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Review

Application of Non-Thermal Plasma on Biofilm: A Review

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Abstract: The formation of bacterial biofilm on implanted devices or damaged tissues leads to biomaterial-associated infections often resulting in life-threatening diseases and implant failure. It is a challenging process to eradicate biofilms as they are resistant to antimicrobial treatments. Conventional techniques, such as high heat and chemicals exposure, may not be suitable for biofilm removal in nosocomial settings. These techniques create surface degradation on the treated materials and lead to environmental pollution due to the use of toxic chemicals. A novel technique known as non-thermal plasma has a great potential to decontaminate or sterilize those nosocomial biofilms. This article aims to provide readers with an extensive review of non-thermal plasma and biofilms to facilitate further investigations. A brief introduction summarizes the problem caused by biofilms in hospital settings with current techniques used for biofilm inactivation followed by the literature review strategy. The remainder of the review discusses plasma and its generation, the role played by plasma reactive species, various factors affecting the antimicrobial efficacy of non-thermal plasma and summarizes many studies published in the field.

Keywords: biofilm; decontamination; dielectric barrier discharge; infection; jet plasma; non-thermal plasma

1. Introduction

Biofilms refer to a group of microorganisms adhered to a substrate within a polymeric matrix [1]. Biofilms possess unfavorable conditions like biofouling, pipe plugging, damage of equipment, prosthesis colonization, and a number of diseases [2]. Due to their greater resistance to antimicrobial treatment than planktonic cells of the same species, biofilms are usually challenging to eradicate [3–5]. Approximately 13 million people suffer from biofilm-related infections in the United States [6]. The Centers for Diseases Control and Prevention (CDC) estimates that approximately 65% of human bacterial diseases are due to biofilms, with a higher estimate (80%) proposed by the National Institutes of Health (NIH) [7]. More than 60% of hospital-acquired infections (HAI) are led by the attachment of a number of microorganisms to medical implants/devices like catheters, prostheses, fracture-fixation devices, dental implants, and cardiac devices [5,8–14]. In Europe and USA, the World Health Organization estimates that about 4.5 million and 1.7 million patients are impacted by healthcare associated infections that lead to 100,000 and 37,000 deaths per annum [15]. Most of the infections that occur in nosocomial settings are due to the growth of bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyrogens*, and *Candida albicans* in biofilm form [16].

Critical requirements for developing an antimicrobial tool against biofilms in hospital settings are robustness and the disinfection and sterilization ability without any human side effects or damage to the

medical devices and implants. Traditional sterilization methods such as heat and chemical treatments (quaternary ammonium compounds, aldehydes, alcohols and halogens or radiation, chlorhexidine and silver salts, peroxygens, glutaraldehyde, and ortho-phthalaldehyde) [8,17,18] cannot be considered as perfect bacterial decontamination techniques because of their potential to eventually degrade the treated surface. Moreover, chemical usage can be toxic and lead to environmental pollution. As biofilms are significantly more resistant to antimicrobial treatment than in the planktonic form of the same species [3], removing them by conventional treatments would be problematic. Also, antibiotic treatment may not always inactivate overall bacterial cells present in the biofilm as they are more resistant to antibiotics. In addition to their limited efficacy, other drawbacks of current sterilization and disinfection methods include their environmental impact, clinical downtime and economic costs [19]. Therefore, a novel antimicrobial treatment technique, such as a non-thermal plasma (NTP), one which is safer, efficient, and cost-effective in clinical settings, will be of great interest.

The main scope of this paper is to provide a review of the published research in the area of biofilm decontamination and sterilization using plasma. The emphasis here is on the potential of NTP for biofilm inactivation. The basics of this novel technology is reviewed, and a brief overview is given of extensive published research in the field. Other sections provide explanations and reviews of biofilms and their formation, the varieties of NTP, plasma generation, various plasma reactive species, factors influencing the antimicrobial efficacy of plasma, and summaries of several significant publications in the field.

2. Literature Review Strategy

This review was prepared through an extensive literature survey. PubMed and Google Scholar were used to search for literature, with no date restriction in the field of NTP and biofilm. The following keywords and phrases were used to find relevant published articles: “medical biofilm”, “hospital infection by biofilm”, “biofilm formation”, “dielectric barrier discharge”, “NTP and biofilm”, “NTP towards biofilm decontamination/sterilization”, “antimicrobial efficacy of NTP”. Since the articles were of heterogeneous nature, it was impossible to apply rigid selection criteria. Instead, articles were chosen based on their biofilm focus, including investigations of NTP usage for biofilm sterilization, or decontamination. In addition, the reference lists of each article found during the primary search were reviewed to identify other relevant literature.

3. Biofilm and Its Formation

Understanding the characteristics and formation of biofilms would be the first step towards their decontamination or sterilization via NTP. Knowing the structure of biofilms is important as it serves to protect them against several antibiotics and sterilization procedures. A biofilm refers to a group of microorganisms adhered to biotic or abiotic surfaces. These adherent microorganisms are enclosed within a self-produced matrix of extracellular polymeric substance (EPS). The biofilm matrix is composed of 97% water and mainly polysaccharides, proteins, and extracellular DNA (eDNA) which provide the biofilm structure and also act as a reservoir for nutrients [20]. Out of the total organic materials present in the biofilm, 75–90% is EPS, 10–25% is microbial cells and 1–2% is proteins, polysaccharides, peptidoglycans, lipids, phospholipids, DNA, and RNA [21]. Biofilms are present in diverse environments such as in households, water sources, pipes, industry, and hospitals. Their presence in hospitals can lead to diseases, prostheses colonization, product contamination, biofouling, and equipment damage [22]. Various bacterial species such as *Pseudomonas*, *Staphylococcus* and *Candida* are frequently isolated in clinically relevant biofilm form [2]. Commonly isolated microorganisms from indwelling medical devices are shown in Table 1.

