Plasma interaction with biofilm

Sohier M. Syame

Department of Microbiology and Immunology, National Research Center, Dokki, Giza, Egypt

In recent year's pharmaceutical companies and many research papers are searching for alternative and efficient antimicrobial agents due to resistance of pathogenic organisms to many antibiotic present. Moreover, persistent infections were also observed due to improved resistance of bacteria in biofilm that formed a microbial multicellular lifestyle and are defined as organized communities of bacteria, collaborating among themselves and being attached to an inert or living surface contained in a self-produced polymeric matrix made principally of exopolysaccharide that known as biofilm. The structural nature and the characteristics of the sessile cells in biofilms, produce resistance towards the antimicrobial agents, leading to a protected environment against adverse conditions and the host´s defences .Biofilms partly protected bacteria, with the efficiency of protection dependent on biofilm thickness .So the conventional sterilization and disinfection methods didn't have any effect with biofilms, which are Complex and stable , hard-to-destroy microbial communities embedded in a matrix . So this problem recently can be prevented by the electrical discharge atmospheric plasma gas that have a potential physical agent for biological decontamination and sterilization ,since plasma contain a mixture of UV radiation ,charged particle and chemically reactive species its used to destroy bacterial endospores and has attracted much interest for the decontamination of potential biological warfare agents . This review focuses on the development of biofilm, its mechanism of resistance to antimicrobial agents, different strategies for control biofilm in food industry, wound biofilm in vitro and vivo basic methods for testing biofilm in static systems. Finally the germicidal effects of non-equilibrium atmospheric pressure plasma generated by a novel resistive barrier discharge on representatives of the classes of bacteria ,application of non-thermal plasma on sterilization of water, food processing, in medicine are discussed.

Keywords: Biofilm; Quorum sensing; Mechanism of resistance of biofilms; food industry; cold plasma

1. The development of biofilm

Biofilm defined as "structural communities of microorganisms embedded in an extracellular matrix of polymeric substances produced by these cells and irreversibly attached to this surface [1] biofilms are not at all just simple bacterial layers on different surfaces but its high organization level in biological systems , where bacteria are developing functional and coordinated communities [2]. Biofilm formation is a dynamic complex multicellular process that is carried out in several different stages: adhesion stage, aggregation stage, maturation stage, mature biofilm stage, and dispersion stage as seen in fig (1).

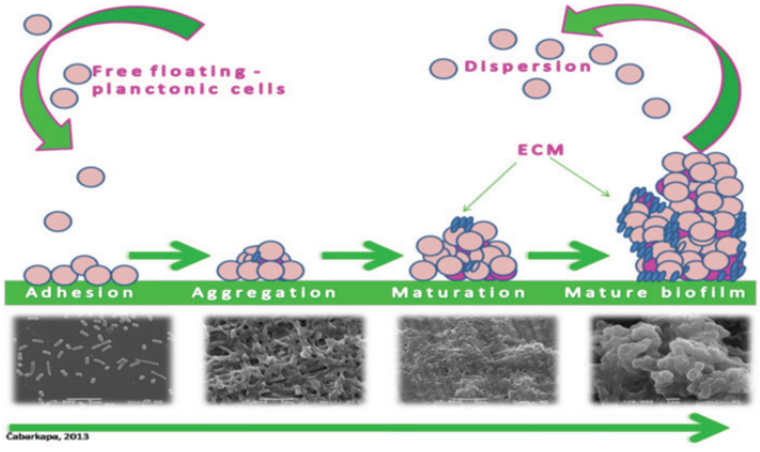


Fig. 1 Stages of biofilm development.

The biofilm formation is a developmental process mediated by many cellular mechanisms combines with each other as bacterial motility, adhesion mechanisms and quorum-sensing (QS) phenomenon. The best method for eradication of biofilms is the prevention of their development which is important targets for the discovery of new drugs [3].

