INHIBITION OF BIOFILM FORMATION ON MEDICAL SURFACES BY NOVEL PLASMA SYSTEM

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To my beloved family, who offered me love and support throughout my life…

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ABSTRACT

INHIBITION OF BIOFILM FORMATION ON MEDICAL SURFACES BY NOVEL PLASMA SYSTEM

Bacterial infections are a global challenge all around the world which affects millions of people every year. Therefore, the utilization of alternative and effective methods to decrease bacterial adhesion on medical surfaces is an issue of highly interested. The plasma technology has been widely used, an effective and economical technique for surface modification of materials, aiming to change surface physicochemical properties and reduce biofilm formation due to a decrease of bacterial attachment.

This study was aimed to investigate the anti-adhesive and anti-biofilm effects of Non-thermal Atmospheric Pressure Plasma Jet (APPJ) treatment on Stainless Steel L316 (SS) and Titanium (grade 5) (Ti) surfaces in the presence of various gases. Optical emission spectrometry was operated to identify the excited reactive species during APPJ treatment by nitrogen, oxygen and argon gases. SS and Ti surfaces were treated by the use of nitrogen, oxygen and argon APPJ system for 15 minutes, separately for each gas. Following treatment, the change of surface modifications was characterised by the attenuated total reflection fourier transform infrared, atomic force microscope, water contact angle goniometer, and scanning electron microscope. Early adhesion and biofilm formation of *Pseudomonas aeruginosa* PAO1 on the APPJ treated surfaces were investigated by culture plate method.

APPJ treatment showed different effects on the native surface properties and also surface roughness parameters according to the presence of different gases. APPJ treated surfaces exhibited increasing hydrophilicity and significantly reducing *P. aeruginosa* adherence. This reduction was sufficient to decrease of biofilm formation on nitrogen and oxygen APPJ treated SS surfaces, however, biofilm formation was not significantly decreased on argon APPJ treated SS surface and also APPJ treated Ti surfaces.

The present study highlights the potential benefits of APPJ treated SS and Ti surfaces with different gases. These positive effects of APPJ treatment could enhance the biological activity of medical surfaces over time.

ÖZET

YENİ PLAZMA SİSTEMİ KULLANILARAK TIBBİ YÜZEYLERDE BİYOFİLM OLUŞUMUNUN ÖNLENMESİ

Bakteriyel enfeksiyonlar her yıl tüm dünyada milyonlarca insanı etkileyen küresel bir sorundur. Bu nedenle, tıbbi yüzeylerde bakteri tutunmasını önleyen etkili stratejilerin bulunması önemli bir konudur. Plazma teknolojisi, yüzeylerin fizikokimyasal özelliklerini değiştirmeyi ve bakteri tutunmasını azaltarak biyofilm oluşumunu önlemeyi hedefleyen, yüzey modifikasyonu için etkili ve ekonomik bir teknik olarak yaygın şekilde kullanılmaktadır.

Bu çalışmanın amacı, farklı gazlarla Termal-olmayan Atmosferik Basınçlı Plazma Jet (APPJ) uygulamasının paslanmaz çelik L316 (SS) ve titanyum (grade5) (Ti) yüzeylerde bakteri tutunması ve biyofilm oluşumunu önleyici etkilerini araştırmaktır. Optik emisyon spektrometresi azot, oksijen ve argon gazları ile APPJ uygulaması sırasında açığa çıkan reaktif türlerin belirlenmesi için kullanılmıştır. SS ve Ti yüzeylerine, azot, oksijen ve argon APPJ sistemi, her bir gaz ayrı olarak 15 dakika süreyle uygulanmıştır. Uygulama sonrası, yüzeylerde oluşan değişiklikler zayıflatılmış toplam yansıma- fourier dönüşüm kızılötesi, atomik kuvvet mikroskop, su temas açısı goniometre ve taramalı elektron mikroskop ile karakterize edilmiştir. APPJ uygulanan yüzeylerde *Pseudomonas aeruginosa* PAO1'in tutunması ve biyofilm oluşturması üzerine etkileri plaka kültür yöntemiyle incelenmiştir.

APPJ uygulanan SS ve Ti yüzeylerin doğal yüzey özelliklerinde ve pürüzlülük parametrelerinde farklı gazların varlığına bağlı olarak değişiklikler olduğu tespit edilmiştir. APPJ uygulaması sonrası yüzeylerde hidrofilik özelliğin arttığı ve *P. aeruginosa* tutunmasının önemli ölçüde azaldığı görülmüştür. Bu azalma nitrojen ve oksijen APPJ uygulanmış SS yüzeylerde biyofilm oluşumunun azalmasında etkili olmuş, fakat argon APPJ uygulanmış SS ve azot, oksijen ve argon APPJ uygulanmış Ti yüzeylerde istatistiksel olarak biyofilm oluşumunu azaltmada aynı etkiyi göstermemiştir.

Bu çalışma farklı gazlarla APPJ uygulamasının SS ve Ti yüzeylerde neden olduğu faydalar vurgulamaktadır. APPJ uygulamasının bu olumlu etkileri zamanla tıbbi yüzeylerin biyolojik etkinliğini arttırabilir.