Table 1. Biofilm-associated microorganisms commonly isolated from selected indwelling medical devices [23].

Indwelling Medical Device	Organisms
Central venous catheter	Coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*
Prosthetic heart valve	Viridans *Streptococcus*, coagulase-negative staphylococci, enterococci, *Staphylococcus aureus*
Urinary catheter	*Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Proteus mirabilis*
Artificial hip prosthesis	Coagulase-negative staphylococci, b-hemolytic streptococci, enterococci, *Proteus mirabilis*, *Bacterioides* species, *Staphylococcus aureus*, viridans *Streptococcus*, *Escherichia coli*, *Pseudomonas aeruginosa*
Artificial voice prosthesis	*Candida albicans*, *Streptococcus mitis*, *Streptococcus salivarius*, *Rothia dentrocariosa*, *Candida tropicalis*, *Streptococcus sobrinus*, *Staphylococcus epidermidis*, Stomatococcus mucilaginous
Intrauterine device	*Staphylococcus epidermidis*, *Corynebacterium* species, *Staphylococcus aureus*, *Micrococcus* species, *Lactobacillus plantarum*, Group B streptococci, *Enterococcus species*, *Candida albicans*

The biofilm formation is initiated after free floating planktonic bacteria attach on a substrate. Within a few hours of attachment, these bacteria bind irreversibly and begin to multiply resulting microcolonies on the surface and produce a polymeric substance around them as shown in Figure 1. This biofilm when matured becomes resistant to antibiotics. Production of EPS is the maturation stage of the biofilm that bind cells together on the surface [24]. EPS also forms a physical barrier which is responsible for limiting the transport of chemicals into and out of the biofilm [24].

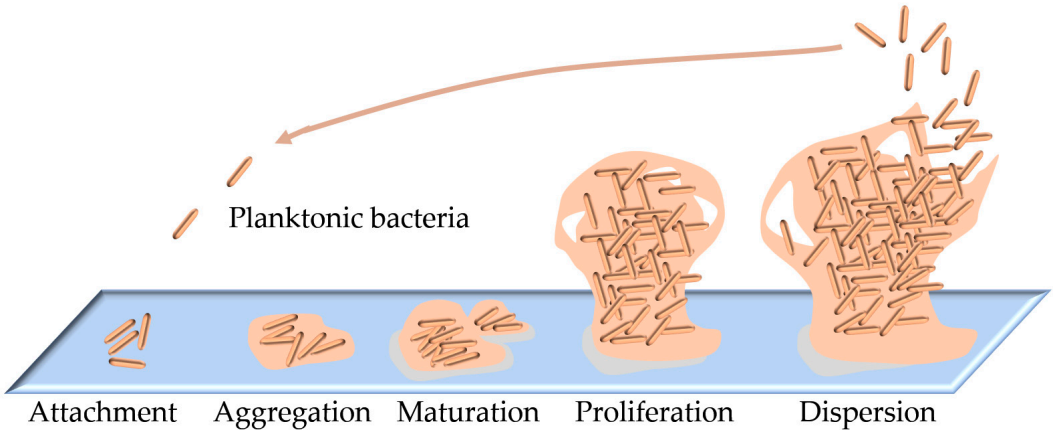

Figure 1. The life cycle of biofilms beginning with the initial cell attachment and ending with biofilm dispersal.

Nutrient depletion, metabolic product accumulation, and other stressors cause cells from the biofilm to disperse. Ultimately, the dispersion of biofilm refers to the final stage of its life cycle and consists of three phases—detachment from the existing biofilm, translocation to other areas, and adherence/colonization on other surfaces [25]. Dispersion of the biofilm spreads the infection to other areas and increases the contamination of a medical device [21]. Enzymes that are responsible for degrading the extracellular matrix, for example, dispersin B and deoxyribonuclease may play roles in this dispersal [26].

Quorum sensing (QS) is a communicative system for biofilm which allows the bacterial cells inside the biofilm to act as a community. This facilitates the growth, survival, and colonization of bacterial cells inside the biofilm [21]. The concentration of other bacteria present within a limited microenvironment is sensed through QS [27]. The bacteria then respond by activating specific genes which then produce virulence factors such as enzymes or toxins [27]. The QS molecules are N-acyl-L-homoserine lactones (AHL) in gram-negative bacteria and peptides in gram-positive bacteria [27].

Many investigations of the mechanism of biofilm resistance to antimicrobial agents have been carried out. A primary reason is the biofilm matrix preventing the access of antimicrobial agents, such as antibiotics, to the embedded bacterial cells [28]. Other reasons include slow penetration, resistant phenotypes, and altered microenvironments [29]. Because of slow penetration, antibiotics may fail to penetrate deeply into the biofilm. Some of the bacteria in the biofilm may develop into a protected phenotype resistant to antibiotics. Similarly, the accumulation of waste and nutrient depletion in the deeper biofilm layers may contribute to antibiotic resistance.

4. Non-Thermal Plasma

Non-thermal plasma (NTP) is a novel and emerging antimicrobial tool that demonstrates the possibility of improved biofilm decontamination and sterilization [30–33]. Plasma is the fourth fundamental states of matter after solid, liquid, and gas. The various ingredients present in plasma such as free radicals, reactive oxygen, and nitrogen species [34], and positive and negative ions [35,36] are believed to play a significant role as antimicrobial agents. Thermal (equilibrium) and non-thermal (non-equilibrium) plasma are the two plasma types. They are differentiated based on the relative energy levels of electrons and heavy particles of the plasma [37]. A thermal plasma is generated at high pressure and power and contains electrons and heavy particles at the same temperature. In contrast, NTP is generated at low pressure and power, and contains electrons at higher temperature and heavy particles at room temperature [37,38].

