structure is weakened and prone to fractures [74]. An alternative painless and non-destructive method is ozone treatment [70–72]. Ozone is a powerful oxidizer, which is used as a disinfectant in the food industry, medicine and water treatment [75]. Recently, ozone gas has been used for caries treatment in dentistry (e.g. HealOzone®, USA).

Ozone is known to be a powerful oxidizer that effectively kills bacteria. Ozone also removes the byproducts and metabolites of bacteria in the carious lesion (e.g. it is able to break up acidic products of cariogenic bacteria). This has two effects: 1) no further demineralization, 2) removal of metabolites, which denies other bacterial types their nutrients [71]. Inaba et al. [76, 77] and Jorgensen et al. [78] found that an oxidant (sodium hypochlorite) removes the organic components in a lesion to enhance remineralization. Similarly, it is believed that ozone removes proteins in the carious lesion. After treatment, calcium and phosphate ions will diffuse into the lesion, resulting in remineralization.
Another painless and tissue-saving approach, which differs from the ozone therapy, is the use of cold atmospheric plasma. The principle of plasma treatment is the inactivation of bacteria in dental biofilms in a non-contact way [6, 79–81]. This plasma treatment differs from the ozone therapy in the nature of active species that are produced. The plasma generates ozone, but also short-living active species like O and OH radicals that are more powerful oxidizers/disinfectants than ozone [75]. The advantage of plasma treatment is that it allows irregular structures and narrow channels within the diseased tooth to be cleaned. The treatment is superficial and non-destructive; it does not cause bulk material removal. In contrast to lasers, plasmas can access small irregular cavities and fissure spaces; besides, the use of plasmas is relatively inexpensive.

2.2.6 Model systems

The prevention and treatment techniques can be tested against bacteria in planktonic state or in dental biofilm model systems. An overview of the model systems is given in the next section.

Bacteria in planktonic state

Survivor curves and D-values

Survivor curves are plots of the number of colony forming units per unit volume versus the treatment time. Colony forming units are colonies arising from pairs, chains, clusters or single bacterial cells. One common parameter that describes inactivation efficiency is the decimal reduction time (D-value), also called the ‘log reduction time’. The D-value is the time required to reduce an initial concentration of bacteria by 90 %, which is one decade on a log plot.

In the beginning of the plasma needle project we started culturing *Escherichia coli* bacteria, because *E. coli* organisms are relatively easy to culture and study. In the first bacterial inactivation experiments, the feasibility study (see Chapter 4), we used droplets of an *E. coli* suspension.

The plasma can be exposed to a droplet of an *E. coli* suspension. After exposure, the droplet is spread onto an agar plate with growth medium. The bacteria are grown for 24 hours at 37 °C on the agar plates. After this incubation time it is possible to count the colony forming units (CFUs). Each *E. coli* organism can form a single colony. So by counting the CFUs it is possible to calculate the initial concentration of *E. coli* from the control groups and the amount of inactivated *E. coli* bacteria.

Unfortunately the method of counting the inactivated colonies is not possible for the *S. mutans* bacteria, because these bacteria stick together and therefore a colony does not represent one single bacterial cell. Another disadvantage of the CFU method is that it
takes a lot of time and that it is prone to errors.

Another method we used was the treatment of *E. coli* films. This method is almost the same as the CFU method, except that one first spreads the bacterial suspension on the agar plate and allows the bacteria to attach for 1 hour. During this attachment the medium evaporates, so the bacteria are more exposed than in the case of a droplet. Just after attachment of the bacteria, the samples are directly exposed to the plasma. The plasma treatment of *E. coli* films results in formation of bacteria-free voids after 24 hours of incubation at 37 °C.

Bacteria in planktonic life show different properties from those in biofilms [82]. Conventional methods of killing bacteria are often ineffective against biofilms. So after the performing initial planktonic experiments, we started to use the more realistic dental biofilm or plaque model systems. The biofilms were cultured, treated and then analyzed *in vitro*.

**Dental biofilm model systems**

Complex model systems have been developed to predict the antimicrobial susceptibility of bacteria grown in oral biofilms. In these models dental plaque biofilms can be replicated and studied *in vitro*. Several of these artificial dental plaque model systems are described by Sissons *et al.* [83]. These include the growth-rate-controlled biofilm fermenters (GRBF), constant-depth film fermenters (CDFF), chemostat-based systems, and most recently the artificial mouth model systems (MAM). In this project we used two dental biofilm model systems: the CDFF and the MAM.

- **Artificial mouth model system (MAM)**
  
  In the artificial mouth model system, multi-plaque microcosms can be grown. A microcosm is defined as "a laboratory subset of the natural system from which it originates but from which it also evolves" [84]. The microcosms used in the artificial mouth model system by Sissons *et al.* contain hundreds of species of bacteria and are presently the closest laboratory replication to plaque in the human mouth. This model is labor-intensive and requires specialist equipment. However, the plaque biofilms that are generated appear to reflect the complexity, diversity and heterogeneity of *in vivo* plaques [85, 86]. In Chapter 7 of this thesis a description and the results of a simplified MAM can be found. This simplified model is called the 'Microplate Biofilm Microcosm' (MBM) model.
• **Constant-depth film fermenter (CDFF)**

The CDFF allows reproducible dental biofilms of fixed thickness and diameter to be grown. The advantages of the CDFF are:

- almost any substratum material can be used
- media flow rate and supplements can be introduced
- replicates of equivalent biofilms can be obtained
- environmental conditions can be controlled

During the project we performed a pilot study with a constant-depth film fermenter (CDFF) at the Academisch Centrum Tandheelkunde Amsterdam (ACTA). Because of lack of significant data we were unable to make decisive conclusions. The results are not presented in this thesis.

### References


Plasmas in dentistry: an overview


Chapter 3

Characterization of the plasma needle

Abstract– In this chapter we characterize a low-power radio-frequency atmospheric plasma (plasma needle) in terms of dissipated (input) and emitted power per unit surface (power outflux). The plasma is a non-thermal source, used for treatment of biological tissues and other vulnerable surfaces. A calibrated thermal probe is used to determine the power emitted from the plasma towards treated surfaces. Transmission of the emitted plasma power through various media (solid layers, fluids and physiological media) is studied for a broad range of plasma conditions. These data give insight into various contributions to the power outflux (thermal conduction, radiation and energetic species), as well as the penetration depth of the plasma into treated objects. The power outflux is shown to be a very important parameter, which determines the performance of the plasma tool. For the effectiveness and reproducibility of the process the power outflux is much more important than the nominal power setting. Thus, a thermal probe should become a standard control unit in surface processing reactors.

3.1 Introduction

Plasma treatment offers many possibilities of refined surface modification. Plasma tools are fast, efficient and extremely versatile: the effects range from surface activation and superficial cleaning to deep etching, from deposition of monolayers to massive material spraying. Just because of this versatility, fine tuning of plasma conditions is crucial to achieve the desired surface modification. In the industrial process control, external parameters like voltage and forwarded power are monitored. However, control of these external settings does not always guarantee the reproducibility of plasma conditions. Therefore, extensive studies were performed to translate the control parameters into the true plasma characteristics.

One of the issues often encountered is the power dissipation in the plasma. The actual plasma power is often different from the externally imposed value, and its accurate determination can be a difficult task. The problems arise especially when the voltage and current waveforms cannot easily be monitored, and when the electric circuit contains dissipative elements other than the discharge. Most surface-processing plasmas are generated by radio-frequency (RF) excitation. The circuit is an RF transmission line, terminated by the load (plasma). To assure optimal power dissipation in the plasma (no reflected power), the impedance of the load must be matched to equal the impedance of the source (generator). This is achieved by inserting a tunable matching network between the source and the plasma. Nominally, the matching network should not dissipate any power, because it consists of imaginary impedances (capacitors and coils). However, in real situations stray resistances are present and consequently power losses in the matching network can be substantial. In low-pressure capacitively coupled reactors the losses can be up to 20 % [1]. In micro-plasmas, the energy coupling is particularly inefficient: the power dissipated by the electronic components can be orders of magnitude higher than the actual plasma power. Hence, in such cases plasma power measurements are less reliable and do not give a good characterization of the plasma. Since plasma power is often used as an input parameter in numerical models, discrepancies arise between experimental and simulation results.

In this chapter we consider another means to describe a discharge. Instead of power coupled into the plasma (input power), we propose to monitor the power emitted out of the plasma per unit surface (power outflux). Naturally, there is a relation between these two magnitudes. However, at a constant input power, the outflux can vary dependent on plasma composition, pressure, convection (gas flow) and spatial position. This power outflux is much more relevant in surface treatment, because it provides direct information about the energy doses received locally by the surface. Moreover, power outflux measurements allow the separation of the various mechanisms of energy transfer to the surface: heating by thermal conduction/convection, radiation heating and
energy transfer from active particles (electrons, ions, radicals and excited species).

Power outflux can be measured by a thermal probe. This simple and powerful method has been developed by Kersten et al. [2] and applied to many low-pressure discharges for surface processing [3, 4]. The probe is versatile: it can be used in low-pressure as well as atmospheric discharges, and also at elevated temperatures. The shape and size of the probe can be adapted for the local reactor geometry, so as to minimize its effect on the discharge. Here, the efficiency and convenience of a thermal probe for characterization of atmospheric plasmas will be illustrated for the example of a small RF discharge (plasma needle) [5]. A plasma needle is used for refined medical treatment of living organisms, human/animal cell manipulation and bacterial disinfection in the body. The reliability and reproducibility of the medical therapy requires extremely careful dosage of plasma; the actual energy received by living cells conditions the success of treatment. It will be shown that a thermal probe offers an excellent method to monitor this energy and thus to control the medical instrument.

3.2 Experiment

3.2.1 The plasma set-up

For treatment of biological samples (cells in culture) a metal/plastic plasma chamber, shown in Figure 3.1, is used. The RF-powered needle (13.56 MHz) is a sharpened tungsten wire with a thickness of 0.3 mm, mounted in the sidewall of the chamber. For the generation of the RF signal a Hewlett Packard 33120A waveform generator and a RF amplifier (Amplifier Research 75AP250RF) are used. The plasma operates most readily in helium, but admixing other gases (O$_2$, N$_2$, N$_2$O) up to a few per cent is possible. The helium flow supplied to the chamber and controlled by a mass flow meter (Brooks series 5850E) is typically 2 l min$^{-1}$. The gas is supplied through an orifice in the sidewall opposite to the one where the needle is mounted. The volume of the chamber is about 0.5 liter; gas refreshment time is thus 15 s. The chamber is not vacuum tight; the estimated amount of air due to leakage is about 0.5 %. This amount of air is sufficient to create active radicals that modify biological samples; there is no need to admix other gases.

This geometry is adapted for treatment of cells in culture [6]. The samples (Petri dishes and microscope glasses) are placed on the platform at the bottom of the chamber. The chamber is supplied with two external manipulators: one to move the sample platform horizontally, and another one to adjust the vertical distance between the needle and the sample surface. This vertical distance has a substantial influence on the plasma. Typical images of the glow are shown in Figure 3.2. When the (grounded) sample is
remote (more than 2 mm away from the RF needle), the discharge is feeble and concentrated at the needle tip. In the close vicinity of a grounded object the glow spreads over its surface and increases in brightness. Note that the vertical distance is not measured along the needle axis: the needle is inclined at about 35° to the platform. Nevertheless, the orientation of the needle does not affect plasma behavior: the glow always seeks the shortest distance to the surface.

Figure 3.2: Two typical appearances of the glow. Note that this plasma is not created in the plasma chamber, but at the tip of the portable needle. Left: the glow confined at the needle tip, right: the glow in contact with a surface.
3.2.2 The power measurement

Power transmission from the RF source to the load (plasma) is maximized by means of an adjustable matching network. An Amplifier Research PM 2002 power meter with a dual directional coupler is placed between the source and matching network to monitor the forwarded and reflected power. The nominal power (the difference between the forwarded and reflected powers) contains the power consumed by the plasma, but also RF radiation and losses due to ohmic heating in the matching network and cables. These elements are supposed to be low-loss, but still their contribution to the total power consumption can be on the order of several watts. In standard RF discharges (10-100 W) it can result in substantial errors in power determination (10-30 %). In micro-plasmas, which dissipate only milliwatts of power, losses in the electronics are overwhelming. Therefore, accurate determination of the actual power dissipated in the plasma is a troublesome task.

The plasma power can be evaluated using the subtraction method proposed by Horwitz [7] and Godyak and Piejak [8]. The difference in the nominal power with and without plasma at the same electrode voltage is a good estimate for the dissipated power. With this method, the discharge voltage must be measured using an attenuating voltage probe placed as close as possible to the powered electrode; the voltage-power curves must be determined and subtracted. However, for the micro-plasma (needle) circuit the voltage probe is a substantial dissipative element, so its usage is not recommended. Fortunately, the needle voltage does not change much when the plasma is extinguished. This is because the discharge forms a relatively small load, which does not have a large influence on the circuit. Therefore, the needle voltage has not been monitored during all power measurements. The discharge is switched off by opening the chamber and thus admitting large amounts of air into the plasma.

3.2.3 The thermal probe

The thermal probe is a round copper substrate with a diameter of 3 mm and weight of 0.13 g, which is spot welded to a thermocouple (type j). The copper substrate is mounted at the bottom of the chamber through a special opening in the platform. The temperature is recorded by the thermocouple as a function of time while the discharge is switched on and off. The power flux towards the probe (\( J \)), i.e. the local plasma power outflux at the position of the probe, is calculated from the following:
plasma on (temperature rise):

\[ c_p m_p \frac{dT}{dt} = J - J_{\text{loss}} \]  

(3.1)

plasma off (cooling):

\[ c_p m_p \frac{dT}{dt} = -J_{\text{loss}} \]  

(3.2)

A typical time–resolved temperature curve is shown in Figure 3.3. The rise and fall parts of the curve are fitted with exponential functions. The cooling part allows the determination of the heat losses \( J_{\text{loss}} \). Since the mass \( m_p \) and thermal capacity \( c_p \) of the probe substrate are known \( (c_p m_p = 0.05 \text{JK}^{-1}) \), the power flux \( J \) is readily determined. The temperature rise is low \( (2-3 \, ^\circ\text{C}) \), so that the thermal capacity can be assumed constant. The measurement takes several seconds; it is assumed that the fluxes do not vary at this time scale. Measurements and fitting have been repeated several times; the uncertainty is 10 - 20 %.

Figure 3.3: A time–resolved response of the thermal probe. The temperature rises at the onset of the plasma.

### 3.3 Results and discussion

This experiment had two goals. The first one is the characterization of the plasma: the power outflux must be known in order to control the conditions under which vulnerable samples are treated. This is especially important in the treatment of living cells, which may not receive high energy doses. Thus, measurements of power outflux under various conditions relevant for cell treatment have been performed and analyzed.
Special attention was given to the transmission of the plasma power through various media. Mechanisms of power dissipation in a medium are discussed. The second goal is understanding the mechanism of energy transfer from the ionized gas to the substrate. The power outflux from the plasma consists of several contributions. It will be shown that their relative importance can to some extent be deduced from the thermal probe measurements.

The power outflux from the plasma can be split into the following terms:

\[
J = \kappa \nabla T + J_{unst} + J_{radiation}
\]

(3.3)

\[
J_{unst} = J_e + J_+ + J_{radicals}
\]

(3.4)

The first contribution (\(\kappa \nabla T\)) is the usual energy transfer by thermal conduction. \(J_{unst}\) denotes the contribution of unstable particles (species with a short lifetime): electrons, ions, active radicals, excited atoms and molecules. Ions and electrons heat the substrate by transferring their kinetic and recombination energies. Excited species can transfer their excitation energy, radicals can undergo exothermic association or other chemical reactions. Unstable plasma species heat the surface which is in direct contact with the active zone (glow). They are typically not present in remote afterglow zones (with the exception of certain metastable species such as the oxygen singlet \(^1\Delta_g\)), and can be discarded by putting a shield between the plasma and the thermal probe.

Figure 3.4 describes the behavior of the input power and power outflux at various vertical distances from the needle tip (\(d\)). Note that the input power increases significantly when the needle approaches a grounded surface. This effect is related to the increase in the imposed field strength, which roughly obeys \(E = V_{RF} / d\). A higher field results in enhanced ionization and higher electron density. Since in RF discharges electrons are responsible for the energy uptake from the field, the power dissipated in the discharge must also become higher. The effect of increased input power is significant at short needle to surface distances. This can be coupled with a visual observation: at \(d\) shorter than 1.5-2 mm the glow extends to the surface and increases in intensity. The power outflux varies with distance as \(d^{-3.3}\) (Figure 3.5). Since this is not an \textit{a priori} obvious relation, we have compared it with the calculated ‘homogeneous power outflux. The latter is based on an assumption that the outflux equals the power input \(P_{in}\) (See Section 5.2.2) distributed equally in space: \(J_{hom} = P_{in} / (4\pi d^2)\). Since \(P_{in}\) changes with distance, \(J_{hom}\) varies as \(d^{-3}\). Both ‘homogeneous and actual power curves are plotted in Figure 3.5. It is evident that when the needle-surface distance is large, the power is emitted homogeneously. At \(d = 2\) mm, coincident with the visual changes to the glow (extension towards the surface), the power outflux becomes directional. In Figure 3.6, the power transfer efficiency (ratio of the power absorbed by the substrate to the input
power $P_{in}$) is shown. At $d < 1.5$ mm, nearly all plasma energy is deposited on the surface.

Transmission of plasma power through various media is an important issue for medical plasma applications. Living cells or tissues are always covered with a layer of physiological fluid, otherwise they would be damaged due to desiccation. In the following paragraph we will investigate how the plasma power outflux is transmitted through a fluid layer. Two liquids have been studied: demineralized water and phosphate-buffered saline (PBS). The latter is a physiological electrolyte, containing 0.1 mM NaCl. Heat conduction and chemical reactions are the same in both liquids, but PBS and water have significantly different electric conductivities. The latter may possibly have an influence on the plasma operation. In each case, 10 µl of liquid has been pipetted onto the probe to produce a 1.5 mm thick layer. Power outflux collected by the probe has been measured as a function of the distance between the needle and the probe surface, with and without the liquid layer (at the same input power). These data provide a direct estimate of power losses in the liquid medium, as compared to the gas phase. Figure 3.7 shows the fluxes through water/PBS, and the corresponding data without liquid from Figure 3.4 (fitted). Power flux transmitted through the liquid appears to be somewhat lower than through the gas. Furthermore, there is no evident difference between water and PBS. Several mechanisms of power dissipation in the medium are discussed in the following paragraph.

In the next experiment, power outflux has been recorded as a function of the input power. Two situations have been considered: a 1.5 mm thick layer of liquid and the needle at a fixed distance from the probe surface $d = 3$ mm (the distance between the needle and the water/PBS surface is thus 1.5 mm), and $d = 1.5$ mm in the absence of the liquid. The latter situation, without the medium, allows the determination of the flux incident at the water/PBS surface. A comparison of these two cases gives an estimate of the losses in the 1.5 mm layer of liquid medium. The results are shown in Figure 3.8 (no liquid) and Figure 3.9 (water and PBS). To discard the experimental scatter, these data have been fitted and divided by the incident flux (given in Figure 3.8). The resulting transmitted flux is shown in Figure 3.10. It is clear that more than 90 % of the incident energy is lost in the medium. In helium only, without water, the flux at $d = 3$ mm is 11.5 % of the one at $d = 1.5$ mm. The power losses in the liquid are thus higher than in the gas, in agreement with the data presented in Figure 3.7.
3.3. Results and discussion

Figure 3.4: The power outflux collected by the probe in a helium plasma (●), and the input power (○), determined for various distances between the needle tip and the probe surface.

Figure 3.5: The measured power outflux (●) and the predicted ‘homogeneous’ power outflux (○), for various needle-to-probe distances.
Figure 3.6: The percentage of the input power absorbed by the substrate (probe) as a function of the needle–to–probe distance.

Figure 3.7: Power outflux recorded under the same conditions as in Figure 3.4, but in the presence of a 1.5 mm thick layer of liquid. • – water, ◦ – PBS, –– power flux at the same position without liquid.
3.3. Results and discussion

![Graph showing power outflux vs power](image1)

**Figure 3.8:** The power outflux in helium (no liquid) determined at the needle-to-probe distance $d = 1.5$ mm, for various input powers.

![Graph showing power outflux with liquids](image2)

**Figure 3.9:** The power outflux in the presence of liquids, determined at $d = 3$ mm (1.5 mm liquid and 1.5 mm helium), for various input powers. ● – water, ○ – PBS.