1.1 Biofilm matrix or (EPS)

Diverse surface structures, such as flagella, fimbriae, and pili, which play the key role in the adhesion phenomenon, modifying and strengthening of the bond between bacteria and substrate to produe extracellular polymeric substance biofilm matrix or (EPS), which is the main component of biofilm structure ,can be found on the surface of bacterial cells . Biofilm matrix is very hydrated substance, which consists of exopolymer, water, microorganisms, and products of their metabolisms and activities. Exopolymers are usually composed of polysaccharides (40-95%), nucleic acids (1-10%), proteins (1-60%), and lipids (1-40%).Its composition depends on many environmental parameters as microorganisms, temperature, and presence of nutrients [4]. From the previous EPS is the basis of three-dimensional biofilm matrix structure and it is responsible for adhesion of biofilm to surfaces as well as for its consolidation (cohesion) as seen in (table 1).

Table 1 Functions of extracellular polymeric substances in bacterial biofilms.

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Facilitates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids

1.1.1 Quorum sensing

Quorum sensing is signal molecules serve as an intercellular communication between bacteria it is marked as autoinducers (AI) [5]., its depend on population density ,it occur once the appropriate population density (quorum) is reached in the later stage of biofilm formation ,so it was called quorum sensing. Quorum sensing is the key process in regulation of expression of genes that responsible for different physiological activities in the bacterial cell as production of antimicrobial peptides ,motility, virulence regulation, competition, symbiosis, sporulation, bioluminescence, and also some genes responsible for biofilm formation [6]. Quorum sensing present in four classes of intercellular communication in several species as (*Vibrio fisheri, Vibrio harveyi, Enterococcus faecalis, Myxococcus xanthus, and Streptomyces spp*.).These classes are autoinducer polypeptides (AI-P) in Gram-positive bacteria ,N – Acyl – Homoserine Lactones (AHL) or autoinducer-1 (AI-1) in Gram-negative bacteria, autoinducer-2 (AI-2) in Gram-negative and Gram-positive bacteria, and autoinducer-3/epinephrine/norepinephrine (AI- 3/epi/norepi) in some species of enterobacteria [7].

From all mentioned the development process of biofilm is slow, biofilm can be reached as mature after several days [8]. The complete mature biofilm formed by bacterial cells, EPS, and interstitial water channels that enable the exchange of nutrients and elimination of waste metabolic products out of biofilm [9]. At the last stage of biofilm development as the response to the changed environmental conditions, irrespective if they are caused by the lack of nutrients or by other unfavorable impacts the dispersion mechanism occur by deadhesion of microorganisms from the biofilm structure[10].this mechanism can be carried out via erosion, sloughing, seeding, and abrasion [11] as in fig(2).

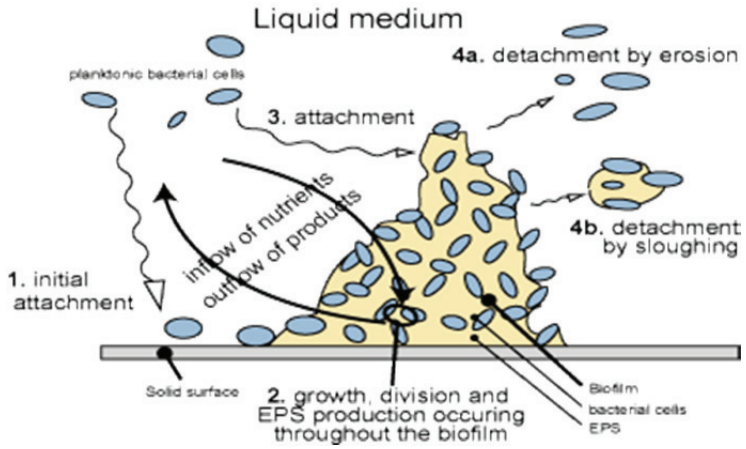


Fig. 2 Schematic representation of processes involved in biofilm formation.

2. Mechanism of resistance of biofilms to antimicrobial agents

The planktonic cultures of microorganisms differ from the same microorganism enclosed into the biofilm matrix as the last show antimicrobial agents may be up to 1,000 times higher [12].

As response to diverse environmental conditions such as DNA damages ,sudden change of temperature, starvation oxidative stress, genetic transfer between the cells and lake or low water activity, the bacteria are adapted themselves by development of diverse mechanisms which mediated by extracellular matrix [13] as in (fig 3).