Previously, thermal plasma was used for tissue removal, sterilization, and cauterization [39]. The problem with this type of plasma treatment is the high heat production and damage to tissues and other surfaces. Conversely, NTP can carry out the same functions without causing any such harm or side effects [40]. NTPs such as Dielectric Barrier Discharge (DBD) and jet plasmas are gaining much interest because of their non-thermal nature. It enables new applications in biological and medical fields where substratum to be treated are mostly living tissue, cells, and biomaterials [41]. As listed in Table 2, recent investigations with NTP show promising results for the sterilization and decontamination of biofilms of different bacterial species with this novel technology.

Table 2. Uses of non-thermal plasma (NTP) for biofilm decontamination or sterilization.

Bacterial Strain	Plasma Type and Parameters (Voltage, Frequency, Power, Working Gas, and Flow Rate)	Inactivation Yield	Substrate for Biofilm Formation with Time for Decontamination or Sterilization	Ref.
Neisseria gonorrhoeae	Jet: 10 kV and 10 kHz, He at 2 L/min	7 log reduction	Coverslips (20 min)	[42]
Enterococcus faecalis	Jet: 18 kV and 10 kHz, Ar/O_2 (2%) at 5 L/min	No CFU detected	Root canal (10 min)	[43]
Streptococcus mutants	DBD: 580 kHz and 2 W/cm^3 power density, He at 2 L/min	98% killed	Tooth slices (30 s)	[44]

Table 2. *Cont.*

Bacterial Strain	Plasma Type and Parameters (Voltage, Frequency, Power, Working Gas, and Flow Rate)	Inactivation Yield	Substrate for Biofilm Formation with Time for Decontamination or Sterilization	Ref.
Weissella confusa	Jet: 20 kHz	2.63 and 2.16 log reduction with and without sucrose	Cellulose ester membrane (20 min)	[45]
Porphyromonas gingivalis	Jet: 8 kV and 8 kHz, He/O_2 (1%) at 1 L/min	All cells killed in 15 µm biofilm	Cover slip (5 min)	[46]
Staphylococcus aureus	FE-DBD: 120 V and 0.13 W/cm^2 power	All biofilms were sterilized	Cover slip and 96 well plate (<2 min)	[47]
Pseudomonas aeruginosa	Jet: 6 kV with 20 and 40 kHz, He/O_2 (0.5%) at 2 L/min	4 log reduction at 20 kHz and complete eradication at 40 kHz	Peg lid of Calgary biofilm device and polycarbonate coupon (4 min)	[3]
Pseudomonas aeruginosa	DBD: 120 kV and 50 Hz	Biofilm reduced to undetectable level	96 well plate and coverslips (5 min)	[48]
Staphylococcus aureus	Jet: 20 kV and 38 kHz, He at 6.7 L/min	3.06 log reduction	Borosilicate slices (10 min)	[49]
Staphylococcus aureus, epidermidis and *Escherichia coli*	Low power gas discharge: 60 W power, oxygen, argon, and nitrogen at flow rate of 2.4 ft^3 h^{-1}	All biofilms killed	Polyethylene terephthalate (PET) films, silicon wafers and cover-glass chambers (25–30 min)	[50]
Burkholderia cenocepacia and Pseudomonas aeruginosa	MicroPlaSter B device plasma with argon gas	0.005% and 2% bacteria survived	Coverglass (10 min)	[51]
Staphylococcus aureus, Pseudomonas aeruginosa and *Candida albicans*	Jet: 5 kV and 61 kHz, 2.5–3.5 W power, Ar+O_2	27%, 39%, and 35% cells survived of *S. aureus*, *P. aeruginosa*, and *C. albicans*	96 well plate (3 min)	[52]
Streptococcus mutans and saliva multispecies	Jet (KINPen 09): Ar (5 slm) and Ar+1%(O_2) at 0.05 slm, HDBD (hollow DBD): 8.4 kV and 37.6 kHz, Ar and Ar+O_2 at 1 and 0.01 slm, and VDBD (volume DBD):10 kV and 40 kHz, Ar at 0.05 slm	5.38 for *S. mutans* and 5.67 for saliva biofilm	Titanium disc (10 min)	[31]
Pseudomonas aeruginosa and *Staphylococcus epidermidis*	Surface dielectric barrier discharge (SBD)-Structured electrode planar SBDA: (13 kV and 20 kHz) and a wire electrode SBD-B: (8 kV and 30 kHz with compressed air at 0.5 slm)	7.1 and 3.8 log reduction by SBD-A in *P. aeruginosa* and SBD-B and 3.4 and 2.7 log reduction by SBD-A and SBD-B in *S. epidermidis*	Polycarbonate disc (10 min)	[53]

Table 2. *Cont.*

Bacterial Strain	Plasma Type and Parameters (Voltage, Frequency, Power, Working Gas, and Flow Rate)	Inactivation Yield	Substrate for Biofilm Formation with Time for Decontamination or Sterilization	Ref.
Candida albicans	Jet(KINPen09): 220 V and 50/60 Hz, 8 W power, Ar at 5 slm and Ar+(1%)O_2 at 0.05 slm, HDBD:9 kV and 37.6 kHz, 9W power, Ar at 6 slm and Ar+(1%)O_2 at 0.06 slm and VDBD: 10 kV and 40 kHz, 16 W power	5 log reduction	Titanium disc (10 min)	[54]
Candida albicans	Surface microdischarge plasma technology (SMD): 9 kV and 1 kHz	6 log reduction	6 well plate (8 min)	[55]
Pseudomonas aeruginosa	Atomflo 300 reactor plasma Jet: 13.56 MHz and 100 W R, He at 20.4 L/min and N_2 at 0.135 L/min at 35 W	Complete biofilm inactivation	Borosilicate coupon (30 min)	[56]
Pseudomonas aeruginosa and *Staphylococcus epidermidis*	Jet (Kinpen09): 2–6 kVpp and 1.1 MHz, 3.5 W power, Ar and Ar+(1%)O_2 at 5 slm	5.41 and 5.10 log reduction in Ar and Ar + O_2 plasma for *P. aeruginosa* and 3.14 and 2.21 log reduction in Ar and Ar + O_2 plasma for *S. epidermidis*	Microtiter plate (5 min)	[57]
Enterococcus faecalis	Plasma dental probe:6 kV and 1 kHz, 0.7 W power, He/(1%)O_2 at 1 slm	93.1% biofilm killing	Hydroxyapatite discs (5 min)	[58]
Bacillus cereus, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*	Jet:6 kV and 20 kHz, He/(0.5%)O_2 at 2 slm	Complete biofilm eradication	Peg lid of Calgary Biofilm Device (<4 min and 10 min)	[59]
Streptococci	Atmospheric pressure air plasma of corona discharge: Positive corona (PC) −8 kV and 20 kHz, negative corona (NC) −7 kV and 0.25 to 2 MHz	3 log reduction	Tooth surfaces (10 min)	[60]
Candida albicans	Jet (KINPen 08): 2–6 kVpp and 1.7 MHz, Ar at 5 slm and Ar/[1%]O_2 at 0.05 slm	Complete biofilm removal with Ar+O_2	Polystyrene (PS) wafers (5 min)	[61]
Pseudomonas aeruginosa	Atomflo 300 reactor plasma Jet: 13.56 MHz and 100 W RF, He at 20.4 L/min and N_2 at 0.135 L/min at 35 W	100% ianctivation	CDC biofilm reactor on borosilicate coupons (5 min)	[62]