In an equilibrium situation, the absorbed plasma energy should be transformed into heat. From the thermocouple data it is easy to estimate the power loss due to heating of the liquid. The consumed power $P_{\text{heat}}$ can be calculated from the measured temperature increase of water ($\Delta T$) after a certain time $t$ ($P_{\text{heat}} = c_{H_2O}m_{H_2O}(\Delta T/t)$). The temperature increase after $t = 2$ s is 0.5–2°, dependent on the input power. The consumed power can be normalized to the power flux incident at the liquid surface (from Figure 3.8), to obtain the fraction needed to heat the liquid. This fraction is $60 \pm 5\%$, independent of the power input. Thus, heating can account only for a part of the total power loss (90 %), and the remaining $30 \pm 5\%$ must be consumed in other processes.
Characterization of the plasma needle

Figure 3.10: Fitted data from Figure 3.9 – fluxes in helium at 1.5 mm and with 1.5 mm water/PBS at 3 mm (---). The ratio of the flux in liquid to the flux in helium (--) yields the power fraction transmitted through the liquid. ●: ratio of flux at 3 mm to that at 1.5 mm, in helium without liquid.

The above data confirm the expectation that the plasma is not an equilibrium system. Energetic plasma particles, which contribute to the energy flux ($J_{\text{unst}}$ in equation 3.4), can perform endothermic reactions and create new species. For example, helium ions carry a potential energy of 24.56 eV (recombination energy) and a kinetic energy of several electronvolt (typical plasma potential). This is enough to dissociate several molecules. In aqueous solutions, helium ions can produce OH radicals and peroxides. The same is valid for helium metastables and for UV radiation under 200 nm. Part of the power used in (photo)chemical reactions can eventually be converted into heat. However, in our situation at least 30% of the incident power is dissipated in a non-thermal way. This can occur in endothermic (photo)chemical reactions with the liquid.

Chemical reactions in the liquid can occur only when the active particles from the plasma (ions, radicals, etc) can reach the liquid surface. These species are unstable; they are lost in the gas phase due to recombination and inelastic collisions with He atoms. Their density decreases drastically with increasing distance from the source. Thus, the larger this distance, the smaller the contribution of chemical reactions. In Figure 3.11, the flux transmitted through the 1.5 mm liquid layer for several distances $d$ between the probe and the needle is compared with the flux incident at the surface (data without liquid at $d = 1.5$ mm). The losses in the medium are substantially higher at short distances from the needle. This supports the hypothesis of power dissipation due to reactions with unstable plasma species.

Finally, the contribution of plasma radiation in the visible and the near UV/IR region has been evaluated. For this purpose, active plasma species have been blocked by a glass plate with a thickness of 1 mm (76 × 26 mm). The plate has been placed
in front of the probe surface, but without contact with the surface. The measurements have been performed at the needle–to–probe distance \( d = 1.5 \text{ mm} \), as a function of the input power. Subsequently, a thin non-transparent sticker has been placed under the glass plate. The sticker does not significantly change the heat capacity/conductivity of the system, but it efficiently blocks plasma radiation. The results are shown in Figure 3.12. The contribution of visible plasma radiation increases with increasing input power and reaches up to 30% of the thermal outflux.

Summarizing, the above data show that the power collected by the substrate strongly depends on absorption in the medium. About 90% of the plasma power outflux incident at the medium surface is blocked: about 60% is consumed in heating, and about 30% is dissipated in (photo)chemical reactions. The remaining 10% transmitted through the medium accounts for heat conduction and radiation (visible and near UV/IR). The contribution of radiation to the transmitted flux can be up to 30%; for water this number may be higher than for glass, because water is transparent in a broader spectral range than glass. In all investigated cases, the energy doses received by the sample can be considered low. In treatment of vulnerable living cells, it should not lead to serious cell damage, in agreement with [6].
Characterization of the plasma needle

3.4 Conclusions

A thermal probe is a powerful means of characterizing the plasma. Unlike other measurable parameters, such as input power, current and voltage, the power outflux from the plasma towards a substrate gives direct information about plasma performance. In surface processing, especially in the case of sensitive materials, reliability of the source and reproducibility of the treatment are crucial. The power outflux appears to be a good gauge: it allows the monitoring of local plasma conditions, which are not reflected by the global parameters (e.g. input power). Here we use the power outflux to characterize a micro-plasma, for which input power measurements are troublesome and not very accurate. A thermal probe provides much more information than the mere magnitude of the power outflux: it allows determination of its spatial distribution and direction. In this work an atmospheric micro-plasma (plasma needle) was taken as an example. It was shown that the power outflux from this plasma is directional, and that most of the plasma energy can be deposited on the surface. Moreover, estimates can be made of various contributions to the power outflux: thermal conduction, radiation and chemical reactions. Power dissipation in various media (e.g. liquids) can be measured and the energy consumed in chemical modification of the medium can be separated. This information is extremely important in the treatment of biological samples, such as cells and tissues. In the plasma needle treatment it is shown that the energy received by the sample is too low to cause serious damage; this is confirmed by previous experiments where no cell necrosis was observed. The response of the sample is caused by chemical interactions between the (plasma modified) medium and the cells.
3.5 Acknowledgments

This work is supported by the Netherlands Organization for Scientific Research (NWO).

References


Chapter 4

The plasma needle in dentistry: a feasibility study

Abstract—Much effort is invested in the development of tissue-saving methods in dentistry. Cleaning and sterilization of infected tissue in a dental cavity or in a root channel can be accomplished using mechanical or laser techniques. However, with both approaches, heating and destruction of healthy tissue can occur. Recently, a non-thermal atmospheric plasma (plasma needle) has been developed. In this work, the interactions of this plasma with dental tissue are studied, and its capability of bacterial inactivation is tested. A plasma needle is an efficient source of various radicals, which are capable of bacterial decontamination; however, it operates at room temperature and does not cause bulk destruction of the tissue. Plasma treatment is potentially a novel tissue-saving technique, allowing irregular structures and narrow channels within the diseased tooth to be cleaned.

4.1 Introduction

Dental cavities as a result of caries are common ailments, and the improvement of treatment methods is a major issue in dentistry [1]. Preparation of cavities prior to filling is done by removing necrotic, infected, and non-remineralizable tissue by means of mechanical drilling or laser techniques [2]. With both methods, heating takes place. Moreover, there are also vibrations during mechanical drilling [3]; this usually is painful for the patient, because heating and vibrations can stimulate the nerve. Moreover, these methods are often destructive; an excess of healthy tissue must be removed to ensure that the cavity is free of bacteria, and fractures can occur.

Our goal is to find a less destructive (no fractures and no unnecessary material removal), and less painful (no heating) method to prepare dental cavities. This may be done by using a non-thermal atmospheric plasma.

A plasma, which is generated by an electrical discharge in a gas, contains free electrons and ions, various active species (e.g., atomic or molecular radicals such as O and OH, and excited molecules) and energetic UV photons [4]. The sterilizing properties of plasmas are well-known [5], [6]. Non-thermal atmospheric plasmas operate at room temperature and do not cause pain and bulk destruction of the tissue [7]. Plasmas can treat and sterilize irregular surfaces; hence, they are very suitable for decontaminating dental cavities without drilling. The advantage of this novel tissue-saving treatment is that although the plasma itself is superficial, the active plasma species it produces can easily reach the inside of the cavity. In contrast to lasers, plasmas can access small irregular cavity and fissure spaces. Moreover, the use of plasmas is relatively inexpensive.

In this study, temperature in the tooth during plasma treatment and the efficiency of the plasma in killing bacteria are investigated.

4.2 Experimental set-up

The portable plasma needle (Figure 4.1) consisted of a tungsten needle (0.3 mm diameter) confined in a Perspex tube (4-mm inner diameter) [8]. Because the voltage (200 V peak-to-peak) needed to ignite a plasma is lowest in a helium atmosphere, the plasma operates in helium-air mixture. The Perspex tube was filled with helium delivered at a flow rate of 2 l min$^{-1}$, controlled by a mass flow controller (Brooks Instrument, model 5850E).

A radio-frequency (RF) voltage was applied to the needle at 13.05 MHz. The signal was produced by a waveform generator (Hewlett Packard, model 33 120A) and an RF amplifier, about thousand times amplification, (Amplifier Research, model 75AP250), with a home-built matching network (Figure 4.2). The matching network consisted of a
4.2 Experimental set-up

Figure 4.1: Portable plasma needle consists of a tungsten wire in a Perspex tube attached to a source of helium. A radio frequency voltage is applied to the tungsten electrode to generate a plasma.

4.4 µH inductor placed in series with the plasma capacitance. A shunt resistance of 50 Ω was placed in parallel with the inductance and the plasma in order to properly terminate the coaxial cable. The dissipated power was monitored using a P6103 Tectonics probe (Amplifier Research) connected via a dual-directional coupler to a power meter (Amplifier Research, model PM 2002). The maximum dissipated power was 90 mW.

Figure 4.2: Schematic overview of the experimental set-up includes the electronics used to apply a radio-frequency voltage to a tungsten wire electrode, which is placed in a Perspex tube filled with helium.

The helium-air mixture is important for the intended application in dentistry because the plasma must be applied in the air-filled oral cavity. Moreover, the presence of air guarantees the formation of active radical species that play an important role in sterilization [5]. In the present configuration, the air content was adjusted by pushing the needle inside or outside the Perspex tube. The more the needle tip protruded from the tube, the more air was present in the plasma.
4.3 Temperature measurements

One of the two primary goals of the study was to determine the temperature change in the pulp chamber during plasma treatment of the enamel surface. In order to preserve the viability of the pulp, it is important to limit temperature rises within the tissue [9]. According to Zach and Cohen [10], an increase in intra-pulpal temperature of less than 2.2 °C falls within a safe range of thermal stress. However, a rise in internal temperature of 5.5 °C causes pulpal necrosis in 15% of the cases, and a temperature increase of 11 °C results in pulpal necrosis in 60% of the cases.

Thus, in order to establish whether the plasma needle can be safely applied to a tooth, temperature measurements were made inside the pulpal chamber using an in vitro test procedure with extracted human third molars (Figure 4.3). The College of Dental Science of the University of Nijmegen supplied these extracted human third molars.

Platinum resistance temperature detectors (RTDs) were used to measure the temperature (Labfacility, model PT-100, DM-503). RTDs contain a sensor element, which is an electrical resistor that changes resistance with temperature. Platinum RTDs have resistance versus temperature characteristics that are temporally very stable.

For calibration, the detector was placed in a climate cabinet (Grenco B.V., model GKSD220.40) and heated to temperatures ranging from 20 °C to 60 °C. A temperature transmitter (Status Instruments, model SEM210) converted the output of the PT-100 to a milli-Ampere transmission signal. This current was converted to a voltage using a 50 Ω resistance. Changes in voltage as a function of temperature were recorded with a sample frequency of 2 Hz using a TUeDACS/1 recorder (Eindhoven University of Technology, The Netherlands) connected to a personal computer. The calibration showed that $T(°C) = 6.18 \cdot V(V) - 2.86$.

To make the pulp chamber accessible for a PT-100 temperature detector, the distal root was amputated and the distal canal was enlarged. Subsequently, a portion of the pulp tissue was removed to allow placement of the temperature sensor [11] (Figure 4.3). The sensor was fixed in place using Cavit (3M) temporary restorative material.

Two variables were investigated: RF voltage and duration of treatment. Preliminary results show that temperatures increase somewhat with increasing plasma voltage (Figure 4.4).
4.3. Temperature measurements

Figure 4.3: (Left) Placement of detector in pulp chamber. (Right) Temperature measurement during plasma treatment.

Figure 4.4: Temperature recorded by a PT-100 temperature sensor inserted into the root channel of an extracted human tooth as a function of time and applied voltage. The curves correspond to different peak-to-peak RF voltages (from bottom to top curve) of 120, 140, 160, 180, 200, and 220 mV (before amplification). Plasma was switched off at $t = 60\, \text{s}$. The peak in temperature occurred at 100 s; afterwards, the tissue cooled down passively. The distance between the needle tip and the tooth was approximately 1.0 mm.
At 220 mV (before thousand times amplification), the temperature rise is about 2.3 °C, which should be safe according to Zach and Cohen [10].

In general, these data suggest that the temperature increase in the pulp of a tooth can be kept within a safe range.

The temperature was determined in a stationary situation, without blood flowing through the tooth. The latter might affect the results due to conduction effects. In the next step, temperature measurements in an irrigated tooth preparation were performed. We expected that conduction would further reduce the heating of the tooth.

### 4.4 Bacterial decontamination

The second aim was to examine the effects of different treatment times on the survival of *Escherichia coli*.

#### 4.4.1 Preparation of cells for exposure

*Escherichia coli* (Cat. no. 69825, Novablue competent cells, Novagen) were inoculated from a glycerol stock into 4 ml of LB-medium, which contained 0.5 % (w/v) yeast extract, 1.0 % (w/v) pepton from casein, 1.0 % (w/v) sodium chloride (Merck, Germany), and demineralized water; the mixture was shaken at 200 rpm for 15-20 h at 37 °C.

Then, 1 ml of the cell suspension was resuspended in 30 ml of the same LB-medium. The culture was grown at 37 °C with shaking (200 rpm) for 2 h. The growth curve of the culture was monitored photometrically by reading the optical density (OD) at 600 nm (UV/VIS Spectrophotometer, Lambda 3B, Perkin Elmer). When an OD\textsubscript{600} = 0.5 was observed, indicating the beginning of the log-growth phase, 50 µl of a 500× dilution of the cell suspension was pipetted onto a nutrient agar media in a sterile petri dish. The media include 0.5 % (w/v) yeast extract, 1.0 % (w/v) pepton, 1.0 % (w/v) sodium chloride, 2.0 % (w/v) Bacto\textsuperscript{TM} agar (Becton, Dickinson and Company, Franklin Lakes, NJ), and demineralized water.

Immediately after seeding, the samples were directly exposed to the plasma for 10, 20, 30, 40, 50, 60, 90, and 120 s, at both 190 and 220 mV. These voltages were chosen in order to keep the temperature increase within the safe range. The distance between the plasma needle and the substrate was approximately 1.0 mm.

After plasma treatment, the dishes were incubated at 37 °C for 24-48 h. Colony-formed *Escherichia coli* bacteria on the agar plates were counted to determine the number of survivors.
4.4.2 Sterilization rate (killing curves)

Bacterial viability was substantially reduced after exposure to the plasma. The preliminary sterilization results obtained for two plasma conditions; those produced with voltages of 190 mV and 220 mV (Figure 4.5) show that the number of colony forming units of *E. coli*, plotted logarithmically, decreases as the treatment time increases.

The Decimal Reduction Time (D-value) is a common parameter that describes decontamination efficiency. It is defined as the time needed to kill 90% of cells, which is one decade on a log plot. D-values are 39 s at 190 mV and 45 s at 220 mV. The difference in killing rates for these plasma conditions is not significant.

For the sterilization of dental cavities, it is necessary to inactivate two decades (99%) of bacteria [12]. This implies that treatment time with the needle should be about 1.5 min. Possibly, for the deactivation of plaque/caries bacteria (e.g., *Streptococcus mutans*), shorter exposure times would be sufficient. These oral bacteria are known to be much more vulnerable than *Escherichia coli* [6], which normally is resident in the lower intestinal tract.

These results demonstrate that the plasma needle is capable of bacterial sterilization. These D-values for the plasma needle, 39 and 45 s, are higher than those for other atmospheric plasmas [5]. However, the other atmospheric plasma sources described in

**Figure 4.5: Number of colony forming units of E. coli as function of time of treatment by He plasma. 190 mV (▲, solid line) and 220 mV (■, dashed line)(N = 1).**
literature are large in size and consume more electric power. Furthermore, their action is less refined; the electric voltages and the gas temperatures are too high and many chemically aggressive species are produced. Therefore, even though they are more efficient in sterilization, they cannot be applied \textit{in vivo}. In contrast, the plasma needle is well adapted for treatment of small and vulnerable objects.

In summary, the non-thermal character and good sterilizing properties of the plasma needle make it a very promising tool for preparation of dental cavities.

### 4.5 Conclusion

Application of non-thermal plasmas in treatment of caries is a challenging, multidisciplinary research problem, requiring expertise from both plasma physics and dentistry. Recently, a novel plasma source, the plasma needle, has been developed. Because it is non-destructive for human tissues, it can be applied \textit{in vivo}. We have demonstrated that the plasma does not cause significant heating of the dental pulp, and it has a good capability for killing bacteria.

### References


Chapter 5

Deactivation of *Escherichia coli* by the plasma needle

**Abstract**– In this chapter we present a parameter study on deactivation of *Escherichia coli* (*E. coli*) by means of a non-thermal plasma (plasma needle). The plasma needle is a small-sized (1 mm) atmospheric glow, sustained by radio-frequency excitation. This plasma will be used to disinfect heat-sensitive objects; one of the intended applications is *in vivo* deactivation of dental bacteria: destruction of plaque and treatment of caries. We used *E. coli* films plated on agar dishes as a model system to optimize the conditions for bacterial destruction. Plasma power, treatment time and needle-to-sample distance were varied. Plasma treatment of *E. coli* films results in formation of a bacteria-free void with a size up to 12 mm. $10^4 - 10^5$ colony forming units are already destroyed after 10 s of treatment. Prolongation of treatment time and usage of high powers did not significantly improve the destruction efficiency: short exposure at low plasma power is sufficient. Furthermore, we studied the effects of temperature increases on the survival of *E. coli* and compared it with thermal effects of the plasma. The population of *E. coli* heated in a warm water bath started to decrease at temperatures above 40 °C. Sample temperature during plasma treatment was monitored. The temperature can reach up to 60 °C at high plasma powers and short needle-to-sample distances. However, thermal effects cannot account for bacterial destruction at low power conditions. For safe and efficient *in vivo* disinfection, the sample temperature should be kept low. Thus, plasma power and treatment time should not exceed 150 mW and 60 s, respectively.

---

5.1 Introduction

Deactivation of harmful bacteria can be performed by applying chemical or physical factors. Conventional methods of sterilization involve heat (steam and dry autoclaves), chemical agents (active gasses like chlorine and ozone, or solutions) and irradiation (UV, gamma). Most of these techniques can cause damage to the treated substrate.

An alternative method of sterilization is treatment with non-thermal plasmas [1–3]. These plasmas operate under moderate temperatures and use non-toxic gases, so thermal and chemical damage to the substrate is limited. Bacterial deactivation has been reported in low-pressure as well as atmospheric plasmas. Plasmas can be used in the sterilization of medical instruments, in air cleaning systems or as counter-weapons in biological warfare. The bactericidal factors in plasmas are active radicals (e.g. O, OH), energetic ions or metastables and vacuum UV (VUV) radiation. The latter is said to play a significant role in low-pressure sterilization, but it is of less importance in atmospheric plasmas [2]. In particular VUV photons (≤ 180 nm) at atmospheric pressure are strongly reabsorbed, which prevents them from reaching the samples.

Atmospheric non-thermal plasmas are suitable for the treatment of heat-sensitive biological tissues. Recently, the effects of these plasmas on living cells and tissues have been studied. It appears that plasma treatment may find many medical applications, like high-precision surgery (controlled tissue removal), improvement of wound healing and disinfection. A plasma source for biomedical applications (plasma needle) has been developed at the Eindhoven University of Technology. It is small-sized and flexible, and it can be operated in the open air. These features are necessary for in vivo treatment of biological tissues, like wounds and dental cavities. Responses of living mammalian cells to plasma treatment have been studied and a method of cell removal without necrosis was found [4–6]. Applications of plasma treatment in dentistry were studied by Sladek et al. [7]. Tests on plasma deactivation of dental bacteria [8] and biofilms [9–11] are conducted to develop a new technique for non-destructive treatment of dental caries and periodontal pockets [12, 13].

Although gas heating in non-thermal plasmas is limited, it always remains an issue that has to be carefully considered in the treatment of living organisms. Cells and tissues are extremely heat-sensitive; e.g. heating of dental pulp is painful and causes serious damage [14]. Therefore the heat produced by the plasma has to be minimized by choosing appropriate operating parameters. In this chapter we present the results of plasma- and heat-induced deactivation of Escherichia coli bacteria. We have chosen E. coli as a model, because these micro-organisms are easy to culture and fairly resistant to various factors; yet, they are heat-sensitive [15–17]. In our deactivation experiments we use bacterial films plated on agar dishes. These samples are comparable with thin films of dental plaque deposited on the tooth surface. Ideally, we would like to estab-
lish conditions in which the bacteria are killed at a temperature close to that of the body, because these conditions can be safely used in dental practice. Furthermore, it is interesting to perform a dose-response study in order to obtain insights into the efficiency, area of reach and possible mechanisms of plasma-induced bacterial deactivation.

5.2 Experimental procedure

5.2.1 The plasma needle set-up

The portable plasma needle (Figure 5.1) consists of a metal wire (0.3 mm diameter) with a sharpened tip, confined in a Perspex tube (4 mm inner diameter). Because, the voltage needed to ignite the plasma is lowest in helium, the plasma operates in helium-air mixtures. The Perspex tube is filled with helium delivered at a flow rate of 2 l min$^{-1}$, controlled by a mass flow controller (Brooks Instrument, model 5850E). A scheme of the whole set-up is given in Figure 5.2. Radio-frequency (RF) voltage is applied to the needle at a frequency of 13.05 MHz. The RF signal is produced by a waveform generator (Hewlett Packard, model 33120A) and amplified by an RF amplifier (Amplifier Research, model 75AP250). A home-built matching network optimizes the power dissipation in the plasma. The network consists of a 4.4 $\mu$H inductor placed in series with the plasma; a shunt resistance of 50 $\Omega$ is placed in parallel with the inductance and the plasma. The dissipated power is monitored using a P6103 Tectonics probe (Amplifier Research) connected via a dual-directional coupler to a power meter (Amplifier Research, model PM 2002).