The extracellular biofilm matrix acts as a barrier for antimicrobial agents, prevents their passage to bacteria via the interaction with antimicrobial agents or production of enzymes inactivate and dissolve antimicrobial agents. The extracellular matrix surrounds the biofilm represent also diffusion barrier, molecular sieve for protection of biofilm against antimicrobial agents of larger molecular mass. And cells of immune response causing slow down the penetration and reduction of the concentrations antimicrobial agent when it pass through deeper layers so the cells form new genes for resistance before attacked by these agents also only surface biofilm bacteria are exposed to lethal concentrations of antimicrobial agent [14] . After their death they act as the source of nutrients and increase of biofilm attrition rate.

The biofilm for [15] microorganisms is a perfect phenomenon for resist pathogens with new virulence factors. Increase capacity of survival in the environment, horizontal transfer of genetic material [16]

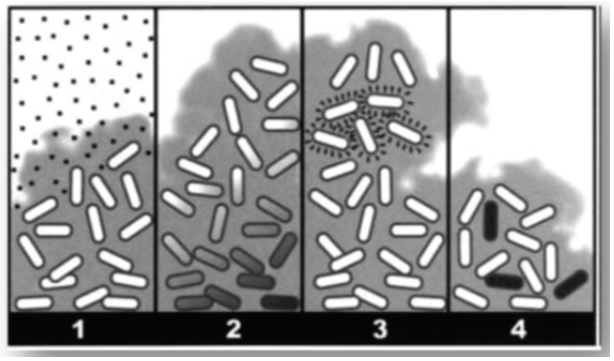


Fig. 3 Hypothesized biofilm resistance mechanisms [15]. 1) The antibiotic penetrates slowly or incompletely; 2) a concentration gradient of a metabolic substrate or product leads to zones of slow or non-growing bacteria (shaded cells); 3) an adaptive stress response is expressed by some of the cells (marked cells); 4) a small fraction of the cells differentiate into a highly protected persisted state (dark cells).

3. Biofilm in food industry

Long term microbial contamination occur during production,processing, packaging and transport of food that cannot be reduced or affected by thermal treatment to lead of the biofilm formation . These contamination occur by both bacteria causing their spoiling and food-borne species such as ., *Escherichia coli .,Salmonella* spp., *Campylobacter* spp and *Listeria monocytogenes* .

Hydrophilic (such as glass and stainless steel)and hydrophobic(polymeric materials) interactions between microbial cell charge and contact surfaces have great role in biofilm formation so the selection of materials for installations used in the food industry is of great importance to provide important new strategies to prevent and avoid listerial biofilm formation [17].

As shown in table (2) Chemical strong oxidizing agents as chlorine, hypochlorous acid, iodine, peroxyacetic acid, hydrogen peroxide quaternary ammonium chloride, ozon and anionic acids have a strong a broad antimicrobial spectrum so can used for sanitation procedures and also has great effect *on L. monocytogenes* biofilm when studied and compared at different experimental conditions for establishment the most efficient treatment [18].also physical methods such as ultrasound, irradiation is another alternative new approaches to control the biofilm formation and planktonic cells in the food industry [19].

Different microbial pathogens including *Listeria monocytogenes* biofilms affected by aerosolized sanitizers as reported by [20].While [21] recorded the use of nanostructured titanium dioxide combined with UVA irradiation for the same purpose.

In the last years, with the increasing of consumers prefer environment- friendly treatments to avoid chemicals that is known as new green emerging strategy, active compounds of microbial origin, used now in wide range as illustrated in Table (3) due to their low toxicity and high biodegradability , for example a glycolipid(Rhamnolipid and surfactin) produced by *Pseudomonas aeruginosa* and a lipopeptide from *Bacillus subtilis* were investigated to prevent the adhesion of *L.monocytogenes* on different pre-conditioned surfaces such as polypropylene and stainless steel [22].