Non-thermal jet plasma devices employing atmospheric pressure plasma are commercially available [63]. kINPen® is a pen-like device developed for biomedical applications that allows precise

and arbitrary movements in 3D [64]. The plasma is generated after applying a high-frequency (HF) voltage coupled to the pin-type electrode [63]. It is electrically safe to use as it is certified and complies with EU standards [64]. The feed gas used is argon with the capability of using other gases in smaller amounts [65]. The kINPen® MED (Leibniz Institute for Plasma Science and Technology-INP Greifswald and neoplas tools GmbH, Greifswald, Germany) is a predecessor device of KINPen® 09 and is the first atmospheric pressure plasma jet device to be accredited as a medical device (Class IIa) for patient use [64]. However, both kINPen® 09 and kINPen® MED are essentially similar [64]. kINPen® plasma has been clinically used for antimicrobial efficacy and for wound healing in animal and clinical observational studies [64]. To our knowledge, only three other plasma sources have been accredited as medical devices. These are SteriPlas (AdTec Ltd., Japan), PlasmaDerm (Cinogy GmbH, Duderstadt, Germany), and PlasmaOne (Medical Systems GmbH, Bad Ems, Germany) [64]. These plasma sources and several others have been used in various biomedical applications such as wound healing and chronic leg ulcers [66,67], reducing bacterial populations in wounds [68], and biofilm decontamination or sterilization [69,70].

Plasma has enormous potential in several biomedical research areas including sterilization of implant surfaces, surface modification [71], in-vitro blood coagulation [72,73], wound healing and disinfection [74,75], tissue regeneration [39,73], treatment of various infections [39], bacterial decontamination and sterilization [69,76,77], dental cavities [78,79], and cancer treatment [80–82]. The methods used for plasma generation are DBD, atmospheric pressure plasma jet (APPJ), plasma needle, and plasma pencil [83]. These plasmas can be produced in air or with various gases, such as oxygen, helium, argon, and nitrogen. Plasma can be produced by power sources with different frequencies such as low frequency, radio frequency, microwave frequency, high voltage AC or DC, to generate atmospheric and low-pressure glow discharge, corona, magnetron, microwave, gliding arc, plasma jet, and DBD discharge [84–86].

DBD has been known for more than a century. Floating-electrode dielectric barrier discharge (FE-DBD) is a DBD-based plasma source that is regarded as the starting point of modern plasma medicine [87]. FE-DBD is an electrical discharge between two electrodes at atmospheric pressure and air where one electrode is grounded, and the other is supplied with a high voltage (Figure 2). The high voltage electrode is enclosed within a dielectric material that limits the discharge current and the formation of an arc. Different dielectric materials used are quartz, glass or silica glass, polymers, ceramics, thin enamel, or polymer layers that block DC current [88]. The distance between the two electrodes ranges from micrometers to centimeters depending on the operating voltage, process gas and the plasma configuration employed [89]. DBD are usually operated at frequencies up to several tens of kHz. Two DBD configurations that have been employed for most of the applications are parallel and concentric configurations. The operating principle of DBD is shown in Figure 3a–d below. Three different types of DBDs are filamentary, patterned, and diffuse DBD [90]. These DBD types depend upon the construction and operating conditions of the plasma sources.

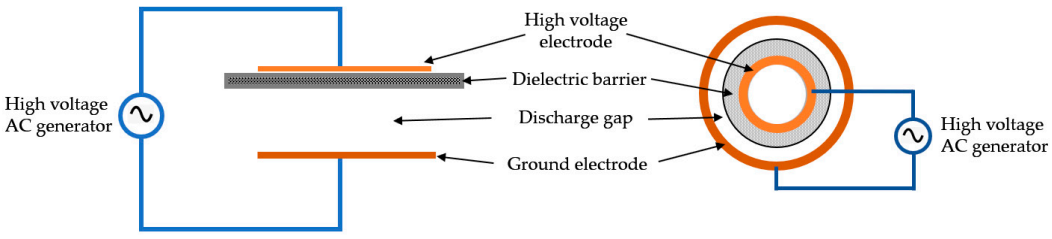

Figure 2. Parallel and concentric dielectric barrier discharge (DBD) configurations [91].