The usage of a flexible, open system is essential for the intended application in dentistry, because the plasma must be applied in the air-filled oral cavity. Moreover, the presence of air guarantees the formation of active radical species, which play an important role in sterilization. In the present configuration, the air content is adjusted by
Deactivation of *E. coli* by the plasma needle

pushing the needle inside or outside the Perspex tube: the more the needle tip protrudes from the tube, the more air can enter the plasma region.

In the experiments described in this chapter, the tip of the metal wire is at the same distance as the outside of the Perspex tube ($x = 0$) (Figure 5.2).

![Experimental set-up](image)

Figure 5.2: Experimental set-up. In the experiments $x = 0$.

### 5.2.2 Power measurements

Power transmission from the RF source to the plasma is maximized by means of the matching network. A power meter with a dual directional coupler is placed between the source and matching network to monitor the forwarded and reflected power. The nominal power (the difference between the forwarded and reflected powers) contains the power consumed by the plasma, but also RF radiation and losses due to Ohmic heating in the matching network, cables and the needle. The power dissipated by the plasma ($P_{\text{plasma}}$) is given by

$$P_{\text{plasma}} = (P_{\text{forwarded}} - P_{\text{reflected}})_{\text{on}} - (P_{\text{forwarded}} - P_{\text{reflected}})_{\text{off}}$$

The plasma is switched off by terminating the helium flow; the RF signal is continued and recent $I$–$V$ probe measurements show that the needle voltage does not significantly change when the plasma is extinguished.

In the current arrangement the power is not fixed, and it can vary depending on the external conditions (e.g. geometry, gas composition, etc.). In Figure 5.3 it is shown that the dissipated power decreases with increasing distance between the needle and the sample surface. Thus, the power has been determined for each condition of *E. coli* treatment.
5.2. Experimental procedure

5.2.3 Temperature measurements

Temperature recording strips (VWR International) have been used to measure the temperature of the substrate during plasma treatment (Figure 5.4). Each spot on the strip is treated for 60 s at a fixed distance of 1 mm between the strip and the tip of the needle. The temperature of the substrate, as a function of the distance to the plasma needle tip, was also measured (Figure 5.5).

5.2.4 Bacterial sample preparation

*E. coli* (Cat. no. 69825, Novablue competent cells, Novagen) are inoculated from a glycerol stock into 4 ml of LB-medium, which contains 0.5 % (w/v) yeast extract, 1.0 %
Deactivation of *E. coli* by the plasma needle

Figure 5.5: Temperature at 150 and 200 mW (at 1.0 mm) as a function of distance.

(w/v) pepton from casein, 1.0 % (w/v) sodium chloride (Merck, Germany) and demineralized water; the mixture is shaken at 250 rpm for 15-20 h at 37 °C. Then, 1 ml of the cell suspension is re-suspended in 30 ml of the same LB-medium. The culture is grown at 37 °C with shaking (250 rpm) for 2 h. The growth curve of the culture is monitored photometrically: the optical density at the wavelength of 600 nm (OD$_{600}$) is determined using a Genesys 10 UV, Thermo Spectronic reader. The relation between colony forming units (CFUs) and the optical density is depicted in Figure 5.6. At the OD$_{600}$ = 0.5, which corresponds to the beginning of the log-growth phase, 100 µl of undiluted cell suspension is spread onto a nutrient agar medium in a sterile Petri dish. The agar medium contains 0.5 % (w/v) yeast extract, 1.0 % (w/v) pepton, 1.0 % (w/v) sodium chloride, 2.0 % (w/v) Bacto™ agar (Becton, Dickinson and Company, Franklin Lakes, NJ) and

Figure 5.6: Optical density at 600 nm as a function of CFUs ml$^{-1}$
demineralized water. The Petri dishes are placed in a stove at 37 °C for 1 h to let the bacteria attach to the agar.

5.2.5 Sample treatment by plasma

Just after attachment of the bacteria, the samples are directly exposed to the plasma. The time, power and distance between the plasma needle and the agar dish have been varied, from 10 s to 60 s, 50 mW to 350 mW and 1 mm to 10 mm, respectively. After plasma treatment, the dishes are incubated at 37 °C for 24–48 h.

5.2.6 Sample treatment by heating

The *E. coli* suspension is prepared as described earlier, but now 100 µl samples of the undiluted suspension (∼ 1 x 10^8 CFUs ml\(^{-1}\)) are heated in a warm water bath (Julalbo, ecotemp TW12, Labyrinth Holland B.V.) to temperatures of 37 °C, 40 °C, 43 °C, 46 °C, 49 °C, 52 °C, 55 °C and 58 °C for 5 min. After treatment, the samples containing 100 µl undiluted suspension are diluted 10^5 times in a LB–medium. Each diluted sample is spread onto a nutrient agar media in a sterile Petri dish. The dishes are incubated at 37 °C for 24–48 h. The experiment is performed in duplicate.

5.2.7 Observations

Plasma needle treatment

After the overnight incubation at 37 °C the sample is inspected. An untreated sample is uniformly covered by a whitish, opaque bacterial film. At the treated spot, the bacteria are absent and the (transparant) bottom of the Petri dish is exposed. This circular void in the bacterial film can be readily seen with the naked eye. The sizes of the voids are measured using a simple ruler. The number of CFUs seeded per square centimeter (CFUs cm\(^{-2}\)) is determined for each experiment, by dividing the amount of CFUs plated on the agar media by the area of the Petri dish. Thus, the number of CFUs destroyed by the plasma can be calculated from the void size.

Heat treatment

Colony-formed *E. coli* bacteria on the agar plates have been counted after incubation to determine the number of survivors.
5.3 Results

5.3.1 Deactivation by plasma needle

Plasma treatment causes formation of a circular void at the exposed spot of the bacterial sample. The sample was viewed under a microscope to look for remnant bacteria in the void, but no survivors were found. The border of the void is very sharp: no gradual change in the CFU density is observed. This means that for a given exposure time, plasma treatment is localized and can be performed with high-precision. In the first experiment the void size has been studied as function of treatment time (Figure 5.7). The void size and the corresponding number of destroyed CFUs increase with treatment time, but there is saturation around 50 s. Further increase of the time to more than 60 s does not improve the deactivation. Ten seconds of plasma exposure is already enough to deactivate $10^4$ – $10^5$ CFUs.

The dependence of the void size and the amount of removed CFUs on the applied plasma power displays similar features (Figure 5.8). It can be seen that saturation starts at 260 mW.

In order to determine the area of reach of the plasma needle, the power-dependent measurements have been performed for different needle–to–sample distances. The results, shown in Figure 5.9, are similar to the ones presented in Figure 5.8. At longer distances, saturation is observed at high power levels. The saturation value seems to be the same for most distances: the maximum void size is 12 mm.

![Figure 5.7: Void size and deactivated CFUs as function of treatment time. The distance between the plasma needle and bacteria is 1 mm and the dissipated power is 180 mW.](image-url)
5.3. Results

Figure 5.8: Deactivated CFUs and void size as a function of dissipated power at 1.0 mm. The treatment time is 60 s.

Figure 5.9: Void diameter as a function of dissipated power at different distances. The treatment time is 60 s.

To cross-check these results, we have performed measurements as a function of distance to the sample at various power levels. As expected, the void size and the number of removed CFUs (Figure 5.10) decrease with increasing distance, at a rate that is similar for all power levels. The distance at which deactivation is possible is surprisingly large: at a sufficiently high power, bacteria that are 8 mm away from the needle are still killed.

5.3.2 Deactivation by heat

The deactivation of *E. coli* at elevated temperatures is shown in Figure 5.11. The onset of deactivation lies around 40 °C; the number of survivors decreases linearly with temperature, until the temperature of 49 °C is reached, at which all bacteria are killed.
5.4 Discussion

In the light of the above data, we can state that the efficiency of bacterial deactivation is determined by treatment parameters like exposure time, plasma power and the distance between the electrode and the bacterial sample. Apparently, there is a limit in the area of reach of the plasma: the maximum size of the spot that can be disinfected by the needle is about 12 mm. This limit can be approached in many ways. For example, treatment at long distances will require higher plasma powers and/or treatment times to reach the same result as in the short-distance treatment. To avoid using harsh conditions, a power of about 100–150 mW and a distance of 1–3 mm are recommended.

Controlling the distance to the sample is particularly important for future applica-
tions in dentistry and surgery. The distance from the RF electrode to the grounded sample (the electrode gap) has a major influence on the plasma operation. One can visually observe that the plasma increases in brightness while approaching a grounded object; the glow expands and spreads over the surface. In a previous work we have determined the power flux from the plasma towards the treated surface [18], and calculated the power transfer efficiency (the fraction of the total dissipated plasma power that is deposited on the surface). At a distance of 1 mm, almost all of the plasma power is absorbed by the substrate [18].

It should be noted that changing the needle-to-sample distance is not a single parameter variation, because the power dissipated in the plasma also depends on the electrode gap (Figure 5.3). This is related to the fact that at the given voltage, the electric field ($E$) increases with decreasing electrode gap, and causes an increase of the ionization rate in the plasma. This is reflected by a higher electron density and conductivity ($\sigma$), and increased power consumption $\sigma E^2$ [19]. With respect to bacterial deactivation, this means that increasing distance reduces the efficiency in a double manner: by separating the sample from the energetic plasma source, and by reducing plasma activity by decreasing the power.

The mechanism of bacterial inactivation in the plasma is an essential issue that is being discussed by many researchers. Several factors can be identified, like heat, UV radiation and plasma chemistry. UV radiation may be the most important factor in low-pressure deactivation, because in low-pressure plasmas the samples are dry and VUV (which is a deadly agent) can easily reach them. At atmospheric pressure, VUV emission is suppressed because the relevant transitions are optically thick; furthermore, when liquid samples are treated, short-wavelength radiation is cut off by water. Studies of optical emission from the plasma needle [19] have shown some UV radiation in the range of 250–400 nm, e.g. an intense OH$^+$ peak at 305 nm and the radiation from the second positive system of N$_2$. Thus, UV in this range could contribute to the destruction of bacteria. According to Laroussi [3], active radicals and anions like O, OH and HO$_2^-$ play the most important role in the destruction of micro-organisms in atmospheric pressure plasmas. These active species destroy the cell membrane, because the latter is constituted of unsaturated fatty acids, which are susceptible to attacks by oxygen-containing radicals. Active species can be supplied to the cells in two ways. Radicals generated in the plasma can diffuse into the liquid sample, or they can be produced locally in the liquid, by conversion of energetic plasma species (ions, metastables, and possibly some UV photons). In this work, we cannot decide on the actual bactericidal agent, but some conclusions can be arrived at by comparing the plasma action to the damage caused by heat alone. Since heat is one of the factors present in the plasma, some of the observed effects can be attributed to thermal destruction of bacteria. However, note that the plasma temperature is significantly elevated only at high power levels and short needle–to–sample
distances. Moreover, plasma exposure has been shorter than incubation at elevated temperatures (1 versus 5 min). The response of the sample to the plasma and to heat is also completely different. If heat produced by the plasma were responsible for killing \textit{E. coli}, the number of survivors on the treated spot would decrease steadily with increasing temperature, as in the case of incubation (Figure 5.11). Thus, the sample would display a smooth profile in the CFU density, reflecting the local temperature. The lowest survivor number at the spot of incidence (shortest vertical distance to the needle), and gradual increase in the radial direction would be observed. No saturation in killing efficiency at high plasma powers would occur, because the plasma temperature increases steadily with increasing power. The fact that plasma treatment produces voids that are completely free of bacteria, and that no transition zone is observed between inactivated and intact areas, suggests that heat is not the only killing factor. Deactivation by heat can play a role only at plasma powers higher than 200 mW, where the temperature rises above 47 °C. Well-localized voids with sharp edges indicate that the bactericidal agents have very short lifetimes in a liquid medium. These active species are fairly stable in the gas phase, because bacterial deactivation is observed even at quite long distances from the needle (several millimeters). Under these conditions the glow, which corresponds to the active plasma zone, does not reach the sample. Bacteria are thus killed by species that are still present in the remote dark region. Once these particles cross the gas-liquid interface, they are immediately consumed in chemical reactions with the components of the solution. Their action in the liquid is thus strictly localized. These features suggest that plasma-produced species like radicals and (helium) metastables play an important role in bacterial deactivation. The mechanism of deactivation by metastables is not clear: they can either directly attack the bacterial membrane, or dissociate water to produce OH radicals, which further react with bacteria. Most probably there is no unique deactivation mechanism and different factors are important under different conditions. For example, when the sample is in contact with the active zone, energetic ions may play a role. At high power levels thermal effects can also contribute to deactivation. In the future work, some of these factors will be separated and/or eliminated. Thermal effects can be suppressed by choosing low power conditions, metastable and radical densities can be measured and correlated with deactivation efficiency, and the radical density can be enhanced by introducing admixtures in the plasma.

For practical purposes, one strives for most efficient deactivation at the conditions that are safe in a clinical environment. We can state that, at present, moderate conditions can be found, for which disinfection is quite efficient. Temperature measurements of the plasma and experiments on bacterial destruction by heat have been performed to establish the ‘worst case scenario’ and determine the limits within which safe medical treatment can be performed. In fact, the results presented show that using harsh plasma conditions does not improve the disinfection results. This means that bacterial destruc-
tion by the plasma can be performed without overheating the biological substrate.

5.5 Conclusions

Bacterial films (E. coli) can be efficiently deactivated using a non-thermal plasma (plasma needle). Samples exposed to the plasma show characteristic voids at the spot of incidence. The voids are circular regions that are completely free of micro-organisms. Their edges are extremely sharp. Thus, bacterial deactivation by the plasma occurs locally and is most likely caused by interactions with short-living plasma species. For safe and efficient disinfection it is necessary to control plasma parameters. It has been established that the best conditions correspond to low power levels (around 100–150 mW) and distances to the sample of 1–3 mm.

Since E. coli is a heat-sensitive micro-organism, deactivation by heat has been studied separately. Heating of bacterial samples results in a linearly decreasing number of survivors with increasing temperature. The sample temperature during plasma treatment has been monitored; at high power levels, the temperature can rise up to 60 °C and heat may possibly contribute to bacterial destruction. However, at moderate (low power) conditions this factor is not present, while disinfection efficiency is equally high. Applying high powers and long treatment times is not favorable, because it unnecessarily heats the sample, while it does not improve the results of bacterial deactivation. For future applications in dentistry, treatment should be preferably conducted at body temperature. The current results show that this condition can be satisfied by treatment with the non-thermal plasma needle.

References


Deactivation of *E. coli* by the plasma needle


Deactivation of *E. coli* by the plasma needle
Chapter 6

Plasma needle treatment of *Streptococcus mutans* biofilms

**Abstract**—A non-thermal atmospheric plasma, designed for biomedical applications, was tested for its bactericidal activity against biofilm cultures of a key cariogenic bacterium *Streptococcus mutans*. The *S. mutans* biofilms were grown with and without 0.15% sucrose. A chlorhexidine digluconate rinse (0.2%) was used as a positive antimicrobial reference. Sucrose concentration and the frequency of plasma application during growth were shown to have a significant effect on the response to treatment and bactericidal activity. A single plasma treatment for 1 min on biofilms cultured with no sucrose caused a bactericidal effect. However, with either single or repeated plasma treatments of 1 min, on biofilms cultured with 0.15% sucrose, growth was only reduced. In summary, there may be a role for non-thermal plasma therapies in dental procedures. Sucrose and associated growth conditions may be a factor in the survivability of oral biofilms to treatment.

6.1 Introduction

Dental plaque biofilms consist of complex communities of oral bacteria with hundreds of species present [1, 2]. Dental plaque induces one of the most prevalent diseases of mankind, caries, the localized destruction of tooth tissues by bacterial fermentation of dietary carbohydrates [3]. The acids produced by the fermentation of these dietary carbohydrates demineralize the enamel, which results in the formation of cavities. *Streptococcus mutans* is an acidogenic, gram positive, facultative anaerobe, which is found in the oral cavity of humans. Epidemiological studies have implicated *S. mutans* as a significant cariogenic organism in childhood caries, caries in young adults, and of root caries in the elderly, and nursing (or bottle) caries in infants [3]. Preparation of a cavity for filling is achieved by removal of the often painful necrotic, infected and demineralized tissue with a mechanical drill or laser [4, 5]. Usually an excess of healthy tissue must be removed to ensure that the cavity is free of bacteria and often the integrity of the remaining tooth is weakened [6].

Atmospheric plasmas produce ozone and also short-living reactive species like O and OH radicals [7]. These radicals, that are more powerful disinfectants than ozone, are very short-living compounds that have a stronger oxidation mechanism than that of ozone. This is because the O and OH radicals have a higher oxidation potential (e.g. hydroxyl radical (OH) = 2.56 V, ozone molecule (O$_3$) = 2.07 V) [8]. Plasmas have been shown to deactivate non-oral pathogens [9, 10]. A new painless and non-destructive approach could involve bacterial inactivation of plaque by treatment with an atmospheric plasma source [11–15]. To date, there have been few investigations regarding the treatment of dental plaque biofilms with non-thermal atmospheric plasmas. The aim of the investigation was to evaluate the bactericidal activity of a non-thermal atmospheric plasma treatment against biofilm cultures of a key cariogenic bacterium *S. mutans* grown under different sucrose conditions. The plasma treatment was compared to a chlorhexidine digluconate mouthrinse.

6.2 Materials and methods

6.2.1 The atmospheric plasma

The non-thermal plasma source designed for biomedical applications (radio-frequency driven plasma needle) was developed at the Eindhoven University of Technology [13]. This plasma needle is small, flexible, and can be operated in the open air (Figure 6.1). The plasma needle is suitable for the treatment of heat-sensitive objects like biological tissues [7].

The portable plasma needle (Figure 6.1.), used in the present study, consisted of a
tungsten wire (0.3 mm diameter) with a sharp tip, confined in a Perspex tube (4 mm inner diameter). Because the voltage needed to ignite plasma is lowest in a helium atmosphere, the plasma operated in helium-air mixture. The Perspex tube was filled with helium (ZG Helium, Purity > 99.995 %, BOC, New Zealand) delivered at a flow rate of 2 l min\(^{-1}\), measured by an electronic volumetric flow meter (Omega, FDP 10). A radio-frequency (RF) voltage was applied to the needle at 13.56 MHz. The signal was produced using a custom-built power generator with a matching network. The matching network consisted of a 4.4 µH inductor placed in series with the plasma. A shunt resistance of 50 ohms was placed in parallel with the inductance and the plasma in order to properly terminate the transmission line. The dissipated power was monitored using a P6103 Tectonics probe (Amplifier Research) connected via a dual-directional coupler to a power meter (Amplifier Research, model PM 2002).

Plasma operation in air guarantees the formation of the active oxygen radical species \([16, 17]\). In the current study no additional gas (e.g. O\(_2\)) was added to the helium stream, but the diffusion of surrounding atmospheric air resulted in air admixture in the plasma of less than 0.5 %. The typical radial reach of this plasma is 9 mm \([18]\).

The dissipated power from the plasma was calculated before each experiment to be 100 mW using the following equation \([18]\):

\[
\text{Dissipated power} = (P_{\text{forwarded}} - P_{\text{reflected}})_{\text{plasma on}} - (P_{\text{forwarded}} - P_{\text{reflected}})_{\text{plasma off}}
\]

For safe in vivo disinfection, sample temperature should be maintained under 43 °C \([7]\), corresponding to plasma power and treatment time not exceeding 150 mW and 60 s, respectively. The optimal working distance of the plasma ranges from 1 to 3 mm \([18, 19]\).
6.2.2 Growth and plasma treatment of *S. mutans* biofilms

Sterile 9 mm coverslips (Thermanox™, Nunc Inc., Naperville, IL, USA) were inoculated with 100 µl of an overnight culture of *S. mutans* R9 in the wells of a 24-well microplate (Sarstedt, Sarstedt Australia Pty Ltd., Ingle Farm, Australia) for 1 h. Excess media was removed and 1.8 ml Brain Heart Infusion (BHI, Becton Dickinson, Franklin Lakes, NJ, USA) with and without 0.15 % sucrose added. The plates were incubated anaerobically (Coy™ anaerobic hood) in an atmosphere of 80 % N\textsubscript{2}, 10 % CO\textsubscript{2} and 10 % H\textsubscript{2} at 35 °C. The growth media was removed from each well by gentle aspiration and replaced by fresh media daily. The pH of the supernatant, pooled sample from each replicate well, from the untreated biofilms was monitored every 24 h (CD 660 Digital pH meter, Walden Precision Apparatus, Linton, Cambridge, UK).