A valuable alternative approach in the field of green-based biofilm control strategies recently applied by using a probiotic [23] investigated that co-culture with *Lactobacillus rhamnosus* and *Lactobacillus paracasei* reduce the biofilm formation of *Listeria monocytogenes*. *Enterococcus durans and Lactococcus lactis* in poultry can reduce *L. monocytogenes* biofilm cells [24].

Finally the use of bacteriophage is another promising approach [25] especially after the approvement of Food and Drug Administration (FDA) for using bacteriophage preparations employing phage P100 as ingredients to control *L. monocytogenes* contamination [26] . In a recent study, phage P100 can significantly reduce *L. monocytogenes* biofilm on both polystyrene microtiter wells and stainless steel coupons surface [27]regardless of any present factors as biofilm levels, growth conditions and serotype.

In addition, bacteriocin-producing strains as *Enterococcus faecium and Enterococcus faecalis* producing enterocin AS-48 can reduce the initial adhesion of planktonic and sessile *L. monocytogenes* and control biofilm formation of it on abiotic surface [28] for at least 24 h at a bacteriocin concentration of 25 mg/ml [29].

Table 2 Some chemical and physical-based approaches proposed to prevent and remove *L.monocytogenes* biofilms and other pathogenic microorganisms from different contact surfaces.

Treatment	Contact surface	Microorganism	reference
Ozone Ultrasound Ultrasound + Ozone	Stainless steel	*Listeria monocytogenes*	[30]
Chlorine dioxide	Stainless steel	*Listeria monocytogenes*	[31]
Chlorine dioxide gas Chlorine dioxide aqueous Sodiumhypochloriteaqueous	Stainless steel	*Listeria monocytogenes*	[32]
Quaternary ammonium compounds ChlorinePeroxide	Polyvinyl chloride drainpipes	*Listeria monocytogenes*	[33]
Chlorine Quaternary ammonium compounds Peroxyacetic acid	Stainless steel coupons	*Listeria monocytogenes*	[34]
Peroxyacetic acid Nisin	Stainless steel coupons	*Listeria monocytogenes*	[35]
Titanium dioxide and UV irradiation	Stainless steel,glass	*Listeria monocytogenes*	[36]

Table 3 Some of emerging green "strategies approaches proposed to prevent and remove *L.monocytogenes* biofilms and other pathogenic microorganisms from different contact surfaces.

Treatment	Contact surface	Micoorganism	reference
Yarrow essential oil	Polystyrene,stainless steel, high density polyethylene	*Listeria monocytogenes* *Listeria innocua*	[36]
Cymbopogon sp. essential oils	Stainless steel	*Listeria monocytogenes*	[37]
Isothiocyanates	Polystyrene	*Listeria monocytogenes* *Escherichia coli* *Pseudomonasaeruginosa* *Staphylococcus aureus*	[38]
Chitosan	Teflon	*Listeria monocytogenes* *Bacillus cereus Salmonellaenterica* *Staphylococcus aureus* *Pseudomonasfluorescens*	[39]
Rhamnolipid Surfactin	Polystyrene	*Listeria monocytogenes* *Salmonella enteriditis* *Staphylococcus aureus*	[40]
Probiotic strains	Polystyrene	*Listeria monocytogenes* *Salmonella typhimurium*	[23]
Bacteriophage P100	Stainless steel	*Listeria monocytogenes*	[27]
Enterocin AS-48 Enterocin AS-48 + biocides	Polystyrene	*Listeria monocytogenes*	[29]

5. Basic methods for biofilm testing in static systems

Several methods used to study biofilms. These methods are easy to do as equipment and accessories are mostly available in most laboratories. The testing used to study early stages of biofilm formation is called static systems as it study the adhesion of microorganisms onto the substrate and intercellular signalisation, the disadvantage of these method is the low amount of available nutrients and biofilm aeration that affects in formation and maturing of biofilm structure that differ from systems with continuous flow of liquid[41].