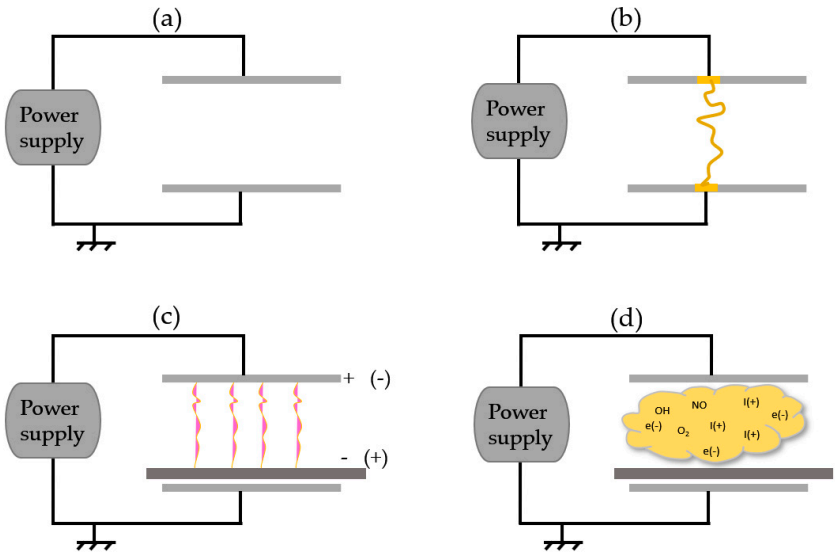

Figure 3. Schematics of DBD plasma operation. (**a**,**b**) arc production between two electrodes without insulation. (**c**,**d**) NTP generation after placement of dielectric barrier layer in one of the electrode [41].

Plasma jet is another type of NTP configuration. It is a tube with electrodes which are either placed inside or around it, through which gas flows and ionizes when it is subjected to the electrical field between the electrodes [92]. Figures 4b and 5b show the pictorial representations of volume DBD and jet plasma. In volume DBD, plasma is ignited in the gap between the high voltage electrode and the sample that is connected to the ground electrode. Whereas in the jet configuration, plasma is ignited inside the nozzle/tube and transported outside the object to be treated by a gas flow [93]. Usually, the DBD plasma uses atmospheric air as the working gas, while jet plasma uses different working gases such as helium, nitrogen, argon, oxygen, etc. Different types of plasma jets can be designed with various electrode configurations, working gases and applied electrical parameters [94].

4.1. Plasma Generation and Treatment

The two most commonly used NTP (DBD and Jet) configurations are shown in Figures 4 and 5, respectively. However, the three different approaches that can be used to treat samples with NTP are explained below:

Direct plasma treatment—It uses the target area as a counter electrode where there is a homogenous generation of plasma with high concentrations of plasma-generated species [95], e.g., DBD plasma.

Indirect plasma treatment—In this treatment technique, the plasma produced between two electrodes are later transported to the target area either by a carrier gas or diffusion [95], e.g., plasma needles, jets and torches.

Hybrid plasma treatment—In this approach, the plasma is produced in multiple micros and nano-discharges in a grounded wire mesh electrode, e.g., surface micro discharge (SMD).

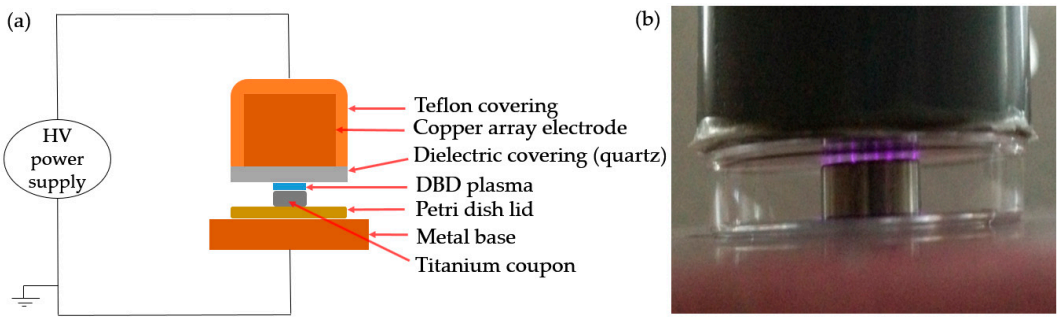

Figure 4. Schematic diagram and photograph of the regular DBD plasma setup. (**a**) demonstrates the schematic diagram of the regular DBD plasma and (**b**) shows the actual experimental setup of DBD plasma [70].

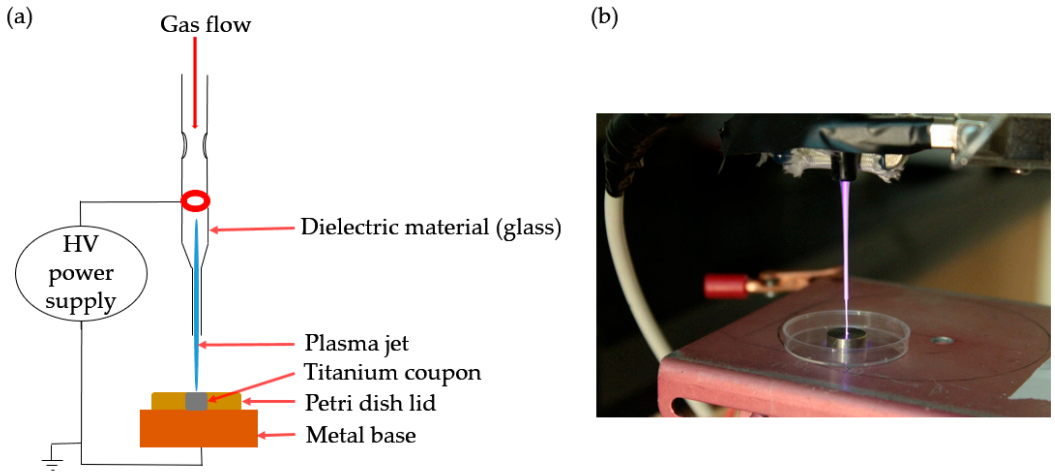

Figure 5. Schematic diagram and photograph of the jet plasma setup. (**a**) demonstrates the schematic diagram of the jet plasma and (**b**) shows the actual experimental setup of jet plasma [69,96].