Two treatment plasma procedures were evaluated. In the first procedure, the 24 h biofilms were treated once, and the effects assessed up to 24 h after treatment. In the second evaluation the 24 h biofilms were treated once a day for 2 days, representative of periodical treatment, and the effects assessed up to 24 h after treatment. The coverslip with adherent biofilm was removed from the well and placed on a stand. The plasma was applied for 60 s, 1 mm away from the biofilm surface. Biofilms were also treated with helium only, as the plasma control and to ambient air conditions as the negative control.

6.2.3 Treatment of *S. mutans* biofilms with chlorhexidine digluconate

*S. mutans* biofilms were treated with chlorhexidine digluconate (CHX) as positive antimicrobial reference. After complete removal of the supernatant, 1 ml CHX (0.2 %) (Sigma-Aldrich Inc, New Zealand) was added to the 24 h biofilms for 10 min. The CHX was removed and the biofilms rinsed twice with 1 ml of sterile water for 1 min. The coverslips with adherent biofilm were placed into a new sterile plate, fresh media added and incubated for a further 24 h. Biofilms were also treated with sterile water for 10 min as the CHX control.

6.2.4 Analysis of bactericidal effects of treatment on biofilm growth

The bactericidal effect of all the treatments was estimated by monitoring growth spectrophotometrically of a biofilm suspension. After treatment the biofilms were prepared as suspensions by placing the coverslip and adherent biofilm in 1 ml sterile water and vortexed. 50 µl of biofilm suspension was transferred to a 96-well microtitre plate (Sarstedt) in 150 µl of fresh BHI with or without 0.15 % sucrose. The absorbance at 595 nm was measured immediately and after 24 h incubation using a microtitre plate reader (Bio-
6.3. Results

Rad Benchmark, Hercules, CA, USA). Absorbance (optical density) values are used to monitor the growth of bacteria in suspension cultures. Absorbance is defined as a logarithm of the fraction of transmitted light through a liquid: \( \text{OD} = \ln \left( \frac{I_0}{I_{\text{trans}}} \right) \), where \( I_0 \) is the intensity of the incident light beam and \( I_{\text{trans}} \) is the intensity of the transmitted light beam. According to the Lambert-Beer law, it is proportional to the thickness of the bacterial film.

For growth profiles of the biofilm suspensions the microtitre plates were prepared as above and incubated in the microtitre plate reader, at 35 °C, programmed to take absorbance measurements (A\text{595 nm}) every 60 min for 12 h.

6.2.5 Statistical analyses

All treatments were arranged in triplicate, descriptive statistics calculated and results analyzed for significance (\( P < 0.05 \)) using a one-way analysis of variance (ANOVA) (SPSS, version 12.0.1, Chicago, IL, USA).

6.3 Results

6.3.1 Antimicrobial effects of plasma and chlorhexidine treatments on growth of \textit{S. mutans} biofilms

Sucrose and frequency of plasma application were shown to have a significant effect on the degree of response to treatment and bactericidal activity (Figure 6.2 and 6.3). The 24 hourly pH of the supernatant from the untreated biofilms was 5.2 without sucrose and 4.66 in BHI supplemented with 0.15 % sucrose. The pH of the un-inoculated BHI was 7.28.

Growth of the biofilm suspension was not observed in either sucrose conditions for up to 12 h after treatment with either plasma or CHX (Figure 6.2). After the control treatments, \textit{S. mutans} biofilms cultured without sucrose demonstrated minimal growth for up to 12 h (Figure 6.2a and c). In comparison to biofilms cultured with 0.15 % sucrose onset of growth was observed after 5-8 h (Figure 6.2b and d). \textit{S. mutans} biofilms cultured in 0.15 % sucrose exhibited a faster recovery rate after repeated treatment with the controls, compared to a single application (Figure 6.2b and d).

The degree of bactericidal activity of the plasma was shown to depend on sucrose concentration and application frequency (Figure 6.3). \textit{S. mutans} biofilms treated once with the plasma and cultured without sucrose showed a bactericidal effect, as evident by no increase in optical density. This was comparable to CHX (\( P < 0.05 \)) after 24 h incubation (Figure 6.3a and b). With repeated plasma treatment, bactericidal activity
was not as apparent (Figure 6.3c and d). Treatment with CHX for 10 min completely inhibited growth of *S. mutans* biofilms.

Figure 6.2: Growth profiles of *S. mutans* biofilms over 12 h incubation, with or without sucrose and after (A) single treatment with no sucrose; (B) single treatment with 0.15 % sucrose; (C) repeated treatment (twice) with no sucrose and (D) repeated treatment (twice) with 0.15 % sucrose. Treatments included plasma (Plasma), helium as the plasma control (PC), chlorhexidine (CHX), water as the chlorhexidine control (CHXC), and exposure to air as the negative control (Ne).
Figure 6.3: Growth of S. mutans biofilms after 24 h incubation with or without sucrose and after (A) single treatment with no sucrose; (B) single treatment with 0.15 % sucrose; (C) repeated treatment (twice) with no sucrose and (D) repeated treatment (twice) with 0.15 % sucrose. Treatments included plasma (P), helium as the plasma control (PC), chlorhexidine (CHX), water as the chlorhexidine control (CHXC), and exposure to air as the negative control (Ne). Error bars represent standard deviation (N=3).
6.4 Discussion

Under the conditions described, the results from this investigation have shown that plasma treatments did exhibit growth inhibitory effects against *S. mutans* biofilms, but these effects were limited by application frequency and presence of sucrose.

Plasma sterilization is usually dependent on the synergy of three bactericidal agents: 1) energetic photons in the UVB and UVC range, 2) reactive species such as oxygen radicals, and 3) charged species such as electrons and ions [10]. Their relative importance depends on the type of plasma source as well as on sample preparation, UV photons get easily absorbed in atmospheric air [10]. For the type of plasma used in this investigation, deactivation of biofilm cells is probably associated to plasma induced reactive species, such as atomic oxygen and OH radicals, and a further electrostatic process that disrupts the membranes [20].

In this study plasma effects were evaluated by monitoring growth responses of the biofilms after single and repeated treatments. Bactericidal activity of a single plasma treatment was dependent on sucrose conditions. Without sucrose a single plasma treatment caused a bactericidal effect against *S. mutans* biofilms. Under the other conditions tested, single and repeated treatments, biofilm growth was impaired but not completely inhibited. The degree of inhibition is probably related to the thickness and age of the biofilm cultures. A thicker biofilm would present a greater challenge for the penetration of plasma radicals [10, 18]. *S. mutans* biofilms grown in the presence of sucrose synthesize glucan-exopolymers [21] which result in a thick, ‘sticky’ biofilm [22]. This could also impede the bactericidal activity of the plasma, killing cells only at the surface and inducing a sub-lethal response to cells attached to the surface [18].

Plaque-induced diseases are complex, and appear to be related to increased levels of particular species in the oral microbiota, which relate to changes in the oral environment [23]. Dental caries in particular have been associated with species other than *S. mutans*. Treatment of oral diseases often requires intervention with a chemical therapeutic such as CHX and a successful outcome is dependent on restoring species homoeostasis. Chlorhexidine is one of the most widely used biocides in antiseptic products, in both hand washing and oral products and as a disinfectant and preservative [24]. Chlorhexidine is regarded as the ‘gold standard’ anti-plaque treatment and is an aggressive chemical, which when used topically can lead to desquamation and soreness of the oral mucosa [25]. Its future as a caries treatment is being questioned, and unless heavily applied it is ineffective in treating child-hood caries [26]. A safe and less aggressive alternative treatment to CHX would be highly desirable. Under these conditions, *S. mutans* biofilms were completely inhibited with CHX, which was expected given the extended exposure time. However, rather than an aggressive chemical approach, plasma treatments could disturb and impair growth of pathogenic bacteria in
plaque and promote the growth of health compatible species.

The results from this investigation have indicated that there may be a role for non-thermal plasma therapies in dental procedures and would warrant further investigation. This work has also highlighted the influence of sucrose and associated growth conditions in the survivability of oral biofilms to treatment.

6.5 Acknowledgments

This research project was sponsored by the Netherlands Organization for Scientific Research (NWO). The first author would sincerely like to thank Chris Sissons and Bob ten Cate for making this work possible.

References


Treatment of *S. mutans* biofilms
Chapter 7

Treatment of microplate biofilm microcosms

Abstract—Dental plaque consists of complex communities of oral bacteria. Several complex model systems have been developed to investigate antimicrobial effects. In this chapter we describe an easily reproducible model to replicate dental plaque, the microplate biofilm microcosm (MBM) model.

We investigated the effects of a non-thermal atmospheric micro-plasma (plasma needle) and chlorhexidine digluconate (CHX) on the growth of microplate biofilm microcosms. Plaque biofilms were grown on Thermanox™ coverslips in 24-well microplates from enriched human saliva.

After treatment, the growth of the biofilms was studied by monitoring the pH and the biomass. Photomicrographs of the structure of the individual biofilms were taken using a digital camera. The differential effect of the treatments among the species was analyzed by the DNA-DNA checkerboard technique. Furthermore, the biofilms were placed in fixative and observed under transmission electron microscope (TEM).

The results show a complex response of MBM to plasma and CHX treatments. The TEM images show that the plasma and chlorhexidine treatment damaged bacteria in the biofilms. DNA-DNA checkerboard analyses show that some species were significantly suppressed by the plasma treatment. Photomicrographs show that the chlorhexidine treatment visually changed the structure of the biofilm. The pH of the chlorhexidine digluconate treated samples changed significantly after treatment and went back to the pH value of the blanks. Furthermore, sucrose concentration had a significant influence on the pH, biomass and the species composition of the microplate biofilm microcosm.

7.1 Introduction

Dental plaque is a complex oral biofilm made up of hundreds of oral bacteria organized in communities [1]. These bacterial communities are embedded in an exopolysaccharidic matrix with a complex architectural structure. Bacteria in these communities can cause diseases such as dental caries and periodontitis, but they also play an important role in oral health.

Mechanical removal of dental plaque by efficient oral hygiene can almost completely prevent caries [1]. Other approaches to prevent caries are fissure sealants, antimicrobial agents, fluoride, and dietary sugar substitutes (e.g. xylitol). Cavities will form if the caries prevention is not sufficient.

Treatment of carious lesions involves removing necrotic, infected and non-reminestralizable tissue by means of mechanical drilling or laser techniques [2] prior to filling. In both methods heating takes place, and in mechanical drilling vibrations are induced [3]. Heating and vibrations are usually painful for the patient. Moreover, these methods are often too destructive: an excess of healthy tissue must be removed to ensure that the cavity is free of bacteria. In addition, the remaining tooth structure is weakened and prone to fractures.

An alternative painless and tissue-saving method to treat caries is plasma needle treatment [4–6]. In this new approach a non-thermal atmospheric micro-plasma, the plasma needle [7], is used. The principle of plasma treatment is the deactivation of bacteria in dental biofilms, in a painless, non-contact and non-destructive way [8–11]. The advantages of this novel tissue-saving treatment in dentistry are [12]:

- disinfection of site-specific lesions
- treatment without excessive heating
- usage of short-living bactericidal species that do not remain in the mouth after treatment
- treatment in less than a minute with reproducible results.

The plasma needle was tested on *Escherichia coli* and *Streptococcus mutans* bacteria grown on agar dishes [13, 14] and *Streptococcus mutans* biofilms [6]. The plasma needle treatment killed *E. coli* and *S. mutans* bacteria grown on agar plates and stopped the regrowth of the *S. mutans* grown in biofilms. It should be noted that these studies were primarily concerned with single-species cultures and biofilms. However, the antimicrobial effect of the plasma needle treatment should be studied on more complex, and thus more realistic bacterial samples.
To predict the antimicrobial susceptibility of bacteria grown in oral biofilms, complex model systems have been developed. In these models dental plaque biofilms can be replicated and studied *in vitro*. Several of these artificial dental plaque model systems are described by Sissons *et al.* (1997) [15]. These include the growth-rate-controlled biofilm fermenters (GRBF), constant-depth film fermenters (CDFF), chemostat-based systems, and most recently the artificial mouth model systems (MAM). In the artificial mouth model system, multi-plaque microcosms can be grown. A microcosm is defined as "a laboratory subset of the natural system from which it originates but from which it also evolves" [16]. The microcosms used in the artificial mouth model system by Sissons *et al.*, contain hundreds of species of bacteria and are nowadays the closest laboratory replication to plaque in the human mouth. This model is labor-intensive and requires specialist equipment. However, the plaque biofilms that are generated appear to reflect the complexity, diversity and heterogeneity of *in vivo* plaques [17, 18].

In this chapter we describe an easily reproducible model to replicate dental plaque, the microplate biofilm microcosm (MBM) model [19]. This MBM model is used to test the susceptibility to antimicrobial agents. The MBM model is based on the model of Guggenheim *et al.* [20]. Guggenheim *et al.* developed a biofilm model of supragingival plaque based on 5 species using 24-well microplates. The MBM model is relatively simple to prepare, maintain and analyze. The advantage of the model is that it allows for the short-term and efficient exposure of the biofilm to antimicrobials.

In the MBM experiments the differential effect of the treatments among the species was analyzed by the DNA-DNA checkerboard technique [21]. Checkerboard DNA-DNA hybridization (CKB) is a technique which gives a simultaneous and quantitative analysis of up to 28 plaque samples against 40 key microbial species (Table 7.1), blanks and standards on a 15 cm × 15 cm square nylon membrane [22]. CKB analysis has been successfully applied to study gram-negative bacterial complexes associated with periodontal disease *in vivo* [21, 23]. It enables a more detailed analysis than would be practicable with culture techniques. Wall-Manning *et al.* further developed CKB analysis to focus on a supragingival plaque composition and species associated with dental caries [24].

At present there is little information on the deactivation of complex dental biofilms by non-thermal atmospheric plasmas. Therefore, to check the bactericidal effectiveness of the plasma, microcosm microplate biofilms were grown and treated by the plasma needle. The effect of the plasma needle treatment was compared to a 0.2 % chlorhexidine digluconate mouthwash [25]. The microcosm biofilms were treated 1 and 5 minutes, at 50 or 100 mW respectively. The dynamic behavior (growth of the biofilms) after treatment was studied by measuring the pH and the biomass. In addition, photographs and transmission electron microscopy (TEM) images were taken. We also investigated the composition of the biofilms after treatment by checkerboard DNA-DNA hybridiza-
Table 7.1: The 40 CKB species

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococci</td>
<td><em>Streptococcus mutans, Streptococcus sobrinus, Streptococcus parasangui</em>,</td>
</tr>
<tr>
<td></td>
<td>*Streptococcus mitis 1, Streptococcus oralis, Streptococcus intermedius,</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus vestibularis, Streptococcus mitis 2, Streptococcus gordonii</em>,</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus sanguis, Streptococcus anginosus</em></td>
</tr>
<tr>
<td>Lactobacilli and</td>
<td>*Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus</td>
</tr>
<tr>
<td>Bifidobacter</td>
<td><em>acidophilus, Lactobacillus rhamnosus, Bifidobacterium dentium</em></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>Actinomyces</td>
<td><em>Actinomyces israelii, Actinomyces gerencseriae, Actinomyces</em></td>
</tr>
<tr>
<td></td>
<td><em>naeslundii, Actinomyces odontolyticus, Rothia dentocariosa</em></td>
</tr>
<tr>
<td>Microaerophiles</td>
<td><em>Actinobacillus actinomycetemcomitans, Eikenella corrodens</em></td>
</tr>
<tr>
<td>Aerobes</td>
<td>Neisseria mucosa, Haemophilus parainfluenziale</td>
</tr>
<tr>
<td>Anaerobes 1</td>
<td><em>Fusobacterium nucleatum ss nucleatum, Campylobacter rectus, Veillonella</em></td>
</tr>
<tr>
<td></td>
<td><em>parvula, Capnocytophaga gingivalis, Peptostreptococcus</em></td>
</tr>
<tr>
<td></td>
<td><em>asaccharolyticus, Gemella morbillorum, Prevotella melaninogenica,</em></td>
</tr>
<tr>
<td></td>
<td><em>Leptotrichia buccalis, Eubacterium saburreum, Corynebacterium</em></td>
</tr>
<tr>
<td></td>
<td><em>matruchotii, Prevotella nigrescens/intermedia</em></td>
</tr>
<tr>
<td>Anaerobes 2</td>
<td><em>Porphyromonas gingivalis, Selenomonas noxia, Micromonas micros</em></td>
</tr>
</tbody>
</table>

An extra parameter we studied in the MBM experiments was the effect of sucrose concentration (0, 0.15 and 0.30 %) on the post-treatment biofilm behavior. The results are presented in this chapter.

### 7.2 Materials and methods

#### 7.2.1 The plasma needle apparatus

A non-thermal plasma source for biomedical applications (radio-frequency driven plasma needle) has been developed at the Eindhoven University of Technology [7]. The plasma needle is small and flexible, can be operated in the open air (Figure 7.1), and results in the formation of active radical species that play an important role in sterilization [26, 27]. The plasma needle is suitable for the treatment of heat-sensitive objects and even living biological tissues [28].

The plasma used in the present study consisted of a tungsten wire (0.3 mm diameter)
7.2. Materials and methods

Figure 7.1: The plasma needle (left photo by Bart van Overbeeke).

(Engelhard-CLAL/Drijfhout BV, Amsterdam, The Netherlands) with a sharp tip at the end, confined in a Perspex tube (4 mm inner diameter). Because the voltage needed to ignite the plasma is lowest in a helium atmosphere, the plasma was sustained in helium-air mixture. The Perspex tube was filled with helium (ZG Helium, Purity > 99.995 %, BOC, New Zealand) delivered at a flow rate of 2 l min$^{-1}$, measured by an electronic volumetric flowmeter (Omega, FDP 10). A radio-frequency (RF) voltage was applied to the needle at 13.56 MHz. The signal was produced by a custom-built power generator and a corresponding 4.4 µH inductor network placed in series with the plasma capacitance. A 50 ohms shunt resistance was placed in parallel with the inductance and the plasma in order to properly terminate the RF transmission line. The dissipated power was monitored using a P6103 Tectonics probe (Amplifier Research, Souderton, United States of America) connected via a dual-directional coupler to a power meter (Amplifier Research, model PM 2002). The power dissipated in the plasma during the treatments was 50 or 100 mW.

7.2.2 Growth conditions and experimental protocol

The microplate plaques were established based on the methodology described by Filoche et al. [19]. Approximately 20 ml of stimulated saliva was collected from a donor who had refrained from oral hygiene for 24 h. Ethical approval was granted from the Wellington Ethics Committee, New Zealand. The same donor was used for all the experiments. Di-thiothreitol was added (0.5 mM final concentration) and the saliva filtered through sterile glass wool and collected in a sterile glass beaker and mixed [29]. The prepared saliva (100 µl) was inoculated onto 9 mm diameter sterile Thermanox™ coverslips (Nunc Inc., Naperville, IL, USA) that had been placed in each well of the 24-welled microplates (Sarstedt, Sarstedt Australia Pty Ltd., Ingle Farm, Australia) for 1 h. Af-
Treatment of microplate biofilm microcosms

ter 1 h the saliva was gently aspirated from the coverslips and base of the wells. The plaques were grown in 1.8 ml of chemically defined artificial saliva, defined medium mucin (DMM) [30] supplemented with either 0, 0.15 or 0.30 % sucrose. The plates were incubated in an anaerobic hood in an atmosphere of 80 % N₂, 10 % CO₂ and 10 % H₂ (Coy™, Laboratory Products Inc., Grass Lake, MI, USA) at 35 °C without shaking.

The pH of the 24 h supernatant was measured daily. The plate was gently shaken, the supernatant removed from each replicate well, pooled and the pH recorded (CD 660 Digital pH meter, Walden Precision Apparatus, Linton, Cambridge, UK).

Growth of the microplate plaques was measured as wet-weight accumulation. After removal of the supernatant, the coverslip with the adherent biofilm was removed using sterile tweezers, placed on a sterile tissue to absorb any excess liquid and biofilm biomass measured as wet weight (mg).

Photographs of the individual biofilms were taken using an Olympus Camedia (C-5050 Zoom) digital camera (Olympus America Inc., Pty Ltd., New York USA).

7.2.3 Application of the plasma and CHX

Different parameters for a ‘single’ plasma treatment were investigated, including: plaque age, treatment time and sucrose concentration. In one treatment regimen, microplate plaques were grown for 1 d in DMM supplemented with either 0, 0.15 or 0.3 % sucrose prior to treatment with plasma for either 1 or 5 min. In another treatment regimen, microplate plaques were grown for 2 d in DMM supplemented with 0.15 % sucrose and treated with plasma for 5 min.