Static systems basic protocols for biofilm testing include:

1. Microtiter plate biofilm assay
2. Air-liquid interface assay (ALI)
3. Colony-based biofilm system

5.1 Microtiter plate biofilm assay

This assay widely used since the mid-1990s with different modifications described by [42].The basic principle of the test is as follows: inoculation of bacteria culture into microtiter plate wells and incubated with the substrate at the selected temperature for the desired period of time to allow bacteria to grow, eliminated the suspended bacteria by flushing and washing , crystal-violet dissolution used to stain the bacteria that successfully attached to the substrate and

determine the quantity of bonded stain by spectrophotometry .The ingredient of dissolution crystal violet is (33% of glacial acetic acid ,DMSO, ethanol/acetone (80%/20%), 30% of acetic acid, 95% ethanol) as seen in fig (4). The advantage of this method is the facility for testing of a larger number of isolates, that makes is suitable as a screening method Also its simple, fast test, save many chemicals by using small amount of it ,this test enables different simple several parameters as O2 and CO2 concentrations ,incubation temperature, composition of the medium, pH, so can be modified according to the aim of the studies such as disinfectants and plant extracts

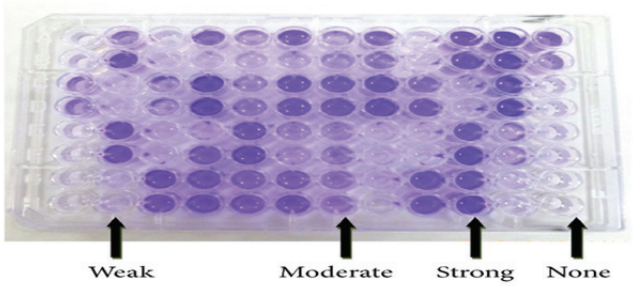


Fig. 4 Microtiter plate method showing none, strong, moderate, and weak biofilm producers differentiated by crystal violet stain in 96-well tissue culture plate.

5.2 Air-liquid interface assay (ALI)

There is biofilm formed on a bounded border of surfaces between air and liquid [43].this kind of biobilm can be analytically with microscope by setting the plates (with 24 or less wells) into oblique position in relation to horizontal plane at the angle of 30o to 50o that form air-liquid inter-phase in the center of the well bottom, while formed the biofilm after staining and washing the wells can be analyzed by invert microscope (phase contrast microscopy).If not available, the ordinary microscope can be used after covering the plate well with plastic or glass and make the previous procedures.

5.3 Colony-based biofilm system

In this technique, a semipermeable membrane set on the surface of a fresh substrate (agar) that used for bacterial growth moved to supply nutrients for biofilm formation ,that provide maximum growth for bacteria and there is no detachment of cells typical for biofilms immersed in liquid. Stable nature of biofilms formed in this technique is used for testing their sensitivity to antimicrobial agents. And in that case, the reduction of the number of bacterial cells can be more reliably attributed to the cell death rather than to detachment. It's very important to use an identical test with the addition of redox indicators (tetrazolium salt or resasurine)When actions of antimicrobial substances on biofilm are tested to analyzed the effect of the tested substances on metabolic activity of cells in biofilm [44] from the applied method, it is recommended to see the structure of the formed biofilm using some of the high resolution microscopy techniques such as transmission electronic microscopy (TEM),scanning electronic microscopy (SEM) , or confocal laser microscopy (CLSM).