4.2. Active Plasma Agents

The main active agents present in the plasma are radicals, charged particles, reactive oxygen species (ROS-O, O_2*, O_3, OH), reactive nitrogen species (RNS-NO, NO_2), UV radiation, and electrical field. These active agents in combination are believed to be responsible for the antimicrobial efficacy. The reactive species (ROS and RNS) possess strong oxidative effects on the outer cellular structure [74]. ROS in cellular level leads to lipid peroxidation, DNA damage, protein modulation and programmed cell death in microorganisms [97,98]. Some of the molecular marker that is involved in plasma treatment are listed below in Table 3. UV radiation has fewer effects on bacteria as NTP at atmospheric pressure is a poor source of UV [97]. Similarly, charged particles play a vital role in rupturing the bacterial outer cell membrane. The electrostatic force created by the charge accumulation on the outer cell membrane overcomes its tensile strength and ruptures it [74].

Table 3. Molecular markers involved in plasma treatment.

Molecular Marker Involved in Plasma Treatment	Significance
8-hydroxydeoxyguanosine (8-OHdG) and Ɣ-H2AX	Ubiquitous marker of oxidative stress and a by-product of oxidative DNA damage [99,100]
3-nitrotyrosine	Protein oxidative damage marker causes chemical fragmentation, inactivation, and proteolytic degradation [100]
Proteinase K	Plasma exposure reduces the catalytic activity of proteinase K by damaging the protein [101]
Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)	A marker for oxidative stress that measures lipid peroxidation. It damages DNA and proteins through the formation of covalent adducts [99,102]
Polyunsaturated fatty acids (PUFA)	Causes lipid peroxidation of bacterial cell membrane by extracting H atom from PUFA by plasma Reactive oxygen species (ROS) [98]
Intracellular ATP	Poly(ADP-ribose) polymerase-1 (PARP-1) results in decreased ATP level which signifies cell surface damage caused by leaking cellular proteins/nucleic acids [103,104]

5. Factors Influencing the Antimicrobial Efficacy of NTP

Sterilization of biofilm via NTP is promising. However, it is affected by various factors, and careful consideration should be given while designing the plasma system for this purpose. Some of the factors that affect the efficacy of NTP are discussed in the following sections.

5.1. Plasma Treatment Time and the Distance between the Plasma Source and the Sample

NTP is regarded as dose-dependent, i.e., its efficacy depends on the plasma treatment time and the distance between the plasma nozzle and the sample as shown by one of the study [52]. This study found an increase in survival rate of *S. aureus* biofilm upon increasing the distance between the plasma source and the sample. More biofilm was killed at 8 mm distance in compared to the other distances used, such as 9, 10, and 11 mm. One of the potential reasons could be the reactive species that can reach the sample depend upon the distance, at fixed flow rate of working gas [105]. The antimicrobial efficacy of plasma increases with plasma treatment time and remains constant after a certain exposure period as revealed by several studies [3,59,106]. Different doses of photon and reactive species can be directed at the target sample by varying the distance between the plasma nozzle and the sample [107].

5.2. Frequency and Electrical Input Power (Voltage)

Electrical input power is another parameter which can be optimized to increase the antimicrobial efficacy of NTP [108,109]. The plasma power mainly depends on the distance between the plasma nozzle and the sample [78]. The amount of photons generated per second in a defined volume increases with increasing power [108]. One of the studies demonstrated greater reduction of *E. coli* and *L. monocytogenes* viability when using a higher voltage of 70 kV_{RMS} after plasma treatment [110]. They found significant effects on cell integrity when higher voltage was used with a shorter treatment time of 5 sec. Another study using DBD plasma of 20 kV and 25 kV resulted 2.43 and 4.12 log reduction in bacterial cells while those with 16 kV showed 1.0 log reduction. The reason for this is the production of higher input energy density with higher voltage [98]. Frequency also plays a major role in increasing the plasma efficacy. One research group showed the complete eradication of *P. aeruginosa* biofilm after increasing the frequency from 20 kHz to 40 kHz. This higher frequency results in a higher density of the plasma reactive species delivered to the target by generating more plasma pulses and effective plasma on-time [3].

5.3. Role of the Gas or the Gas Mixture with its Flow Rate

As mentioned earlier, different gases and mixtures have been used in plasma jets to increase antimicrobial efficacy. Argon requires lower ionization energy than helium and it is cheaper, whereas helium possesses better thermal conductivity thereby preventing thermal instabilities [107]. Moreover, the noble gases increase the antimicrobial properties and plasma stability, whereas the addition of oxygen aids in producing chemically active species [111–113]. One study demonstrated more inactivation of spores of *Bacillus* genus when He was combined with 3% O_2 in comparison to 100% He [114]. This is due to the generation of oxygen species such as singlet O and O_3 in a He/O_2 plasma environment, as mentioned by the study [114]. These oxygen species play a significant role in the sterilization process because these species have strong oxidative effects on the outer membrane of the bacterial cell [39,54,115]. The addition of oxygen in the working gas contributes to the deactivation efficacy of the plasma jet by the combined action of plasma-induced endogenous ROS and the plasma generated ROS as reveal by the study [116]. This combined effect damages the bacterial membrane leading to biofilm deactivation [116]. Nitrogen has also been added to the noble gases to increase the formation of reactive nitrogen species [107]. Up to 3% of oxygen maintains the discharge stability, which produces reactive metastables that are long-lived and capable of traveling tens of centimeters at the nozzle exit velocity. This occurs even though the usual lifetime of atomic oxygen is of the order of 1 ms [117]. However, if more oxygen is added to the helium, the discharge becomes unstable due to the quenching effect of the oxygen gas, and this would decrease the plasma efficacy as a result of a decrease in plasma density [111].