After removal of the supernatant the coverslips and adherent plaque were placed on a stand and treated with plasma for either 1 or 5 min, approximately 1 mm from the biofilm surface (optimum plasma performance is between 1 to 3 mm) [13]. The plasma was applied to the center of the 9-mm diameter coverslip with adherent plaque. From the previous works on *Escherichia coli* (see Chapter 5) we determined the effective area of reach of the plasma. The area of reach appeared to be larger than 10 mm, thus we assumed that a 9-mm coverslip (with adherent plaque) can be treated with a reasonable degree of uniformity. The plasma control included exposing the plaque biofilms to a helium flow of 2 l min⁻¹, for either 1 or 5 min. As a negative control, the plaque biofilms were exposed to air for either 1 or 5 min. After treatment the coverslips with adherent biofilm were placed in a new sterile microplate with fresh media. The biofilms were harvested for analysis immediately and 24 h after treatment.

As a positive antimicrobial reference, the microplate plaques were treated with chlorhexidine digluconate (CHX) [25]. After complete removal of the supernatant, 1 ml CHX (0.2 %) (Sigma-Aldrich Inc, New Zealand) was added to the biofilms for 10 min. The CHX was removed and the biofilms rinsed twice with 1 ml of sterile water for 1
7.2. Materials and methods

Biofilms were also treated with sterile water for 10 min as the CHX control. The plaque biofilms were placed into a new sterile plate, fresh media added and harvested for analysis immediately and 24 h after treatment.

7.2.4 Microbiota profiling of the microplate plaques using Checkerboard DNA-DNA (CKB) analysis

Before and after the treatments, plaque suspensions were prepared for Checkerboard DNA-DNA (CKB) analysis by placing the coverslip and adherent biofilm in sterile water and mixing vigorously to remove the adherent biofilm. Aliquots of the samples were dispensed, the cells harvested and the pellets stored at -80 °C until analyzed.

The CKB DNA-DNA hybridization assay was carried out as described previously [24]. For the CKB analysis, cells were re-suspended in 0.25 M NaOH/0.5 × TE buffer to a final concentration of 2 mg ml⁻¹ per sample. A 100 µL aliquot of each suspension was heated at 96 °C for 5 min to lyse the cells and extract the DNA. Samples were neutralized with 800 µL 5M NH₄ acetate and deposited onto a 15 cm × 15 cm positively charged nylon membrane (Roche) using the 30 parallel lanes of a multi-channel immunoblotter (Minislot 30, Immunetics). A UV-Crosslinker (Hoefer) set at 70,000 µJ cm⁻² was used to cross-link the DNA to the membrane. Two sets of DNA standards (equivalent to 10⁵ and 10⁶ cells of the target species), and one positive (plaque) and one negative (E. coli) control were also cross-linked to each membrane.

The plaque and standard DNA on the membranes was cross-hybridized with 40 unique DIG-labeled probes (prepared from whole cell chromosomal DNA) using a 45 channel miniblotter (Immunetics), and left overnight with gentle shaking at 42 °C. Any unbound probe was removed using a high stringency wash (68 °C) in phosphate buffer. Membranes were blocked and then incubated with anti-DIG:AP antibody (Roche) for 1 h at room temperature. Excess antibody was washed off and the probe-DNA hybrids detected via chemi-luminescence using CDP-Star (Roche) in detection buffer. Hybrids were visualized using chemi-luminescent film and digitally using the Chemigenius II and GeneSnap v. 6 software (SynGene).

To quantify the CKB data, the spot intensities of each sample in the digital image were compared to those of the internal standards using software gifted by dr. S. Sockransky. The resultant values were converted to absolute and total percentage probe counts. Failure to detect a signal was recorded as zero. The mean percentage to the DNA probe count was reported.
7.2.5 Analysis of plaque ultra-structure using transmission electron microscopy

Immediately after the treatments, the coverslips with the plaques were placed in TEM fixative and taken for transmission electron microscopy (TEM) analysis. Samples were fixed in half strength Karnovsky’s fixative, post fixed in osmium tetroxide (Sigma Aldrich) with added potassium ferrocyanide (Sigma Aldrich), dehydrated in a graded ethanol series and embedded in Procure 812, an Epon substitute. Sections were cut on a Reichert-Jung Ultracut E microtome, stained with uranyl acetate and lead citrate and examined in a Philips CM100 electron microscope. To determine whether 5 min plasma treatment affected cell size, TEM images were analyzed. The diameter of at least 10 individual, non-aggregated, bacteria was measured from at least three TEM micrographs.

7.2.6 Statistical analysis

All treatments were performed in triplicate, descriptive statistics were calculated and results were analyzed for significance (P < 0.05) using a general linear model univariate analysis of variance (ANOVA) and the post-hoc Tukey’s HSD test (SPSS, v12.0.1 for Windows, SPSS Inc., Chicago, IL, USA). Principal component analysis (PCA) in SPSS was used to identify the compositional relationships, based on the 40 CKB species, between the microplate plaques in response to the different growth conditions, treatments and relative to the composition of the initiating saliva sample [19, 31].

7.3 Results

7.3.1 Growth of the microplate plaques

Sucrose supplementation had a marked effect on the 24 h pH of the supernatant (Figure 7.2), ranging from pH 6.86, 4.20 and 3.94 with 0, 0.15 and 0.30 % sucrose respectively. Only treatment with CHX reduced the pH drop, from 4.25 to 6.83 (Figure 7.3).

After 24 h plaque biomass was significantly greater in DMM supplemented with 0.3 % sucrose compared to plaques grown without sucrose (P < 0.05) (Figure 7.4). Plasma treatment for 1 or 5 min did not inhibit growth of the plaques (Figure 7.5). Growth inhibition was observed after CHX treatment of 1 d microplate plaques but not of 2 d microplate plaques (Figure 7.5).

Plaque morphology did not appear altered directly after treatment (Figure 7.6). In comparison, after treatment with CHX plaque coverage of the coverslip was patchy. However, ultra-structure assessment of individual plaque cells using TEM demonstrated
7.3. Results

Figure 7.2: The effect of sucrose supplementation on the 24 h pH of the artificial saliva DMM. Uninoculated = no sucrose and no biofilm. Error bars represent 2× SE.

that cell lysis and enlargement, compared to the untreated plaques (Figure 7.7a) was evident immediately after a 5 min plasma treatment (Figure 7.7b). The average size (N=16) of the plasma, CHX and untreated bacteria was 0.65, 0.47 and 0.52 µm, respectively. Cell lysis was also observed after CHX treatment (Figure 7.7c).
Figure 7.3: pH of pooled DMM as function of biofilm age. Inoculation started at day 0. The treatments were at day 2. Un-inoculated = no sucrose and no biofilm. Biofilms were grown in 0.15 % sucrose. (N=1). Plasma: 100 mW, 5 min treatment.

Figure 7.4: The effect of sucrose on plaque biomass after 24 h. (N=9). Error bars represent SD.
Figure 7.5: Biomass (mean wet weight) of microplate plaques. Plasma: 100 mW. NT = untreated. a) Plaques grown for 1 d in 0.15 % sucrose. The error bars shown are the standard deviations (N=4). b) Plaques grown for 2 d in 0.15 % sucrose. The error bars shown are the standard deviations (N=3).
Figure 7.6: 9-mm diameter coverslips with adherent biofilm immediately after treatment. Biofilms were grown for 1 d in 0.30 % sucrose. Plasma: 50 mW, 1 min treatment. The empty spot in the left image is a tweezers artefact.

Figure 7.7: Effect of plasma (100 mW, 5 min) and chlorhexidine digluconate on the ultra-structure of the microplate plaques immediately after treatment. The microplate plaques had been biofilms grown in 0.15 % sucrose for 1 d prior to treatment.
7.3. Results

7.3.2 Microbiota composition of the microplate plaques

PCA of the microbiota composition of the saliva, collected on two separate occasions 4 weeks apart, was similar (Figure 7.8). However, composition of the initiating saliva was quite different to the untreated 1 d microplate plaque, which was different again from the 2 d untreated microplate plaque (Figure 7.8 and 7.9).

Figure 7.8: PCA analysis of the microbiota composition of the initiating saliva and untreated plaques. The plaques were grown for 1 and 2 d in DMM supplemented with 0.15 % sucrose.
Treatment of microplate biofilm microcosms

Effect of sucrose concentration, plasma and CHX treatment on microbiota composition of 1 d old microplate plaques

Sucrose was shown to have the greatest effect on microbiota composition of the microplate plaques immediately after treatment and yielded plaques of quite different microbial profiles (Figure 7.10). PCA showed that the plaques differentiated according to sucrose concentration rather than to treatment (Figure 7.11). Treatment of the microplate plaques with 0.15 % sucrose showed greatest separation according to treatment (Figure 7.11).

Analysis of plaques (grown in medium supplemented with 0.15 % sucrose) 24 h after treatment, showed that a plasma application of 1 min had a greater effect on microbiota composition than treatment for 5 min (Figure 7.12), and appeared to affect different species than CHX (Figure 7.13 and Table 7.2).

Plasma treatment appeared to selectively reduce the relative abundance of S. mutans, Streptococcus sobrinus, Peptostreptococcus asaccharolyticus, Gemella morbillorum and Leptotrichia buccalis. In comparison, CHX treatment selectively inhibited Streptococcus mitis 2, Streptococcus anginosus, Rothia dentocariosa and Capnocytophaga gingivalis (Table 7.2).
7.3. Results

Figure 7.10: Effect of sucrose on species composition of 1 d old untreated plaque.

Figure 7.11: Effect of sucrose and treatments on species composition in MBM plaque. The MBM was incubated for 1 d with various sucrose concentrations. CKB analysis was performed immediately after treatment. PCA analysis in SPSS (in figure: PC = plasma control, NT = untreated). Plasma: 50 mW, 1 min.
Figure 7.12: PCA analysis of the composition 24 h after the treatments of the MBMs. The MBMs were grown for 1d in 0.15 % sucrose and then treated. Plasma power = 100 mW.
Figure 7.13: The composition 24 h after the treatments of the MBMs. The plaques were grown for 1 d in DMM supplemented with 0.15 % sucrose and then treated.
Table 7.2: % to total DNA. The affected species of the MBMs 24 h after the treatments. The MBMs were grown for 1 d in 0.15 % sucrose and then treated. Plasma power = 100 mW. * = significant reduced species compared to untreated.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gram -/+,-/ aero</th>
<th>Plasma 1 min. (N=5)</th>
<th>Plasma 5 min. (N=4)</th>
<th>CHX (N=5)</th>
<th>Untreated (N=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mutans</td>
<td>+, fac. anaerobe</td>
<td>1.10 *</td>
<td>1.53</td>
<td>1.78</td>
<td>1.82</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>+, fac. anaerobe</td>
<td>0.90 *</td>
<td>0.97 *</td>
<td>1.60</td>
<td>1.30</td>
</tr>
<tr>
<td>S. mitis 2</td>
<td>+, fac. anaerobe</td>
<td>11.71</td>
<td>8.13</td>
<td>2.00 *</td>
<td>8.18</td>
</tr>
<tr>
<td>S. anginosus</td>
<td>+, fac. anaerobe</td>
<td>2.98</td>
<td>2.40</td>
<td>2.35 *</td>
<td>2.52</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>+, anaerobe</td>
<td>0.92 *</td>
<td>1.16 *</td>
<td>0.98 *</td>
<td>1.56</td>
</tr>
<tr>
<td>B. dentium</td>
<td>+, anaerobe</td>
<td>0.00 *</td>
<td>0.00 *</td>
<td>0.00 *</td>
<td>0.34</td>
</tr>
<tr>
<td>A. israelii</td>
<td>+, anaerobe</td>
<td>0.00 *</td>
<td>0.00 *</td>
<td>0.00 *</td>
<td>0.58</td>
</tr>
<tr>
<td>R. dentocariosa</td>
<td>+, aerobe</td>
<td>0.71</td>
<td>1.06</td>
<td>0.30 *</td>
<td>1.15</td>
</tr>
<tr>
<td>F. nucleatum ss</td>
<td>- , anaerobe</td>
<td>1.02 *</td>
<td>1.63 *</td>
<td>0.77 *</td>
<td>2.24</td>
</tr>
<tr>
<td>nucleatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. gingivalis</td>
<td>- , aerobe</td>
<td>1.18</td>
<td>1.37</td>
<td>0.86 *</td>
<td>2.27</td>
</tr>
<tr>
<td>P. asaccharolyti-</td>
<td>+, anaerobe</td>
<td>2.06 *</td>
<td>1.73 *</td>
<td>2.74</td>
<td>3.05</td>
</tr>
<tr>
<td>cus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. morbillorum</td>
<td>+, anaerobe</td>
<td>8.12</td>
<td>6.65 *</td>
<td>6.68</td>
<td>9.58</td>
</tr>
<tr>
<td>L. buccalis</td>
<td>- , anaerobe</td>
<td>2.55 *</td>
<td>4.32</td>
<td>5.47</td>
<td>4.52</td>
</tr>
</tbody>
</table>
Effect of plasma and CHX treatment on microbiota composition of 2 d old microplate plaques

Analysis of the plaques 24 h after treatment showed that a plasma application of 5 min appeared to affect different species than CHX (Figure 7.14 and Table 7.3). The 5 min plasma treatment reduced several species compared to the untreated biofilms (Table 7.3). CHX treatment inhibited *S. mitis* 2 and *Lactobacillus rhamnosus*.

Figure 7.14: The composition 24 h after treatments of the MBMs. The MBMs were grown for 2 d in DMM supplemented with 0.15 % sucrose and then treated.

Both plasma and CHX treatment did appear to have a selective response. However, different species were targeted by the plasma at the biofilms grown for 2 d as compared to the biofilms grown for 1 d (Table 7.2 and 7.3), e.g. *S. mitis* 1, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *C. gingivalis* and *Micromonas micra*. 
Table 7.3: % to total DNA. The affected species of the MBMs 24 h after the treatments. The MBMs were grown for 2 d in 0.15 % sucrose and then treated. Plasma power = 100 mW. ⋆ = significant reduced species compared to untreated.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gram -/+, an-/ aerobe</th>
<th>Plasma 5 min. (N=2)</th>
<th>CHX (N=3)</th>
<th>Untreated (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mitis 1</em></td>
<td>+, fac. anaerobe</td>
<td>2.33 ⋆</td>
<td>4.50</td>
<td>3.61</td>
</tr>
<tr>
<td><em>S. mitis 2</em></td>
<td>+, fac. anaerobe</td>
<td>3.61</td>
<td>1.22 ⋆</td>
<td>4.76</td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td>+, anaerobe</td>
<td>0.22</td>
<td>0.09 ⋆</td>
<td>0.32</td>
</tr>
<tr>
<td><em>B. dentium</em></td>
<td>+, anaerobe</td>
<td>0.00 ⋆</td>
<td>0.65</td>
<td>0.49</td>
</tr>
<tr>
<td><em>A. israelii</em></td>
<td>+, anaerobe</td>
<td>0.54 ⋆</td>
<td>0.83</td>
<td>0.67</td>
</tr>
<tr>
<td><em>A. naeslundii</em></td>
<td>+, anaerobe</td>
<td>0.52 ⋆</td>
<td>0.77</td>
<td>0.69</td>
</tr>
<tr>
<td><em>A. odontolyticus</em></td>
<td>+, anaerobe</td>
<td>0.00 ⋆</td>
<td>0.21</td>
<td>0.18</td>
</tr>
<tr>
<td><em>C. gingivalis</em></td>
<td>-, aerobe</td>
<td>2.03 ⋆</td>
<td>6.55</td>
<td>4.97</td>
</tr>
<tr>
<td><em>L. buccalis</em></td>
<td>-, anaerobe</td>
<td>6.62 ⋆</td>
<td>7.65</td>
<td>8.38</td>
</tr>
<tr>
<td><em>M. micros</em></td>
<td>+, anaerobe</td>
<td>2.47 ⋆</td>
<td>5.36</td>
<td>5.68</td>
</tr>
</tbody>
</table>

7.4 Discussion

Microplate biofilm microcosm is an advanced model system for oral flora, cultured under conditions that are optimal for the growth of a bacterial community. Large variety of species comprises the biofilm; their coexistence may be symbiotic or competitive. Thus, this is an intrinsically complex system and its response to various external factors is also complex and not easy to predict. In principle, MBM is a reliable model, the only source of uncertainty may be introduced by a non-constant film thickness. This thickness can vary, unless they are grown in a CDFF [15]. In our experiments, the approximate thickness of the MBM plaques was 1 mm, which was rather large as compared to a real situation in the mouth.

It should be mentioned that the MBM model does not entirely reflect the *in vivo* situation. The MBMs grown in vitro were only 1 or 2 d old and grown in an anaerobic environment. The biofilms encountered in vitro can be more mature, and they are not grown in a completely anaerobic environment. In mature biofilms the matrix is better developed, so it can efficiently protect the bacteria against antimicrobial agents. Furthermore, if the bacteria grow aerobically, as in the mouth, the aerobic species might be located at the top or surface layer and the anaerobic bacteria at the bottom of the biofilm. In case of MBMs, the distribution of various species is probably random/unorganized. Furthermore, the MBM model is not exposed to exactly the same chemical environment as the oral biofilms. The latter have to survive in a more hostile environment,
which is created by the immune system of the organism. The oral defense system consists of both non-specific factors, such as saliva flow, lysozyme protease (causing cell lysis) and apo-lactoferrin (causing cell death), and specific immune factors (lymphocytes, macrophages, immunoglobulin IgG, IgA, IgM secretion). In the presence of these factors, bacteria may be easier to kill by additional (externally administered) antimicrobial agents.

Keeping in mind advantages as well as shortcomings of this model, we shall attempt to analyze the complex behavior of the biofilm. From the above-presented data, we can draw some general conclusions. The results from this investigation have shown that, under the conditions described, sucrose had a greater effect on the microbiota composition of the microplate plaques than treatment with either plasma or CHX. Sucrose is a very important influencing factor for oral flora and it is transformed into lactic acid. The resulting low pH selects for aciduric organisms, such as *S. mutans* and lactobacilli. The results from our investigation have shown that at 0.3% sucrose biomass was greatest. The results from this investigation imply that sucrose conditions and associated growth conditions may affect the success of treatments with any therapeutic agent. Growth conditions at such low pH may induce growth responses of those acid-tolerant bacteria which subsequently confer resistance to treatment. The effect of plasma or CHX may not be as apparent because of greater biomass.

In treated samples, at a given sucrose concentration, both CHX and plasma show bactericidal action on the biofilm, but the effects of these agents are different. Firstly, CHX changes the MBM structure visibly (Figure 7.6): the biofilm appears partly broken and dissolved. There are no such drastic changes in macroscopic appearance of plasma-treated samples. However, at the microscopic level (Figure 7.7) one can see that both plasma and CHX lead to cell lysis. The effect of plasma on the cell morphology is puzzling: most cells appear swollen and their membranes seem damaged, yet there is no complete cell disintegration. Furthermore, CHX is capable of restoring the neutral pH of the supernatant (Figure 7.3), while plasma-treated samples remain acidic. This means that CHX stops bacterial metabolism, and provides a strong indication of total bacterial inactivation.

Total bacterial inactivation by CHX and plasma seems to be contradicted by the results of biomass measurements. It is observed that the biomass increase in all considered cases: in CHX-treated and control samples the increase is twofold, while in plasma-treated and plasma-control samples the increase is even larger (four times). These results suggest that in all cases the antimicrobials did not lead to total bacterial inactivation. The question that arises is whether the increase of biomass reflects properly the viability and growth of biofilm. The increase of biofilm mass in case of plasma treatment can be also due to other effects, such as structural changes in the matrix, which lead to an increased water absorption. In previous studies it was shown that plasma
treatment increases wettability of various organic polymers. This effect can be responsible for higher water content in treated samples, and in case of MBM, for increased biomass. Besides, if plasma causes cell swelling (Figure 7.3), increase of total biomass is also expected. Alternatively, exposure to vigorous gas flow can loosen the film structure and thus enable more fluid to penetrate into the MBM. This effect would not play a role in CHX-treated MBMs.

The selectivity of plasma and CHX towards various species is also different. Both treatments are not completely lethal to all bacteria: in fact, some species are enhanced while others are reduced. Apparently, 1 minute plasma treatment has a fairly strong bactericidal influence on S. mutans, which is a major pathogen causing caries. We also observed that the plasma treatments reduced the anaerobes in the biofilms compared to the untreated samples.