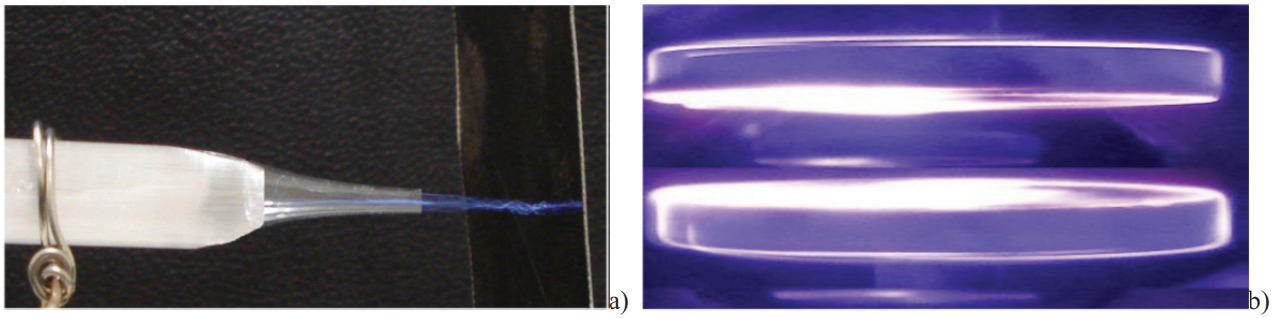
6. Cold Plasma

Sir William Crookes in 1879 was the first discover plasma [45]. The name “plasma” was first used by Irving Langmuir in 1926 who studied this state of matter as: “... near the electrodes, and sheaths containing very few electrons, the name plasma is applied to this region containing balanced charges of ions and electrons [46]. Later, plasma defined as a state of matter in which a number of atom and/or molecules are electrically ionized or charged it does not have a regular volume or shape and form beams under magnetic fields. Plasma can be found in two forms the first of natural phenomena such stars and lightening or man-made as in neon lights, plasma television and in the production of fluorescent and etc.

6.1 Non Thermal Atmospheric Plasma

There are two groups of plasma depending on the source of generation as non-thermal plasma (NTP), and thermal plasma (TP). NTP consists of gas molecules with moderate temperatures and electrons with higher temperatures so defined as cold and non-equilibrium .many technological developments have done to produce cold plasma under atmospheric pressures providing cost effective systems [47]. There is more than one types of NTP’s that have the advantage of work at atmospheric pressure used for decontamination purposes , dielectric barrier discharges (DBD) as seen in fig (5), corona discharges, radio Frequency (RF) torches, and gliding arc discharge plasmas, all of them have the capacity to discharge plasma at moderately low temperatures, at atmospheric pressure so can used to decontaminate

heat sensitive materials [48]. NTP has great antimicrobial effect without changing the properties of material being decontaminated that is due to weakly ionized nature of the cold plasma beams.



a) b)

Fig. 5 a) the atmospheric pressure plasma torch, b) the dielectric barrier plasma discharge.

6.2 The mechanism of sterilization and decontamination

Cold plasmas are composed of a cocktail of different chemical species such as positive ions, negative electrons, excited atoms, UV photons, radicals and reactive neutral species such as reactive oxygen (ROS) and Nitrogen species (RNS) as seen in (Figure 6),these species act directly on the bacterial membrane proteins [49]. Also photodesorption formed by ultraviolet (UV) photons caused degradation of genetic material, the previous deactivation mechanisms causes the death of bacterial cells by several reaction including DNA oxidation, undergo lipid peroxidation of poly-unsaturated fatty acids and amino acids oxidation [50]. NTPs can affect biological objects via a synergetic action of bioactive components such as UV photons, metastable and charged, neutral active species [51]. The most effective action of NTPs is by made modifications to the surfaces in order to prevent surface colonization by bacterial attachment, biofilm formation as seen in table (4)and decrease biofilm-associated infections this is occur by two mechanism the first by penetration, attachment of antimicrobicidal agents [52] such as, antibacterial peptides, antibiotics, chlorhexidine ,silver (Ag) nanoparticles, and nitrofurazone, etc. which destroy attached bacterial cells and thus prevent surface colonization. The second is modification of the different physical, chemical character to prevent bacterial adhesion [53] such as porous distribution, surface chemistry, hydrophility, size, and roughness.

Table 4 Nonthermal plasma activity against biofilms.

Microorganism	Plasma source	Feeding gas	Bactericidal effect (log 10 CFU reduction)	Time to reach the effect	Reference
P. aeruginosa	Plasma jet (13.56 MHz, 100 W)	He/$N_2$20.4/0.135	7	180 s	[54]
	Plasma jet (13.56 MHz, 100 W)	He/$N_2$20.4/0.15	7.12	300 s	[55]
	Plasma jet (20 kHz, 6 kV)	He/$O_2$99.5/0.5	4	240 s	[56]
	Structured electrode planar SBD surface barrier discharge (SBD; 20 kHz, 13 kV)	Air	7.11	600 s	[57]
S. aureus	Floating-electrode dielectric-barrier discharge (500 Hz, 15 kV)	Air	7	60 s	[58]
Staphylococcus epidermidis	Structured electrode planar SBD surface barrier discharge (SBD; 20 kHz, 13 kV)	Air	3.38	600 s	[57]
	Plasma jet (100 kHz)	Ar/air 9/1	9	240 s	[59]
E. coli	Postdischarge plasma afterglow (28 kHz; 10 kV)	N_2/$O_2$80/20	1.6	40 min	[60]
P. gingivalis	Plasma jet (10 kHz, 8 kV)	Ar	>2	300 s	[61]
Chromobacterium violaceum	Plasma jet	He/$N_2$20.4/0.305	>2	300 s	[62]
S. mutans	Plasma brush	Ar /O_2	7.23	13 s	[63]