Many researchers have chosen argon as the working gas because of its inertness and have succeeded in achieving planktonic and biofilm sterilization [51,118–120]. One of the study observed the sterilization effects of microwave-induced argon plasma on *E. coli* and MRSA and suggested the generation of free radicals, and UV light, as well as the etching process, are responsible for the sterilization efficacy [118]. Coupled to effect of the choice of gas, its flow rate determines the velocity at which the active species are delivered to the target [121]. The study by Nishime et al., uses a He plasma with a flow rate of 2 and 4 SLM and found that the shape of the inhibition zone and homogeneity were compromised at higher flows with no significant difference in the size of inhibition zones [121]. With higher gas flow rates, flow dynamics such as turbulent mixing and buoyancy effects play major roles in the formation and distribution of active species [122].

6. Biomedical Applications of NTP on Biofilm

A wide variety of research has been published on the use of NTP in biofilm treatment [3,53,61,62,120,123] with the help of different plasma systems such as corona discharge, microwave discharges, plasma jet, gliding arc, and dielectric barrier discharge within the past few years. The literature published in 2015 by Xu et al. demonstrated complete inactivation of *Staphylococcus aureus* biofilms that was grown on borosilicate slices placed in the 24-well plates for 12 h [49]. They used atmospheric pressure plasma jet (APPJ) with helium as a working gas at 6.7 standard liters per minute (SLM) flow rate. Within 10 min of plasma treatment, the reduction in biofilm cells was found to be more than 99.9% in comparison to the untreated biofilm samples. The effect of plasma treatment on biofilm was observed using a confocal microscope, exhibiting many dead bacteria on the biofilm upper layer in comparison to the bottom layer. Further, intra-bacterial ROS in the biofilm was detected by ROS monitoring probe 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA). Their findings also provide insight into the mechanism of biofilm inactivation by plasma reactive species and plasma-induced intracellular ROS.

Matthes et al. in 2013 studied the antimicrobial efficacy of two surface barrier discharges (SBD) known as SBD-A (structured electrode planar SBD) and SBD-B (a wire electrode SBD), with air plasma in *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms. The biofilm was grown on polycarbonate discs placed into microplate wells for 48 h and treated with plasma from 30–600 s. They achieved a colony reduction factor (CRF) of 7.1 $\log_{10}$ and 3.81 $\log_{10}$ for *P. aeruginosa* by SBD-A and

SBD-B plasma. Whereas for *Staphylococcus epidermidis*, CRF of 3.38 $\log_{10}$ and 2.69 $\log_{10}$ were found out by SBD-A and SBD-B plasma at 600 s of plasma exposure. The study used a positive control as CHX that is used as a treatment for dental biofilm and found around 1 log reduction in biofilm cells. Furthermore, in a similar study, the cytotoxicity of the plasma system was tested on a mouse fibroblast cell line concluding that the average viability of the cells did not decrease below 50% until 150 s of plasma exposure time [53].

Non-thermal plasma has also been used widely in dentistry to remove dental biofilms. A 2014 study used positive corona (PC) and negative corona (NC) discharges on biofilm contaminated teeth surfaces and tested the effect of water electro-spraying for decontamination [60]. Both discharges were able to reduce the biofilm cell concentration by 1–1.3 orders of magnitude and 2.73 logs in 5 and 10 min of plasma treatment time. In addition, 3.16 orders of biofilm cell reduction were achieved after water electro-spraying through the plasma. The impact of those discharges on tooth surfaces was also studied by FTIR and SEM, but no significant changes were observed. In 2010 and 2011 Koban et al. used three plasma devices named as atmospheric pressure plasma jet, a hollow dielectric barrier discharge electrode (HDBD) and a volume dielectric barrier discharge (VDBD) against dental biofilms. Their results showed log reduction of 5.38 and 5.67 for *Streptococcus mutans* (*S. mutans*) and saliva biofilm when compared with the CHX as a control which shows a reduction of 3.36 and 1.50 for *S. mutans* and saliva biofilm [31]. They also achieved 5 log reduction of *Candida albicans* biofilm cells and suggested the plasma to be more effective than CHX in the treatment of single and multispecies dental biofilms.

In comparison to the plasma treated biofilm that has a 5 log reduction, the chemical antiseptics such as CHX or NaOCl had a reduction factor of 1.5, suggesting that this plasma can be used as an alternative to chemical antiseptics for dental practice [54]. This study concluded the plasma could be used as an alternative to chemical antiseptics for dental applications. One of our study [70] showed 2.43 log reduction of biofilm when treated with CHX in compare to more than 3 log reduction after plasma treatment. We also found some disruption of biofilm cells when treated with CHX, however the biofilm destruction caused by plasma was more severe. A 2010 study used glass coverslips for growing biofilm and achieved complete inactivation (7 log reduction) of *Neisseria gonorrhoeae* biofilm after treating with jet plasma for 20 min [42]. A mixture of helium and oxygen were used as a working gas to generate plasma. In the similar study, the effect of plasma on biofilm was visualized by transmission electron microscopy (TEM). The images show disruption and damage to the cell wall and dispersed nucleoid region.

In 2014, Vandervoort et al. established complete inactivation of *Pseudomonas aeruginosa* biofilm after 30 min of plasma jet treatment. The biofilm in this study was grown for 24 h on borosilicate glass coupons under continuous culture system [56]. Their atomic force microscopy (AFM) results show significant loss of the biofilm structure when treated with plasma for a longer time. They also concluded that changes in biofilm structure that leads to the loss of culturability and viability are related to a decrease of the biofilm matrix adhesiveness. Similarly, Zelaya et al. claimed 100% inactivation of the *P. aeruginosa* biofilm cells after 5 min of applying plasma jet. They used batch culture to grow 1, 3, and 7 day old biofilm on borosilicate glass coupons [62]. They also showed a decrease in adhesiveness to borosilicate and biofilm thickness after plasma treatment by AFM.