The mechanisms of plasma and CHX interactions with bacteria are completely different. CHX is a non-specific cell-killing chemical, which attacks all cell membranes. Since the membrane is dissolved, the cytoplasm leaks out and lysis occurs. In contrast, plasma can affect many cell constituents, and the effects may be sub-lethal as well. Membrane damage in plasma interactions is one of the killing mechanisms: the reactive oxygen species (ROS) generated by the plasma can cause lipid peroxidation and lysis. This could explain swelling of plasma-treated bacteria: when the membrane poration is not very large (incomplete destruction), Na, Ca and water enter the cell and cause swelling; this is usually followed by cell death (lysis). Previous tests on eukaryotic cells showed that membrane damage could occur, especially after long plasma treatment times [28]. In case of eukaryotic cells, cumulative necrosis was the principal death mechanisms: the plasma radicals and heat denatured the proteins (thus also proteases and other enzymes), so that dead cells were “fixed” and their structure was preserved. However, necrosis was not the major plasma-induced effect: many delayed and sub-lethal responses were found. The effects on bacteria can be also delayed-lethal, or sub-lethal, when the membrane is not severely compromised. One of the possible mechanisms is the radical-induced damage to intracellular components (e.g. DNA damage). The affected bacteria would have to repair the damage, and in this way deplete their energy resources; this effect also leads to swelling and lysis (in analogy to lytic necrosis that follows ischemic stress). Furthermore, cell cycle arrest occurs when a substantial damage is detected. In fact, delay of bacterial growth, observed in plasma treatment of single-species biofilms (S. mutans) was most likely due to cell cycle arrest [6].

In summary, plasma effects on bacteria are of a more complex nature than mere killing. Additional complication arises from the limited penetration depth of short-living radicals into an organic biofilm. Species present in the top layer will be destroyed, but the underlying layers may undergo sub-lethal transformations. Since the microcosm is a quasi-independent system, where various bacteria compete with each other,
death of one species can result in enhancement of another one. Treatment with antimicrobials, be it plasma or chlorhexidine, does not inactivate all species, but it definitely does disturb the plaque. The current results on plasma treatment in combination with previously established weakening or cell cycle arrest of *S. mutans* [6] give a reasonable indication that plasma treatment would contribute to oral hygiene. Disturbed plaque with weakened bacteria would be easier to destroy by the natural defense system of the body. Naturally, the ultimate proof of effectiveness of plasma treatment in healing of cariolic lesions must be supplied by an *in vivo* study.

### 7.5 Acknowledgments

This research project was sponsored by the Netherlands Organization for Scientific Research (NWO) and by the Center for Plasma Physics and Radiation Technology. The first author would sincerely like to thank Chris Sissons and Bob ten Cate for making this work possible. We also like to thank Margo van Bekkum for the DNA-DNA checkerboard analysis and St John for the TEM images.

### References


REFERENCES


Treatment of microplate biofilm microcosms
Chapter 8

Plasma needle treatment of substrates with respect to wettability and growth of *Escherichia coli* and *Streptococcus mutans*

**Abstract**—In this chapter, we discuss surface modification of various materials exposed to a non-thermal atmospheric plasma. The source used is the plasma needle: a radio-frequency driven, non-thermal atmospheric micro-plasma. A number of substrates (Perspex and polystyrene) were treated with the plasma needle. The modification of materials was subsequently identified as hydrophilization of the surface, and experimentally validated by water contact angle measurements. Furthermore, we studied the effect of this modification on growth of two bacterial species, *Escherichia coli* and *Streptococcus mutans*. Bacteria were cultured on treated and non-treated polystyrene 96-wellplates; the growth of *E. coli* on treated substrates was enhanced, while for *S. mutans* it was reduced. Explanation of these effects is provided.
8.1 Introduction

In the past decades, gas plasma treatment has become increasingly popular in the field of surface processing, both for industrial (electronics and material engineering) and biomedical purposes. It is known that plasma treatment can result in various kinds of surface effects, including highly specific modifications like etching and thin layer deposition. This knowledge has inspired a wide range of biomedical applications. Two main applications have been identified: sterilization of materials [1, 2], and scaffold processing [3]. However, other refined modifications are also possible. In the 1950’s, Barker and LaRocca [4] discovered that plasma treatment could support the growth of cells. Meanwhile it is known that, depending on the plasma conditions, enhancement or inhibition of cellular and bacterial growth can be achieved. The mechanisms that govern these effects are not yet fully understood, but it is expected that the influence of plasma on the adhesion properties of the surface plays an important role.

Such surface modification has a number of applications, including selective culturing of bacteria and cells, increasing the biocompatibility of implants and making surgery tools more resistive to bacteria.

Substrate modification for the sake of controlling bacterial adhesion has been performed using non-thermal plasmas at reduced pressure. It is expected that this technique can be adapted for atmospheric pressure.

Atmospheric non-thermal plasmas are suitable for treatment of heat-sensitive objects and even living biological tissues. Recently, the effects of these plasmas on living cells and tissues was studied. Plasma treatment may find many medical applications, like high-precision surgery (controlled tissue removal), improvement of wound healing and disinfection [5].

A non-thermal plasma source for biomedical applications (radio-frequency driven plasma needle) was developed at the Eindhoven University of Technology [6]. It is small-sized and flexible, and it can be operated in the open air. These features are necessary for in vivo treatment of biological tissues, like wounds and dental cavities. Responses of living mammalian cells to plasma treatment were studied and a method of cell removal without necrosis was found [7–9]. Applications of plasma treatment in dentistry were studied by Sladek et al. [10]. Tests on plasma deactivation of bacteria [11] and dental biofilms [12] were conducted to develop a new technique for non-destructive treatment of dental cavities and periodontal pockets. Some tests on inactivation of bacteria inoculated in a stock were performed using the plasma needle. A parameter study was conducted using thin films of Escherichia coli bacteria sustained on agar dishes [11] in order to determine the ”area of reach” (the diameter of the affected spot) of the plasma needle and to optimize the conditions for bacterial destruction. Efficient bacterial deac-
tivation is found at low-power conditions, i.e. when the plasma power and treatment time do not exceed 150 mW and 60 s, respectively. Under these conditions plasma temperature remains low, so possibly it can be applied *in vivo* without irritating the dental nerve.

One of the important issues in treatment of dental decay is the post-treatment behavior: adhesion and stability of filling materials, re-growth of biofilms, and reoccurrence of caries. In this work we study the influence of atmospheric plasma treatment on surface properties; in particular, we intend to employ the plasma needle to control bacterial adhesion and growth. Plasma needle is suitable for modification of organic (e.g. bone, tissue) as well as inorganic materials (e.g. polymers, metals, dental fillings).

If reduction of bacterial growth can be achieved, the needle may be used for preventive treatment of caries. Furthermore, surface activation of the treated tooth and filling material can improve the restoration of the cavity.

To investigate such possibilities first the influence of plasma on the surface must be identified. Here the modification of surface wettability after plasma needle treatment is examined. First, we have used Perspex (PMMA) and polystyrene as substrate materials and determined their wettability by measurements of contact angle for water droplets. The effective area of reach of the plasma treatment was quantified, and the character of modification (‘long-lasting’ or temporary) established. Furthermore, the growth rate of *E. coli* and *S. mutans* biofilms, cultured on plasma-treated polystyrene 96-wellplates was determined and compared with non-treated surfaces. According to Van Loosdrecht *et al.* [13], there is a clear correlation between adhesion and growth rate of bacteria, although this effect could not be explained in a satisfactory manner. Nevertheless, monitoring growth rate provides a good measure of adhesion efficiency. From the point of view of application, both effects are important: in order to be effective, plasma treatment should prevent both adhesion and growth of dental plaque.

## 8.2 Materials and methods

### 8.2.1 The plasma needle set-up

The plasma needle (Figure 8.1) consists of a metal wire (0.3 mm diameter) with a sharpened tip, confined in a Perspex tube (4 mm inner diameter). Helium flow is directed through the Perspex tube at a flow rate of 2 l min\(^{-1}\), controlled by a mass flow controller (Brooks Instrument, model 5850E). A radio-frequency (RF) voltage is applied to the tip of the needle at a frequency of 13.05 MHz. The RF signal is produced by a waveform generator (Hewlett Packard, model 33120A) and amplified by an RF amplifier (Amplifier Research, model 75AP250) (Figure 8.2). A home-built fixed (not adjustable) matching
network optimizes the power dissipation in the plasma. The forwarded and reflected power is monitored using a P6103 Tectonics probe (Amplifier Research) connected via a dual-directional coupler to a power meter (Amplifier Research, model PM 2002).

In the above-described set-up, the power supplied is not only dissipated in the plasma, but also in the matching network, the electrical circuit and plasma needle. To calculate the power that is solely dissipated in the plasma, the following formula was used [10]:

\[
P_{\text{plasma}} = (P_{\text{fwd}} - P_{\text{refl}})_{\text{on}} - (P_{\text{fwd}} - P_{\text{refl}})_{\text{off}}
\]

In this formula, \(P_{\text{fwd}}\) is the forwarded power and \(P_{\text{refl}}\) is the reflected power; the subscript ‘on’ denotes power measurements when the helium flow is turned on and ‘off’ describes measurements when the helium flow is switched off, while the voltage remains on.

![Image of portable plasma needle](image)

**Figure 8.1:** A picture of the portable plasma needle (left), consisting of a tungsten wire in a Perspex tube attached to a source of helium. When radio-frequency voltage is applied to the tungsten electrode, a micro-plasma is generated (right).

### 8.2.2 Contact angle measurements

The change of wettability of the substrate surfaces is studied after each plasma needle treatment. To prepare the samples, the substrates are thoroughly cleaned with 70 % ethanol and dried in open air. Droplets of de-ionized, double-distilled water of approximately 4 \(\mu\)L (1 mm diameter) are placed on the surface. The water contact angle is determined repeatedly at the surface of the substrate using a Krüss Drop Shape Analysis System DSA10/G10.

Then, water droplets are removed, and the substrate is cleaned with 70 % ethanol and dried again. Subsequently, every substrate is treated with the plasma at a fixed spot, and the contact angles are measured again at and around the treated spot, but at the same locations as before plasma treatment. This serves to prevent a possible error due to substrate inhomogeneity. In a number of cases the substrates are cleaned and
dried, and contact angle measurements are repeated. This step is performed to verify if the plasma-induced surface modification is temporary or long-lasting. All plasma treatments are performed in a vertical arrangement with 1 mm distance between the plasma needle tip and the substrate surface (Figure 8.3).

Series of experiments are performed according to the described procedure, to study the effects of plasma power and treatment time. Power settings are varied by changing the amplitude of the input voltage (180 mV – 200 mV, before amplification). Treatment time is 30 or 60 seconds.
8.2.3 *Escherichia coli*

An overnight culture of *Escherichia coli* is prepared by inoculating a 4 ml of Luria-Bertani (LB) medium from a glycerol stock (*E. coli* Novablue competent cells, Novagen). The LB-medium consisted of pepton (10 g l⁻¹), yeast extract (5 g l⁻¹), sodium chloride (10 g l⁻¹) and demineralized water (1 l). The bacteria are allowed to incubate during 16 h with vigorous shaking (250 rpm) at 37 °C. Thereafter, 1 ml of the suspension is re-suspended in 30 ml of fresh LB- medium. This diluted substance is shaken for approximately 2 h at 250 rpm and 37 °C. The growth curve has been monitored photometrically by measuring optical density (absorbance) at 600 nm with an optical density reader (Genesys 10 UV, Thermo Spectronic reader). When the optical density of the bacterial suspension has reached OD = 0.5, the culture is at the beginning of the log-growing phase and thus ready for use.

8.2.4 *Streptococcus mutans*

An overnight culture of *Streptococcus mutans* is prepared by inoculating 10 ml of Brain Heart Infusion (BHI) medium from a glycerol stock (*S. mutans* C180-2, Academisch Centrum Tandheelkunde Amsterdam, The Netherlands). The BHI medium consists of brain heart infusion (37 g l⁻¹) and demineralized water (1 l). The bacteria are incubated during 16 h in a stove at 37 °C. After the incubation, the mixture is vortexed. The suspension is diluted 1/50 in BPS medium (5/10 BHI + 50 mM PIPES + 0.2 % sucrose) and re-suspended. *S. mutans* form so called biofilms when they are cultured overnight in wellplates [14, 15].

The prepared *Escherichia coli* and *Streptococcus mutans* cultures have been dispensed over both plasma-treated as well as non-treated polystyrene Nunclon™ Δ Surface 96-wells plates. Directly after dispersion, the photometric absorbance at 650 nm of the suspensions in the wells has been determined with a microplate reader (Multiscan Ascent, Thermo Electron Corporation). Absorbance (optical density) values are used to monitor the growth of bacteria in suspension cultures. Absorbance is defined as a logarithm of the fraction of transmitted light through a liquid: \( \text{OD} = \ln\left(\frac{I_0}{I_{trans}}\right) \), where \( I_0 \) is the intensity of the incident light beam and \( I_{trans} \) is the intensity of the transmitted light beam. According to the Lambert-Beer law, it is proportional to the thickness of the bacterial film.

After the absorbance measurements, the plates are placed in a stove at 37 °C, in case of *E. coli* for 2 h under normal atmosphere, whereas for *S. mutans* in an anaerobic atmosphere (80 % N₂, 10 % CO₂, 10 % H₂, Anaerobic gaspack, Becton, Dickinson and Company) for 4 h. After this incubation, the photometric absorbance is determined again to see if any changes have occurred.
8.3 Results

8.3.1 Contact angle measurements

<table>
<thead>
<tr>
<th>Material</th>
<th>Power dissipated in plasma</th>
<th>Contact angle before treatment</th>
<th>Contact angle after treatment</th>
<th>Contact angle reduction</th>
<th>Contact angle after washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perspex</td>
<td>0.299 W</td>
<td>67°</td>
<td>38°</td>
<td>29°</td>
<td>&gt; 67°</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>0.104 W</td>
<td>87°</td>
<td>&lt; 20°</td>
<td>&gt; 67°</td>
<td>34°</td>
</tr>
</tbody>
</table>

In Table 8.1, a summary of the contact angle measurements results are shown. All contact angles are averaged over at least 5 measurements. Angles smaller than 20° can not be measured. It is clear that the contact angle of water decreases in all experiments - plasma needle treatment makes substrates more wettable (hydrophilic). This effect is the most pronounced in case of polystyrene substrates. The persistency of the surface modification has been checked and it appears that the polystyrene substrates become long-lasting (24 h) hydrophilic, whereas Perspex samples show only a temporary change in wettability. However, the effect in polystyrene after washing is smaller than before, which implies that there is also a temporary effect in this material.

Figure 8.4: Area of reach (= diameter of affected spot) of the surface modification for Perspex. The * on the x-axis is the position of the tip of the needle. Power dissipated in plasma = 0.374 W, Treatment time = 30 seconds.
In Figure 8.4, the change of wettability around the treated spot is shown. The star at the x-axis denotes the position on the substrate, where the tip of the plasma needle has been located 1 mm above the surface.

It can be observed that the plasma creates a local hydrophilic area on the substrate with a radius of approximately 10 mm. This feature is material dependent: for polystyrene the radius is smaller (9.9 mm), whereas Perspex had the largest radius (10.5 mm). The observed contact angle reduction is a function of the energy that is absorbed by the substrate. From previous work [16] it is known that at 1 mm distance most of the energy is absorbed by the substrate; the energy absorption is thus approximately equal to input power in the plasma multiplied by the treatment time. In Figure 8.5 the measured relation between energy absorption and contact angle reduction is shown for Perspex. Contact angle decreases with increasing energy absorption, but there is a limit value for the achievable contact angle reduction. The maximum contact angle reduction is approximately 53°.

8.3.2 Bacterial growth and adhesion

Figure 8.6 and 8.7 show the change in absorbance values during *E. coli* and *S. mutans* growth, every 0.5 and 2 hours respectively. Plasma treatment time is kept constant at 60 seconds, while dissipated power was varied. Non-treated wells are used as negative control to compare bacterial growth on treated with non-treated polystyrene well plates. The figures show that plasma needle treatment of the substrates before dispensing of the bacteria stimulates *E. coli* growth. In contrast *S. mutans* growth is decreased in compar-
ison with the negative control groups. The effects seem to be most pronounced for low power values; the optimal conditions for growth manipulation are thus achieved at low powers.

Figure 8.6: Bar diagram of E. coli growth. N = 4

Figure 8.7: Bar diagram of S. mutans growth. N = 4

8.4 Discussion

Chemically reactive species, and especially short-living radicals are of major importance in surface modification. Radicals are abundant in the plasma needle’s discharge. According to Kieft et al. [17], the radical density in the plasma is $10^{19} \text{ m}^{-3}$. The following particles are expected to be produced in the discharge: $\mathrm{O}^-$, $\mathrm{O}_3$, $\mathrm{NO}$, $\mathrm{NO}_x$, $\mathrm{H}_2\mathrm{O}_2$, $\mathrm{HO}^-$.
Wettability and growth of *E. coli* and *S. mutans*

HOO- and ONOO–. Furthermore, the surface is bombarded by energetic ions, but their density is much lower \((10^{17} \text{ m}^{-3})\) [18].

There are also other plasma-related factors that may influence surface properties. When the substrates are exposed to the plasma needle, the surface is bombarded not only by reactive particles, but also irradiated by UV. The depth of UV photons is restricted to a one micrometer thick layer [1]. In other words, UV can cause superficial surface modification, such as a change in plastics wettability and bondability. This means that, apart from reactive particles, also UV might influence the wettability of the substrates. However, in the case of the plasma needle UV is not the major factor because UV emission intensity is very low [19]. Thus, we can assume that surface modification in our case is caused by chemical and not photochemical reactions.

We have observed two types of surface modification: a temporary and a long-lasting increase of the surface wettability. The temporary change is observed with Perspex, while the long-lasting change has been found for polystyrene. This effect can be explained by the nature of the substrate material. The chemical structures of both polymers, and the possible pathways of radical oxidation are given in Figures 8.8 and 8.9.

![Figure 8.8: Plasma-induced change of Perspex structure.](image)

Temporary changes in both polymers can be induced by reactive particles (e.g. \(O_2^\cdot\), \(HO^\cdot\) and \(HOO^\cdot\)) from the plasma, that break bonds such as the C–H, C–C and/or C–O, This creates dangling bonds where the oxygen-containing molecules, such as H\(_2\)O and O\(_2\) from ambient air can loosely adhere. Thus hydrophilic groups are formed temporarily at the sites of dangling bonds. After certain time dangling bonds recombine and the effect vanishes. Possibly some long-lasting hydrophilic sites can be produced as well (e.g. when a C–C bond is cleaved and replaced by C–OH), but this effect is not expected
to be very pronounced.

Perspex contains ester-like bonds, which are polar and thus make the material hydrophilic by nature. In case of Perspex, the creation of dangling bonds is the major mechanism responsible for wettability changes, and thus the effect is not lasting (Figure 8.8).

In contrast, polystyrene is purely hydrophobic, because it contains only covalent hydrocarbon bonds, in saturated aliphatic chains and benzene rings. This is the reason for a very high water contact angle for untreated material. Furthermore, from the chemical formula it can be seen that polystyrene is likely to be a target for attack of aggressive oxygen radicals. The benzene rings can be cleaved and oxidized, so that highly hydrophilic alcohol groups can be implanted in the destroyed rings. The resulting new structure is chemically stable, which implies that the hydrophilization effect is long-lasting, in agreement with the experimental observation (Figure 8.9).

The observed contact angle reduction of Perspex is in agreement with the literature data obtained using different (low-pressure) plasma sources. Kogoma et al. [20] used a CF$_4$–O$_2$ plasma on several materials, including graphite, polypropylene and polytetrafluoroethylene, and reported a similar relation between contact angle and treatment time, presumably keeping the plasma power constant. Initially, the wettability increased rapidly with treatment time, and stabilized at a plateau value for long treatment times. Larrieu et al. [21] treated polystyrene films with O$_2$, an O$_2$ + N$_2$ mixture and N$_2$. The energy applied, or the amount of energy that was dissipated in the plasma, was kept constant for different treatments. The applied energy is related to the energy absorption introduced in this article. They presented several graphics with a dimensionless contact angle change versus treatment time. These dependencies are similar to the ones observed in our experiment.
A plausible explanation for this phenomenon is the fact that after a certain period of treatment, the substrate becomes saturated with reactive species and the upper surface layers of the substrate cannot absorb or react with additional particles. Hence, the contact angle reduction reaches its maximum value. When the plasma power is higher, this situation is reached sooner, because the flux of reactive particles increases with increasing power. The adhesion and growth of bacteria can be partly explained by surface hydrophilization, but the difference in behavior of *E. coli* and *S. mutans* needs an additional explanation.

Both bacterial species are hydrophilic by nature, hence they stick better to hydrophilic substrates. Thus, surface hydrophilization can be responsible for increased growth of *E. coli*, but it is in contradiction with growth inhibition of *S. mutans*. Our preliminary explanation for this effect involves the response of bacteria to active oxygen species. The plasma needle’s discharge produces these species because of operation in humid, open air. Interactions with the substrate can result in higher oxygen content of the surface, e.g. in the form of adsorbed oxygen, peroxide or alcohol groups. It is known that *E. coli* is aerobic, and its growth is not hindered by oxygen, while *S. mutans* is facultative anaerobic and generally dislikes oxygen species. These properties can explain the different growth response of these bacteria to plasma treatment. In summary, one can state that plasma needle treatment chemically alters the upper layers of the surface of substrates. This increases the wettability of the materials.

### 8.5 Conclusion

The atmospheric plasma needle and its effect on surface properties have been described. It was found that plasma treatment can increase the wettability (hydrophilic character) of the surface, which is expressed by reduction of water contact angle.