Microorganism	Plasma source	Feeding gas	Bactericidal effect (log 10 CFU reduction)	Time to reach the effect	Reference
	Plasma jet (2.45 GHz)	$He/O_2/N_2$2/1.2/1.5	4	18 s	[64]
Lactobacillus acidophilus	Plasma brush	Ar/O_2	6.84	13 s	[63]
Neisseria gonorrhoeae	Plasma jet (10 kHz; 10 kV)	$He/O_2$2/0.04	7	20 min	[65]
Streptococcus sanguinis	Plasma jet (1.8 MHz, 170 V)	Ar	0.6	180 s	[66]
C. albicans	Plasma jet (2.45 GHz)	$He/O_2/N_2$2/1.2/1.5	5	12 s	[64]
	Surface microdischarge (1 kHz, 9 kV)	Air	5	7 min	[67]
	Plasma microjet	$He/O_2$98/2	>2	60 s	[68]

The potent action of microbial inhibition by plasma discharge is connected to level of oxygen concentration based active species such as ozone , atomic oxygen and radicals [69] that can begin lipid peroxidation to produce short chain charged fatty [70] that increase the fluidity of the membrane [71] to cause structural membrane integrity.

There is many factors which can effect on NTP biocidal actions as the bacterial strains, species ,the system used and material being treated as vegetative cells are more susceptible to plasma application than spores, [72], also gram-negative are more susceptible to plasma treatment than gram positive ones as they have thinner layer (~ 2 nm) compared to gram-positive bacteria (~ 15 nm) many evident in the various literature studied the inavtivation of NTP systems on vegetative cells, fungi, spores, yeast [49] and biofilm formers [73] . The inactivation of *Streptococcus mutans* [74] and *Escherichia coli* [75] with the use of microplasmas, this type of plasma used then recently in the areas of dentistry and microsurgery. also many studies recorded the use of NTP's in food processing as aresult of the successful inactivation *of Listeria monocytogenes* from processed meat surfaces [76] and on cheese [77] inactivation and destruction *of S. aureus,* [78] *Pseudomonas aureginosa* biofilms with gas-discharge plasmas [79] .

Moreover, the use of plasma discharge has shown to be useful against spore forming, atmospheric plasma applied to *Bacillus subtilis and Bacillus pumilus* [[80].

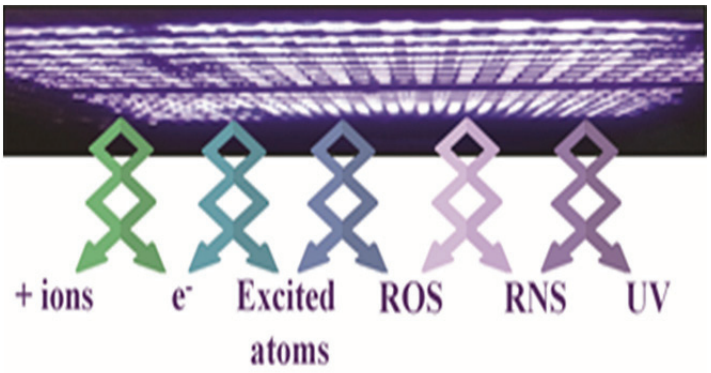


Fig. 6 Cold plasmas are composed of a cocktail of different chemical species such as positive ions, negative electrons, excited atoms, UV photons, radicals and reactive neutral species such as reactive oxygen (ROS).

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