Another study by Alkawareek et al. in 2012 achieved complete eradication and more than 4 log (99.99%) reduction in the number of biofilm cells when treated with 40 and 20 kHz plasma jets. Their confocal microscopy results indicate this from the biofilm thickness after 240 s of plasma treatment suggesting the penetration of reactive species into the biofilm inner layer. This study demonstrated the potential to completely remove biofilms formed on inanimate surfaces employed in the manufacture of indwelling medical devices [3]. Similarly, another study in 2012 showed 6 log reduction of *Candida albicans* biofilms after treating with surface micro-discharge (SMD) plasma for 8 min. This contact-free application of plasma to kill biofilm cells could be beneficial for the eradication of nosocomial infections in hospital settings [55].

Liu et al. in 2017 evaluated the bactericidal effects of non-thermal argon/oxygen plasma on *S. mutans* and/or *S. sanguinis* biofilms [124]. The 7 days old biofilm grown on 48-well plate showed 99% of bacterial cells reduction after 2 min plasma treatment. Their study further suggested the virulence properties of *A. oris* by altering its hydrophobicity and capability to co-aggregate with *S. sanguinis*. A study by Bhatt et al. in 2018 used a novel argon plasma-activated gas (PAG) for the treatment of different biofilm species such as *S. aureus*, *P. aeruginosa*, and *E. coli*. These biofilms were grown on polytetrafluoroethylene channel segments similar to the GI endoscopic channels for 48 h and treated with PAG for 9 min. Their results demonstrated effective killing of biofilm (8 log reduction) that have a potential alternative to the high-level disinfectants (HLDs) and/or ethylene oxide in the endoscope reprocessing procedure [125].

A study by Patenall et al. in 2018 investigated the ability to disrupt and limit biofilms growth of *P. aeruginosa* by helium cold atmospheric pressure plasma (CAP) jet. 4–5 log reduction in viable bacterial cells were found when 8 h grown biofilms were treated with plasma, whereas only 2 log reduction were achieved after treating biofilms grown for 12 h. The plasma treatment time was 5 min. This study concludes that using CAP in a time-dependent manner is important for reducing the formation of biofilms [126]. Another interesting study by Lu et al. in 2018 used helium porous plasma jet on *P. aeruginosa* biofilms grown on the surface of the glass vial for 24 h. The results showed 4.5 $\log_{10}$ reduction of bacterial cells after 5 min of plasma treatment. This could provide an alternative approach for inner surface sterilization and decontamination in the medical device, food, and pharmaceutical industries [127].

Thus, the most commonly used plasma configurations are jet and DBD plasma. In DBD configuration, the plasma ignites from an electrode array, therefore, covering larger area and can treat larger surfaces for planktonic as well as bacteria in biofilms. This plasma is more suitable in clinical settings to treat the biofilm contaminated medical devices and implants. However, jet plasma has been widely used when compared to the DBD as previously mentioned. Jet configuration is relatively smaller and is suitable for treating bacteria in nooks and crannies of a surface, for example, in dentistry. This is possible since a plasma source with jet configuration can be customized in a desired shape and size depending upon the application. It is also possible to use different gases for plasma generation in this configuration leading to the generation of more ROS and RNS. When comparing the inactivation yield, jet configuration has demonstrated better efficacy than the DBD configuration. The jet plasma can yield complete sterilization of biofilm [56], however, the maximum inactivation yield by DBD plasma was only 7.1 log reduction [53]. In both cited studies, the bacterial strain used was *Pseudomonas aeruginosa*. On the other hand, it has been also found that the performance of NTP depends upon the parameters used. For example, the study [42] used 10 kV and 10 kHz to generate jet plasma and yielded 7 log reduction, whereas, another study [3] yielded only 4 log reduction when treating with plasma generated by 6 kV and 20 kHz source. A similar difference was observed when comparing the results of other two studies on DBD plasma. One of the studies [53] demonstrated 3–7 log reduction when treated with DBD plasma from 8–13 kV and 20–30 kHz, however, there was only 5 log reduction when treated with 8–10 kV and 37–40 kHz plasma source [54]. Therefore, it can be concluded that the antibacterial efficacy of plasma depends on several parameters like plasma generation, biological, and environmental parameters. Some of the plasma parameters are exposure time, voltage, frequency, distance between plasma source and the sample, working gas type, and the flow rate. The biological parameters that might affect the efficacy could be the bacterial species of interest and gram characteristics, initial inoculum, growth stages, biofilm growth period and mode, and the thickness of biofilm. Similarly, the environmental parameter affecting the inactivation yield by plasma could be the matrix composition, its relative humidity (RH), and the acidity [98].

7. Other Medical Applications

The advent of NTP has opened multiple opportunities for its use in several areas. Apart from biofilm decontamination and sterilization, it is being widely investigated in cancer treatment, blood

coagulation, wound healing, tissue regeneration, and dentistry. NTP causes selective death (apoptosis) of cancer cells as these cells are more sensitive to plasma treatment than non-malignant ones [93]. This anti-cancer effect has also been demonstrated in vivo [93].

Dentistry is another field where plasma has been used to treat dental implants, intraoral disease, and cleaning or disinfecting dental cavity tissue or tooth root canal [86]. Blood coagulation has also been possible using plasma without any thermal effects and bacterial contamination [72,128]. Some of the findings using skin models suggest that using NTP can contribute to decontaminating acute and chronic wounds and accelerate healing [129]. These advances in plasma surgery wound healing, and tissue regeneration is because of the development of a plasma device called "Plazon" [39] that has been in medical use for patients for nine years [130].

8. Conclusions

The extensive studies of recent years clearly show the promising nature of NTP technology in eradicating biofilms in the medical field. However, there is a long unmet need for the development of a robust platform that can utilize the advantages of this technology. Therefore, the development of feasible NTP devices is needed if this technology is to make a paradigm shift in the world of decontamination/sterilization rather than being currently limited to in vitro and a few in vivo studies.

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