There is a correlation between the contact angle reduction and substrate energy absorption; increasing energy absorption leads to a greater reduction in contact angle. However, saturation is observed and a maximal contact angle is reached after sufficiently long treatment time. The final value most likely depends on the particular material under treatment. The area of reach of the helium plasma was determined on the order of 20 mm and is dependent on the material. Polystyrene shows the most localized effect whereas Perspex has the largest area of reach.

The wettability increase is temporary for Perspex, while polystyrene can be made long-lasting hydrophilic. A possible explanation for this phenomenon is deduced from the chemical structures of these polymers.

Finally, it is found that plasma treatment can accelerate the growth of aerobic *E*
*Escherichia coli* and inhibit the growth of anaerobic *Streptococcus mutans*. Inhibition of growth of *S. mutans* is a very encouraging result from the point of view of dental applications.

### 8.6 Acknowledgments

This research project was sponsored by the Netherlands Organization for Scientific Research (NWO). The first author sincerely likes to thank Tom Baede for performing the microbiological experiments. We also like to thank the Academisch Centrum Tandheelkunde Amsterdam (ACTA) for their support.

### References


Wettability and growth of *E. coli* and *S. mutans*


Chapter 9

General discussion
9.1 Introduction

The collected data presented in this thesis gives us several indications for the possible consequences of plasma treatment, together with many hints on how to improve the procedure of treatment and assaying. In the following discussion section, we first discuss the plasma needle design and the operational parameters. Secondly, some facts from literature on plasma-induced bacterial killing are given. We shall attempt to describe the inactivation results in terms of basic mechanisms of plasma interactions with micro-organisms. Then, the advantages as well as the limitations of our in vitro model systems are discussed. A comparison between the planktonic and the biofilm model systems is made. One of the future challenges of the plasma needle project is to examine the depth effects of the plasma species in plaque. In short, we discuss the penetration depth of our plasma species. Furthermore, the plasma needle treatment is compared with ozone treatment. This because ozone therapy is another alternative painless and non-destructive method for caries treatment. Finally, this general discussion chapter ends with conclusions and some future recommendations.

9.2 The plasma needle design and operational parameters

With appropriately designed electrodes, power and gas supply, a stable non-thermal atmospheric micro-plasma can be operated, below the glow-to-arc transition. This generally requires one or more of the following features: inert gas such as helium to lower the breakdown voltage, a significant gas-flow rate for appropriate cooling, radio-frequent power to generate a non-equilibrium plasma, a cathode with a sharp tip to increase the local electric field.

The design of the plasma device changed several times during the project. We started with a closed system. The plasma needle was confined in a closed box filled with helium. In this closed system Stoffels et al. experimented with various needle geometries [1]. As expected, a thin needle with a sharp tip was needed to easily ignite a plasma. Unfortunately, the closed system could not be used for in vivo treatment, so we developed a portable, open system. The first portable plasma needle was relatively long and surrounded with ceramic material (as can be seen in Figure 4.1). The surrounding ceramic was necessary to keep the plasma at the tip of the needle. The ceramic material was very vulnerable, so we replaced it by glass that was slightly thicker and therefore less vulnerable. During the project we discovered that substantial power was lost through RF radiation at the electrode (like an antenna). Therefore the needle was made shorter to reduce this power loss. We also improved the matching network to reduce power loss in the matching network [2]. In this way the power consumption
could be kept low. The plasma needle presented in this thesis is still a prototype. In the near future a new needle will be designed. It is important to control the operational parameters to stay on the safe side of operation. The parameters to be controlled are the power dissipated in the plasma and the power emitted to the substrate. Power and distance need to be controlled to prevent arcing, and to assure stable plasma operation and efficient bacterial inactivation. By controlling the power, also heating of the substrate can be controlled, which is important in preventing thermal damage.

Laroussi et al. uses the so called plasma pencil to inactivate bacteria [3]. The idea is basically the same as the plasma needle, however, Laroussi et al. expose the bacteria to the afterglow of the plasma. They use sub-microsecond high voltage pulses at repetition rates in the 1–10 kHz range to ignite the plasma. The voltages are applied between the two electrodes and a gas mixture (such as helium and oxygen). The gas flows through the apertures in the electrodes. When a discharge is ignited in the electrode gap, a plasma plume of several centimeters is launched through the aperture in the outer electrode and it expands into the surrounding room air. Laroussi et al. say that the advantages of the plasma pencil are plasma controllability and stability, room temperature and atmospheric pressure operation, and low power consumption. However, the plasma pencil seems to be hard to control and not so flexible in practice. The device and the plasma plume are relatively long and therefore difficult to handle. The power consumption is higher than the plasma needle, and the helium flow rates are 5-10 times higher. In comparison, our plasma needle is more flexible and the size of the plasma can be very small. In Figure 9.1 an example is shown of the plasma needle connected to a catheter. It can be seen that the plasma can be made very small.

Figure 9.1: Plasma ignited at the tip of a catheter. Length of plasma is approximately 2 mm.
In this thesis several methods are described to measure power and temperature. Thermocouples, powerheads, resistance temperature detectors and temperature recording strips were used.

The measurement of dissipated power by the so-called subtraction method, is based on measuring the forwarded and reflected power (see Section 5.2.2). Exact power measurements in RF atmospheric micro-plasmas are inherently difficult. Typically, the power dissipation in the matching network originates from the resistive heating of components of the matching network. If there is a significant change in RF current, or a change in the voltage when the discharge is shut off, this formula can give false input powers. Therefore, the subtraction method does not give the precise dissipated powers, but it gives an indication.

The correct measurement of the dissipated power is to measure the current in the matching network and the voltage at the needle. However, such measurements are not practical, because I-V probes cause large disturbances to the plasma. Ewout van der Laan made a model of the plasma needle set-up [2]. In his model he used the matching network impedance, total power and voltages to calculate the dissipated power. He noticed that the calculated power was higher than the measured power with a constant multiplicative factor of about 2. However, his model was based on an ideal matching network (no reflected power). So the real dissipated power should be somewhere in between the measured and the calculated dissipated power, the accuracy being about 50%.

The temperature of a surface exposed to the plasma can be measured with calibrated platinum resistance temperature detectors (RTDs) or temperature recording strips (see Chapters 4 and 5). However, the plasma cannot be directly applied to the RTD because it is made from metal (platinum). Close presence of a grounded metallic surface changes some plasma features. The temperature recording strips are not very accurate, but probably good enough to get a rough idea of the temperature during treatment. Another possibility is to measure the gas temperature (heavy species, i.e. neutrals, ions) by emission spectroscopy. The gas temperature can be determined by analyzing the rotational structure of the $\text{N}_2$ second positive system emission [3]. The accuracy of this method is about 10 degrees.

In the plasma needle project the plasma was ignited in helium with a small admixture of air. Other gasses can be added to the plasma to improve bacterial inactivation efficacy, for example oxygen [4, 5]. However, it is unattractive to add gasses like oxygen to the helium flow of the plasma needle, because this quenches the plasma and diminishes the production of radicals [6]. The plasma activity decreases because electron attachment causes depletion of plasma electrons.
9.3 Inactivation mechanisms

Plasma sterilization relies on three groups of bactericidal agents: 1) energetic photons in the UVB and UVC range, 2) reactive species such as oxygen radicals, and 3) charged species such as electrons and ions. The effect is usually due to the synergy of all these factors [7], but their relative importance depends on the type of plasma source as well as on the sample preparation. In low-pressure plasmas, UV inactivation is shown to be important. Moisan et al. [5] observed characteristic three phases in inactivation of bacteria and spores. The first killing phase was due to DNA destruction by direct UV irradiation. It provided a high killing rate (2–3 decades in about 5 min), but the effect was only superficial because of the limited penetration depth of UV photons into thick samples. The second phase (2–3 decades in 20 min) was due to the (photo-) chemical erosion of spores and debris on the top of the sample. The third phase (2 decades in a few minutes) corresponded to UV destruction of genetic material of exposed bacteria after the debris had been etched away in the second phase.

Bacterial samples used for inactivation under reduced pressure were usually thin and dry (dried out during evacuation). In atmospheric discharges one can treat thick biofilms, droplets and suspensions without drying out and changing the sample structure. The inactivation mechanisms are also somewhat different. The three phases are not clearly distinguished and the role of UV radiation is said to be less important [4, 8]. Laroussi et al. [9] suggested an alternative, electrostatic mechanism of inactivation. The negative charge deposited by the plasma on the membrane can cause its rupture and cell death, especially in the presence of irregularities. This occurs in samples exposed to the active plasma zone (glow) which contains electrons and ions; the effect is superficial. We also observed this rupture of the membranes in the MBM experiments (Figure 7.7b).

The results presented in Chapter 6 show that the plasma has a temporary effect on the S. mutans culture. It can delay the growth by about 10 hours. However, it does not lead to total bacterial deactivation. Re-growth of the film is observed after 24 hours. Our observations suggest that plasma species do not kill bacteria, but weaken them. This can be explained by the biofilm thickness, discussed in Section 9.5.

In contrast, the 0.2 % chlorhexidine digluconate treatment leads to complete inactivation of bacteria. This could be expected, because CHX is an aggressive chemical which disrupts cell membranes (Figure 7.7c).
9.4 Model systems

In this thesis we described several model systems to study bacterial inactivation: droplets, bacterial films (superficial treatment) and biofilms. But what is the most realistic model system to study plasma inactivation? And what are the advantages and disadvantages of these models? In this section we discuss the model systems used.

The first inactivation experiment was performed on droplets of *E. coli* in growth medium (see Chapter 4). The difficulty of this model is that the droplets diminish by evaporation during plasma treatment. Another difficulty is that the shape of the droplet changes during treatment. Both difficulties will result in an inhomogeneous treatment of the droplet. The solution to the inhomogeneity was to spread the droplet on an agar plate and wait for 1 hour. After 1 hour, the bacteria are attached to the surface and the medium is evaporated. This results in a monolayer of bacteria on the agar. A homogeneous treatment is now possible.

However, bacteria in planktonic life and films on agar plates show different properties from those in biofilms. The bacteria in films are directly exposed to the plasma, when they are spread onto an agar plate. This means that there is almost no protection available and the bacteria are relatively easy to inactivate. Therefore we used the more realistic dental biofilms.

But how good are the dental biofilm model systems we used? The structure of the biofilms cultured *in vitro* is very dense. There are no channels or voids like *in vivo*. This suggests that it is even harder to inactivate bacteria in these model systems than in planktonic state or *in vivo* plaque.

In the inactivation experiments we concentrated on dynamic behavior of the biofilm rather than on destruction of individual bacteria. Previous research concentrated mainly on killing as many micro-organisms as possible, but relatively little attention was given to the condition of the treated species. Laroussi *et al.* described several sub-lethal effects that suggested a mechanism of bacterial defense against injury [10]. In our study, we monitored the kinetic growth response of treated biofilms (Chapter 6). For *in vivo* medical applications (caries treatment and other disinfections) this approach appears more relevant. A living organism is a dynamic system, where intruders such as bacteria are continuously fought by the immune system – the action of the defense cells (leukocytes, lymphocytes, etc.) and the corresponding bacterial responses sustain the desired homoeostatic balance. This is a unique environment that is too complex to be reproduced in any *in vitro* model.
9.5 Penetration depth

In case of inactivation with the plasma needle, we observed superficial as well as depth effects. In this section a short discussion about the penetration depth of our plasma species is given.

In thick samples (droplets and biofilms) only chemical inactivation by active radicals could lead to depth effects. Gas phase density of reactive oxygen species (ROS, strong bactericidal agents) was found to be about $10^{19} \text{ m}^{-3}$, and the radicals appeared fairly stable in aqueous solution: the concentration of ROS in treated liquid was about 1–10 $\mu\text{M}$ [11]. The survival curve for *E. Coli* suspension displayed only one single time scale (1 decade in $\approx 40 \text{ s}$) (see Chapter 4).

In our experiments we produced relatively thick biofilms; this was especially the case for older biofilms and biofilms grown in high sucrose concentrations. The thicker the biofilm, the harder it is to penetrate for plasma species, because the organic material (e.g. exopolysaccharide) prevents direct access to the micro-organisms that are embedded within the biofilm structure. Also the reactive plasma species will react with the organic material present; thus, the more organic material, the less species will be available for killing bacteria. Therefore, the upper layer of *S. mutans* was most likely destroyed, like in previous tests on superficial inactivation of *E. coli* (see Chapter 5), but the effect on deeper lying micro-organisms was sub-lethal.

The biofilm composition in depth was probably random during anaerobically incubation for a 1 or 2 days old biofilm. Because of the limited penetration of plasma species, each time the superficial layer of the biofilm was treated. This top layer is each time different, because of the random composition. Therefore, the results of the DNA–DNA Checkerboard analyses were random.

The results of plasma treatment for 1 or 5 minutes showed almost no difference, probably because the treatment is limited to the top layer (limited penetration of reactive species in biofilm). This is an indication that plasma deactivation is based on short-living species, whose lifetime does not allow deep penetration.

A future aim is to examine the depth effects of plasma treatment on dental-biofilms, i.e. on smooth-surface plaque using vital fluorescence staining in combination with confocal laser scanning microscopy (CLSM). In brief, with a vital fluorescence stain it is possible to measure the percentage of living and dead bacteria present in the plaque after plasma treatment. Moreover, with a CLSM it will be possible to produce a three-dimensional image of the distribution of living and dead bacteria in the plaque after treatment [12–14].

The idea is to collect smooth-surface plaque on enamel discs prepared from extracted human molars; the discs typically are 1.5 mm thick and have a diameter of 5 mm. The discs are put on intraoral acrylic splints that can be worn by volunteers. After several
days, the enamel discs will be removed from the splints so the accumulated plaque on the surface of the disc can be treated with the plasma needle. Immediately after treatment, the vital staining procedure can be applied.

9.6 Plasma needle versus ozone treatment

An alternative painless and non-destructive method for caries treatment is ozone therapy [15–17]. Ozone is a powerful oxidizer, which is used as a disinfectant in the food industry, medicine and water treatment. Recently ozone gas has been used for caries treatment in dentistry (HealOzone, USA).

During ozone treatment first the lesion site and its surfaces are thoroughly cleaned. Then a tight disposable silicon cup is fitted on the selected tooth. The cup is used for the exposure of the selected tooth to ozone. The ozone is generated and delivered at a concentration of 2,200 ppm. The cup seals the area on the tooth and therefore prevents leaking of ozone. After 10 seconds of treatment the ozone is sucked away by a vacuum system. Finally the system delivers a liquid reductant that neutralizes the remaining ozone and facilitates the remineralization process.

The question is: what is the real mechanism in the ozone treatment? Is it the preparation before treatment, is it the ozone or is it the liquid reductant after treatment? Or maybe, is it the combination of these? There are studies that showed that ozone is not very effective in treatment of biofilms [16, 18]. The penetration depth of the ozone is limited by the organic material [19]; this situation is similar for active plasma species.

The studies on ozone treatment are mainly empirical. The exact mechanism of ozone action is not clear yet. It may be due to its disinfecting properties, mouthwash applied after treatment, or the combination of all actions performed in this therapeutic procedure. The influence of ozone on the remineralization process is also not understood. Possibly, the mechanism is based on the opening (open-up) of lesions, which facilitates the remineralization by calcium/phosphate provided by the saliva.

Plasma-produced ROS are more powerful oxidants than ozone; thus, smaller amounts and shorter exposures will be required to produce the same disinfecting effect. Furthermore, short-living radicals will disappear in the course of treatment, so they will not have to be removed afterwards. The treatment will be thus strictly topical. Another important feature is the selectivity: in the applied range of conditions, plasma can deactivate bacteria, while it does not cause necrosis in eukaryotic (body) cells [11].

The disadvantage is that the plasma needle can not be used when there are amalgam dental fillings present. When surface conductivity is too high (metals) arcing can occur. This will cause heating, which can be painful for the patient. On the positive side, most fillings are now made of non-conducting composite material.
9.7 Conclusion and future recommendations

The fact that plasma does not cause total sterilization in biofilms is not as disappointing as one might think. On the contrary, dynamic growth studies provide us with an important clue for *in vivo* treatment. In the usual medical applications, such as wound preparation, treatment of dental caries and periodontal pockets, root treatment, etc. one does not need to aim at complete bacterial inactivation. In many cases it would be of no use, because of the constant supply of new invaders from outside. However, it is important to weaken the bacteria at the site of infection so as to trigger an immune reaction from the body. In this sense, plasma may succeed in healing various infections. In the particular case of the oral cavity, the defense system is very rich. It consists of both non-specific factors, such as saliva flow, lysozyme protease (cell lysis) and apolactoferrin (cell killing), and specific immune factors (lymphocytes, macrophages, immunoglobulin IgG, IgA, IgM secretion) [20]. The defense system is strengthened after combating (weakened) bacteria: this is the commonly known working principle of a vaccine. Plasma treatment may thus be regarded as a topical vaccine that addresses the diseased area with high precision.

The *in vitro* caries models (artificially cultured biofilms) suffer from some drawbacks, such as the absence of the normal defense factors in the oral cavity. Hence, the current results give some indications that plasma treatment will be effective in the presence of the immune system, but the final decision can be made only after *in vivo* tests have been completed. The exact procedure of plasma therapy will be subject to many changes; the feedback will arise from medical practice. Once the effectivity *in vivo* has been shown and the role of immune reaction has been resolved, one will find the optimal protocol for treatment. One of the vital parts of this protocol will be the mechanical pre-treatment of the lesion site (debridement or breaking of the plaque). This will expose bacteria in the lesion, lead to better disinfection results and most likely, facilitate remineralization.

References


Summary

Who is not afraid of the dentist? Or to be more specific… who is not afraid of the dental drill?

Caries is a common ailment, that is caused by bacteria in dental plaque. Cavities will form when you do not properly remove the plaque on your teeth. The improvement of caries prevention and treatment techniques is a major issue in dentistry. Preparation of cavities prior to filling is presently done by removing infected and non-remineralizable or demineralized tissue by means of mechanical drilling. During mechanical drilling heating takes place and vibrations are induced. This can cause pain sensation in the patient. Moreover, drilling is often destructive: an excess of healthy tissue must be removed to ensure that the cavity is free of bacteria. In addition, the remaining tooth structure is weakened and prone to fractures.

This thesis is about the use of non-thermal atmospheric plasmas in dentistry. The goal of the PhD project is to clean dental cavities in a non-destructive and painless way. The idea is to inactivate bacteria in plaque to stop caries. This cleaning can be done by use of a non-thermal atmospheric micro-plasma. A plasma needle, which is a hand operating tool, is used to generate the non-thermal atmospheric micro-plasma.

The plasma needle is a portable device consisting of a tungsten needle confined in a Perspex tube. A radio-frequency voltage of 13.56 MHz is applied at the sharpened tip of this needle to ignite the plasma. The plasma is ignited in helium with a little admixture of air at atmospheric pressure. But what is a plasma and why do we want to use a plasma?

A plasma, which is generated by an electrical discharge in a gas, contains free electrons and ions, various active species (e.g. atomic or molecular radicals, for example O and OH radicals, and excited molecules) and energetic UV photons. All these species play an important role in sterilization. The sterilizing properties of plasmas are well-known and extensively described in literature. Non-thermal atmospheric plasmas are for example used in the sterilization of medical instruments, but also in biological warfare (e.g. against Anthrax).

Non-thermal atmospheric micro-plasmas operate at room temperature and do not
cause pain and bulk destruction of the tissue. Plasmas can treat and sterilize irregular surfaces; therefore they are very suitable for decontaminating dental cavities as a result of caries. The advantage of this novel tissue-saving treatment is that although the plasma treatment itself is a superficial treatment, the active plasma species it produces can penetrate into hollows, such as cavities. In contrast to dental lasers, plasmas can access small irregular cavity and fissure spaces. Moreover, the use of plasmas is relatively inexpensive. In the future plasmas can also be used in other dental interventions than just caries treatment. One can think of the plasma use in root canal treatments, in periodontitis or in superficial cleaning of dental tools. There are many applications possible.

First the project started with creating a radio-frequency plasma at the tip of a needle. The first needle was confined in a closed system filled with helium. But this closed system could not be applied in the mouth, so a portable open system was developed. The plasma needle was born.

First we characterized the plasma needle in terms of dissipated and emitted power per unit surface (power outflux) (Chapter 3). A calibrated thermal probe was used to determine the power emitted from the plasma towards treated surfaces. Transmission of the emitted power through various media was studied for a broad range of plasma conditions. These data gave insight into various contributions to the power outflux, as well as the penetration depth of the plasma into treated objects. The power outflux is shown to be a very important parameter, which determines the performance of the plasma tool.

Then the temperature in the tooth during plasma treatment and the efficiency of the plasma in killing bacteria were investigated (Chapter 4). We established whether the plasma needle can be safely applied to a tooth. Temperature measurements were performed inside the pulpal chamber using extracted human third molars. A thermosensor was inserted into the pulp chamber and the temperature was recorded during plasma needle treatment. The effects of different treatment times on the survival of Escherichia coli were studied. Bacterial viability was substantially reduced after exposure to the plasma.

The plasma operating conditions were optimized for bacterial deactivation (Chapter 5). Plasma power, treatment time and needle-to-sample distance were varied. E. coli films plated on agar dishes were used as a model system for this optimization. Plasma treatment of E. coli films results in formation of a bacteria-free void with a size up to 12 mm. $10^4 – 10^5$ colony forming units are already destroyed after 10 s of treatment. Prolongation of treatment time and usage of high powers do not significantly improve the destruction efficiency: short exposure at low plasma power is sufficient. Furthermore, we studied the effects of temperature increase on the survival of E. coli and compared it with thermal effects of the plasma. The population of E. coli heated in a warm water...
bath starts to decrease at temperatures above 40 °C. Sample temperature during plasma treatment has been monitored. The temperature can reach up to 60 °C at high plasma powers and short needle-to-sample distances. However, thermal effects cannot account for bacterial destruction at low power conditions. For safe and efficient in vivo application, the sample temperature should be kept low. Thus, plasma power and treatment time should not exceed 150 mW and 60 s, respectively.

After this optimization and characterization, we tested the plasma needle for its bactericidal activity against biofilm cultures of a key cariogenic bacterium Streptococcus mutans grown under different sucrose concentrations (Chapter 6). A chlorhexidine digluconate (CHX) rinse was used as a positive antimicrobial reference. Sucrose and frequency of the plasma treatment were shown to have a significant effect on the degree of response to treatment and bactericidal activity. A single plasma treatment of biofilms cultured with no sucrose yielded a bactericidal effect. Single and repeated plasma treatments of biofilms cultured in 0.15 % sucrose only impaired growth.

Then we used an easily reproducible model to replicate dental plaque in vitro, the microplate biofilm microcosm (MBM) model (Chapter 7). The effects of the plasma needle and CHX on the growth of MBMs were studied. Plaque biofilms were grown on coverslips in 24-well microplates from enriched human saliva. After treatment, the growth of the biofilms was studied by monitoring the biomass and the pH. Photomicrographs of the structure of the individual biofilms were taken. The differential effect of the treatments among the species was analyzed by the DNA-DNA checkerboard technique. Furthermore, the biofilms were placed in fixative and observed under transmission electron microscope (TEM). The results showed a complex response of MBM to plasma and CHX treatments. The TEM images showed that the plasma and CHX treatment damaged bacteria in the biofilms. CKB analyses showed that some species were significantly suppressed by the plasma treatment. The CHX treatment visually changed the structure of the biofilms. The pH of the CHX treated samples changed significantly after treatment and went back to the pH value of the blanks. Furthermore, sucrose concentration had a significant influence on the pH, biomass and the species composition of the MBM.

Finally, we investigated surface modification of various materials exposed to the plasma needle (Chapter 8). A number of substrates (Perspex and polystyrene) were treated with the plasma needle. The modification of materials was subsequently identified as hydrophilization of the surface, and experimentally validated by water contact angle measurements. Furthermore, we studied the effect of this modification on growth of two bacterial species, E. coli and S. mutans. Bacteria were cultured on treated and non-treated polystyrene 96-wellplates; the growth of E. coli on treated substrates was enhanced, while for S. mutans it was reduced.

The results from this PhD project have indicated that the plasma needle is able to inactivate bacteria in a non-destructive and painless way. Therefore, there will be a role
for non-thermal atmospheric plasma therapies in dental procedures. However, in this project we did not investigate the effect of plasma on bacteria \textit{in vivo}. This should be investigated in future studies.
Samenvatting

Wie is er niet bang voor de tandarts? Of beter gezegd, wie is er niet bang voor die akelige boor?

Cariës is een bekend en pijnlijk probleem, dat veroorzaakt wordt door bacteriën in tandplak. De verbetering van cariës preventie- en behandeltechnieken is een belangrijk onderwerp in de tandheelkunde. Preparatie van ‘gaatjes’ gebeurt momenteel door het geïnfecteerde en gedemineraliseerde weefsel te verwijderen met behulp van een mechanische boor. Tijdens het boren warmt de tand op en ontstaan er vibraties die pijn kunnen veroorzaken bij de patiënt. Boren is vaak destructief: onnodig gezond weefsel wordt verwijderd om ervoor te zorgen dat het gaatje volledig vrij is van bacteriën.

Dit proefschrift gaat over het gebruik van niet-thermische, atmosferische microplasma’s in de tandheelkunde. Het doel is om bacteriën in tandplak te doden met behulp van plasma, en zodoende cariës te stoppen. Gaatjes kunnen zo op een niet-destructieve en pijnloze manier schoongemaakt worden. Het microplasma wordt gegenerereerd door een plasmanaald.

De plasmanaald is een draagbaar en handzaam apparaat bestaande uit een metalen draad (de naald) in een Perspex buis. Om een plasma te ontsteken, wordt een radiofrequent voltage (13,56 MHz) aangelegd op de punt van de naald. Het plasma wordt bij atmosferische druk ontstoken in helium met toevoeging van lucht uit de omgeving. Maar wat is nu precies een plasma en waarom gebruiken we een plasma?


Een niet-thermisch, atmosferisch microplasma werkt bij kamertemperatuur en atmosferische druk. Het veroorzaakt daardoor geen pijn en beschadiging van het weef-
Samenvatting


Het project begon met de ontwikkeling en karakterisatie van de plasmanaald: het bepalen van het gedissipeerde en uitgezonden vermogen (power outflux) (Hoofdstuk 3). Power outflux is een erg belangrijke parameter voor de werking van het plasma.

Vervolgens is er een haalbaarheidsstudie uitgevoerd (Hoofdstuk 4), waarbij werd onderzocht of de plasmanaald veilig in de tandheelkunde gebruikt kan worden. Uit temperatuurmetingen in een tand tijdens plasmabehandeling is gebleken dat het plasma thermisch veilig is. Ook is het effect van het plasma op *Escherichia coli* bestudeerd, waarmee werd aangetoond dat het plasma bacteriën kan doden.

Na de haalbaarheidsstudie is het plasma geoptimaliseerd voor inactivatie van *E. coli* (Hoofdstuk 5). Plasmavermogen, behandeltijd en afstand tussen naald en substraat zijn hierbij gevarieerd. Als modelsysteem is een agar plaat gebruikt, met daarop een dunne laag *E. coli*. Door behandeling van de zogenaamde *E. coli* films ontstaan bacterievrije plekken, waarvan de diameter opgemeten kan worden, als maat voor het aantal gedode bacteriën. Een behandeling van slechts 10 seconden was voldoende om $10^4 - 10^5$ bacteriën te doden. Gebruik van hoge vermogens verbetert de inactivatie efficiëntie niet, dus korte behandeling bij lage vermogens is voldoende. Om de invloed van temperatuur te bepalen, is de overleving van *E. coli* bij verschillende temperaturen onderzocht. De resultaten zijn vergeleken met de temperatuureffecten van de plasmanaald. *E. coli* wordt geïnactiveerd bij temperaturen boven de $40 ^\circ C$. Bij hoge vermogens en korte afstanden, kan de substraattemperatuur tijdens plasmabehandeling oplopen tot $60 ^\circ C$. Om temperatuureffecten uit te sluiten dient het vermogen zo laag mogelijk gehouden te worden; bij lage vermogens treedt thermische effecten namelijk niet op. Ook voor veilig en efficiënt *in vivo* gebruik moet de temperatuur van het te behandelen substraat zo laag mogelijk gehouden worden. Uit experimenten is gebleken dat het vermogen niet hoger mag zijn dan 150 mW en de behandeltijd niet langer dan 60 seconden.

Na karakterisatie en optimalisatie van de plasmanaald hebben we de anti-bacteriële activiteit getest op biofilms (tandplak). Het plasma werd toegepast op single-species (één bacterie) en multi-species (meerdere bacteriën) biofilms. De biofilms zijn gekweekt in kweekmedium met verschillende sucroseconcentraties. De plasmabehandeling is vergeleken met een conventionele mondspoeling: chlorohexidine digluconaat (CHX).

De single-species biofilms worden gevormd door de *Streptococcus mutans* bacterie
(Hoofdstuk 6). *S. mutans* is acidogeen, waardoor het één van de belangrijkste veroorzakers van cariës is. In dit model is gebleken dat sucroseconcentratie en frequentie van plasmabehandeling een significant effect hebben op de *S. mutans* biofilms. Het grootste anti-bacteriële effect was zichtbaar bij een eenmalige plasmabehandeling van biofilms gekweekt in medium zonder sucrose. De bacteriën in de biofilms gekweekt in medium met sucrose werden verzwakt na enkelvoudige en meervoudige plasmabehandeling.

_Hoofdstuk 7_ beschrijft de behandeling van biofilms bestaande uit meer dan één bacterie, de Microplate Biofilm Microcosm (MBM). In het MBM model wordt tandplak gekweekt uit spuug, op een zogenaamde coverslip in een 24–wellsplaat. Om de effecten van plasma en CHX op de groei en compositie van de biofilms te kunnen vergelijken, werden de biomassa en pH bepaald, en zijn er foto’s gemaakt van de biofilmstructuur. De compositie van de biofilms voor en na behandeling is geanalyseerd met de Checkerboard DNA-DNA techniek (CKB). Als laatste zijn de biofilms bekeken met een transmissie elektronen microscoop (TEM). De TEM plaatjes laten zien dat de bacteriën beschadigd worden door zowel het plasma en het CHX. De CKB analyse laat zien dat bepaalde bacteriën gereduceerd werden in de biofilm, voor beide behandelingen. Tevens is gebleken dat de groei en compositie van de biofilms sterk wordt beïnvloed door de sucroseconcentratie van het kweekmedium.

Het bedoelde effect van het plasma en CHX op *E. coli* en *S. mutans* in het MBM model is niet direct te meten op de biofilmstructuur, maar wel op de groei van bacteriën in de biofilms. De CKB analyse laat zien dat bij een enkele plasmabehandeling van biofilms gekweekt in medium zonder sucrose, de bacteriën in de biofilms gekweekt in medium met sucrose werden verzwakt na enkelvoudige en meervoudige plasmabehandeling.

Tot slot hebben we als ‘extraatje’ Perspex en polystyreen blootgesteld aan het plasma (Hoofdstuk 8). Een mogelijke oppervlaktemodificatie zou invloed kunnen hebben op de groei van bacteriën op het oppervlak. De oppervlakte eigenschap die we bestudeerd hebben, is de ‘wettability’ (hydrofiel karakter). De wettability kan bepaald worden door de contacthoek van een waterdruppel met het substraat te meten. Het hydrofiere karakter neemt na plasmabehandeling toe (afname van contacthoek). Na modificatie van het oppervlak hebben we het effect van deze modificatie op de groei van *E. coli* en *S. mutans* bestudeerd. Op behandeld polystyreen nam de groei toe voor *E. coli*, maar af voor *S. mutans*, in vergelijking met onbehandeld polystyreen.

De resultaten van dit promotieonderzoek laten zien, dat niet-thermische, atmosferische plasma’s bacteriën kunnen doden op een niet-destructieve en pijnloze manier, waardoor ze een belangrijke rol kunnen spelen in de tandheelkunde. Wat we in dit onderzoek nog niet hebben kunnen aantonen, is het effect van plasma op bacteriën _in vivo_. Mogelijk kunnen toekomstige studies hierop een antwoord geven.
Samenvatting
Publications related to this work

Papers in international journals


- R.E.J. Sladek, T.A. Baede, E. Stoffels, Plasma Needle Treatment of Substrates with respect to Wettability and Growth of Escherichia coli and Streptococcus mutans, accepted for publication in IEEE Transactions on Plasma Science, 32(4), August 2006


Publications related to this work


Papers in conference proceedings


• T.A. Baede, R.E.J. Sladek, E. Stoffels, Surface modification of substrates for bacteria and cell culture, contributed paper to *XXVII International Conference on Phenomena in Ionized Gases*, Eindhoven, The Netherlands, 10–438, 2005


**Conference abstracts**

• 18th symposium ‘Plasma Physics and Radiation Technology’ Research school Center for Plasma Physics (CPS) and Radiation Technology and the Netherlands’ Physical Society (NNV), 22 and 23 March, 2006, Lunteren, The Netherlands
Oral presentation: Plasma needle treatment of dental biofilms

• 8th Euregional WELT-PP Workshop on the Exploration of Low Temperature Plasma Physics, November 24th and 25th, 2005 Rolduc, Kerkrade, The Netherlands
Oral presentation: Treatment of dental biofilms by the plasma needle.
Poster: Plasma needle treatment of substrates with respect to wettability and growth of *Escherichia coli* and *Streptococcus mutans*

• 7th Euregional WELT-PP Workshop on the Exploration of Low Temperature Plasma Physics, November 25th and 26h, 2004, Rolduc, Kerkrade, The Netherlands
Poster: Deactivation of *Escherichia coli* by the plasma needle

• Second International Workshop on Microplasmas, October 6–8, 2004, Stevens Institute of Technology, Hoboken, U.S.A.
Oral presentation: Plasma treatment of dental cavities

• International Workshop, High-Intensity Physical Factors in Medicine, Biology, Agriculture and Ecology, April 26–28, 2004, Sarov, Nizhny Novgorod region, Russia
Oral presentation: The plasma needle and its biomedical applications
• International Workshop on Cold Atmospheric Pressure Plasmas: Sources and Applications, 14–16th of January 2004, Ghent, Belgium
  Oral presentation: The plasma needle and its biomedical applications

  Poster presentation: Plasma Treatment of dental cavities: a feasibility study

  Oral presentation: Plasma treatment of dental caries: a feasibility study

• Pulsed Power Conference 15–18 June, 2003, Dallas, Texas, U.S.A.
  Poster: Plasma treatment of caries: a novel method in dentistry

  Poster: Plasma treatment of caries: a novel method in dentistry

• Plasma Physics and Short Time-Scale Physics Spring Meeting, DPG 24–28 March 2003, Aachen, Germany
  Poster: Plasma treatment of dental caries

• 5th Euregional WELT-PP, Workshop on the Exploration of Low Temperature Plasma Physics, November 28th and 29th, 2002, Rolduc, Kerkrade, The Netherlands
  Poster: Investigation of possibilities for plasma treatment of dental caries
Dankwoord

Tijdens mijn promotie heb ik met veel plezier samen mogen werken met mensen die direct, maar ook indirect betrokken waren bij mijn promotie. Nu ik aan het einde van mijn promotie gekomen ben, wil ik iedereen hier graag voor bedanken. Ook wil ik de mensen bedanken die ik hier niet bij naam heb genoemd.

Allereerst zou ik in het bijzonder mijn directe collega’s willen bedanken: Eva Stoffels, Ingrid Kieft, Evert Ridderhof, Rik Huiskes en Gerrit Kroesen. Eva, als directe begeleider zou ik je ontzettend willen bedanken voor het tot stand brengen van het project en voor alle goede ideeën. Ik heb veel gehad aan je hulp bij het schrijven van de publicaties en ook de conferenties die ik van je heb mogen bezoeken waren zeer interessant en soms ook avontuurlijk! Ingrid, bedankt voor de fijne samenwerking en de uitwisseling van kennis over de plasmanaald. Evert, bedankt voor de technische ondersteuning. Rik en Gerrit, bedankt voor jullie hulp en steun als promotor.

I want to thank the members of the core committee for reading my thesis and for their useful suggestions.

I would like to thank Ruben Wiese from Greifswald. Thank you for your help with the power outflux measurements.

Tijdens mijn promotie hebben een aantal studenten mij geholpen met experimenten. Tom Baede, Rick Walraven en Ewout van der Laan bedankt! Het was erg prettig en leerzaam om met jullie samen te werken.

De micro–biologische experimenten zou ik niet op de TU/e gedaan kunnen hebben zonder medewerking van mijn collega’s van het VMT-lab (Scheikundige Technologie). Hierbij wil ik de volgende mensen bedanken: Maarten Merkx, Peggy de Graaf, Suzan van den Dongen en Sanne Reulen. Bedankt voor jullie steun, hulp en vooral ook gezelligheid.

I would like to thank my roommates for their company and fun we had: Erik Wage-naars, Gabriela Paeva and Erik Kieft.

Many thanks to my colleagues of EPG: Mark Bekers, Mark Bowden, Tanja Briels, Wouter Brok, Bart Broks, Jan van Dijk, Michiel van den Donker, Arjan Flikweert, Lukas Gabrowski, Maxime Gendre, Bart Hartgers (ontzettend bedankt voor je hulp bij het verbeteren van de figuren!), Tao Jiang, Hjalmar Mulders, Joost van der Mullen, Tanya
Nimalasuriya, Jérôme Remy, Misha Sorokin, Winfred Stoffels, Marc van der Velden, Ed-die van Veldhuizen en Nienke de Vries. Ik heb me dankzij jullie erg thuis gevoeld in de vakgroep.

Mijn speciale dank gaat uit naar de technici binnen EPG: Charlotte Groothuis, Loek Baede en Huib Schouten, bedankt. Speciale dank aan Charlotte! Ik heb veel van je mogen leren, en niet alleen op het gebied van plasmфизica!

Graag zou ik de medewerkers van de werkplaats in N-laag willen bedanken voor het vervaardigen van de plasmanaalden.

En niet te vergeten de secretaresses: Rina Boom bedankt voor de goede gesprekken, de hulp en de lekkere snoepjes. Alice van Litsenburg, bedankt voor alles wat je voor me gedaan hebt.

Verder wil ik de collega’s uit de tandheelkunde bedanken. In het bijzonder de mensen van de ACTA. Bob ten Cate, bedankt voor de samenwerking. Egija Zaura, Suzanne Luppens, Michel Hoogenkamp, bedankt. Dankzij jullie heb ik veel geleerd over het kweken van biofilms. Tevens heb ik via de ACTA de kans gekregen om stage te lopen in Nieuw Zeeland.

It was a real pleasure to work with Chris Sissons, Sara Filoche, Lisa Wong, Margo van Bekkum, Philipa Cashina and Megan Coleman in New Zealand. Chris and Sara, thank you for the wonderful experience. It was awesome!

Ruben Koolhoven en Paul Tielbeek, bedankt voor de tandheelkundige materialen die jullie mij hebben gegeven. Veel succes met jullie tandartspraktijk.

Tot slot wil ik mijn familie en vrienden bedanken. Ik heb ontzettend veel steun gehad van jullie. In het bijzonder wil ik mijn ouders, zus en vriendin bedanken. Pa en ma, bedankt voor jullie steun en aanmoediging. Zonder jullie had ik dit nooit kunnen bereiken. Lieve Jorien, heel erg bedankt voor je geduld en luisterend oor. En niet te vergeten: Theo, Dia en mijn oud-huisgenoten van de Heezerweg, thanks!
About the author

Raymond Sladek was born in Culemborg, the Netherlands, on the 7th of May, 1979. He received his secondary education at the O.R.S. Lek en Linge in Culemborg.

From 1997 until 2002, he studied Biomedical Engineering at the Eindhoven University of Technology. As part of his study he did a three months traineeship at the Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Israel. He worked on the Demineralized Bone Matrix (DBM) model to test biomaterials in vivo. He did his graduation project at the Biomaterials Research and Orthopaedic Surgery group, University of Maastricht. During this graduation project he worked on biomaterials for cartilage and bone tissue engineering. During his study he was also active as a board member of the student outdoor organization All Terrain.

In October 2002 he started his PhD research project in the group of Elementary Processes in Gas discharges (EPG) and Bone Biomechanics at the Eindhoven University of Technology. This dissertation is the result of the PhD project. The subject was the application of the plasma needle in dentistry. As part of the research project he did a traineeship, from February until May 2005, at the Dental Research Group of the Wellington School of Medicine and Health Sciences (University of Otago, New Zealand). He worked in New Zealand on the plasma needle treatment of dental biofilms.
STELLINGEN

behorende bij het proefschrift

PLASMA NEEDLE: NON-THERMAL ATMOSPHERIC PLASMAS IN DENTISTRY

door

RAYMOND SLADEK

I.
Breaking-up dental plaque before plasma treatment will facilitate the inactivation of bacteria in the plaque.

II.
Het bestuderen van afgeschraapt tandplak onder een microscoop is als het kijken naar een vliegtuig nadat het neergestort is.

III.
De kwaliteit van een product kent een optimum in zijn bestaan.

IV.
De vorming van een biofilm op een medisch implantaat is niet te voorkomen.

V.
Technologische ontwikkelingen baren nieuwe regels.

VI.
Een biofilm beschermt je tegen de buitenwereld.

VII.
Je kunt veel meer zien met onzichtbaar licht.

VIII.
Je wordt niet gezonder door je voortdurend bezig te houden met je gezondheid.

IX.
Uiteindelijk worden tandartsen overbodig.

X.
Wie verzorgt straks de zorg in Nederland?
XI.
In het betaald voetbal rollt het geld verder dan de bal.

XII.
Praatjes kunnen gaatjes voorkomen.