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LIST OF SYMBOLS / ABBREVIATIONS

AFM	Atomic Force Microscopy
APP	Atmospheric Pressure Plasma
APPJ	Atmospheric Pressure Plasma Jet
Ar	Argon
ATR-FTIR	Attenuated Total Reflection-Fourier Transform Infrared
CFU	Colony Forming Units
CH_4	Methane
CO_2	Carbon dioxide
DBD	Dielectric Barrier Discharge
E_k	The energy of Upper levels
EPS	Extracellular Polymeric Substances
eV	Electron Volt
He	Helium
K	Kelvin
N_2	Nitrogen
NH_3	Ammonia
NIST	National Institute of Standards and Technology
NO	Nitrous oxide
O_2	Oxygen
OES	Optical Emission Spectroscopy
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SS	Stainless Steel
T_e	Electron Temperature
Ti	Titanium
UV	Ultraviolet
WCA	Water Contact Angle

1. INTRODUCTION

1.1. DEFINITION OF BIOFILM

The first biofilm microcolonies have been discovered from the Early Archean in the 3.3–3.5-billion-year-old cherts from the Onverwacht Group (a series of greenstone belts and volcanic rock formations), South African Kornberg formation in the eastern Transvaal in 2001 [1]. In 2000, from the Pilbara Craton of Australia, the first filamentous biofilm formation have been established in the 3.2-billion-year- old deep sea volcanogenic massive sulphide deposit [2].

Up until the seventeenth century, microorganisms have been described as planktonic suspended bacteria grow in nutritionally rich culture media according to their growth properties. Afterwards, Antonie Van Leeuwenhoek examined using his basic light microscopes the "animalcules" which was microbial biofilms in the plaque on his own teeth [3, 4]. Although he observed that microorganisms attach and grow on living and dead surfaces, the biofilm theory was not propagated until twentieth century. In 1940, Heukelekian and Heller [5] examined "bottle effect" for marine microorganisms and they described it when bacteria attached to a surface, the biological active slime was established and growth rate of bacteria was increased. Another study by Zobell [6] in 1943 was promoted Heukelekian and Heller [5] study and declared that the quantity of bacteria on surface was greater than in the presented liquid medium. Moreover, in 1976, by Marshall [7] was noted "very fine extracellular polymer fibrils" that colligated bacteria to surfaces.

In medical literature, in 1977, the first report relation between chronic infection and aggregation of bacteria surrounded by "slime" was chronic *Pseudomonas aeruginosa* infection in patients with cystic fibrosis [8]. In 1978, Costerton et al. [9] announced that bacteria grow in glycocalyx-enclosed biofilms adherent to biotic or abiotic surfaces in all aquatic ecosystems with sufficient nutrients. Then, the term of "biofilm" firstly was declared by Costerton in 1981 for describing bacterial aggregates and sessile bacterial populations on surfaces [10]. Biofilm was firstly defined as ''it is quite evident that for the most part water bacteria are not free floating organisms, but grow upon submerged surfaces'' in a scientific manuscript in 1983 by Arthur T. Henrici [11].

In 1980s, the improvement of the confocal laser scanning microscope (CLSM) provided the ability to examine the development and behavior of biofilms without limitations and with higher magnifications. This new technology has also displayed detail information on the 3-dimensional structure of biofilm [12–14]. Costerton et al. [13] remarked that biofilm includes microcolonies and single cells, all embedded in a mostly anionic and highly hydrated exopolymer matrix in 1987. Following this, the studies were continued to describe the other unknown aspects of biofilms, including adhesion to surface and interfaces and to each other, definition on microbial aggregates and floccules [15]. Therewithal, a beneficial simple concept of a "biofilm model" were described as microorganisms form microcolonies within an extensive extracellular polymeric matrix and water-filled channels which promote the influx and efflux of nutrients and waste products [15]. Consequently, a new definition of biofilm was determined according to observable and physiological characteristics according to knowledge from all previous studies. Biofilm could be defined as [16]:

"A microbially derived sessile community characterized by cells that are adhere irreversibly to a surface or interface or to each other, are embedded in extracellular polymeric substances which is produced by cells and includes the non-cellular or abiotic components, and exhibit an altered phenotype with respect to growth rate and gene transcription."

1.2. BIOFILM DEVELOPMENT AND STRUCTURE

The formation of biofilm is a phenomenon that occurs in both biotic and abiotic surfaces/environments under diverse conditions. Biofilms are either homogenous or heterogeneous communities include microcolonies and single cells encased in anionic and highly hydrated extracellular polymeric substances (EPS) [17–20]. EPS produced by microorganisms are complex mixture consisting of polysaccharides, proteins, lipids and nucleic acids [19, 21, 22]. The range of composition of biofilm matrix is shown in Table 1.1. In biofilms, the amount of EPS can account for up to 90 per cent, whereas microorganisms account for less than 10 per cent of the dry mass [22]. Studies showed that diverse microorganisms produce vary amounts of EPS. Moreover, the amount of EPS is effected and enhanced according to the age of biofilm, availability of nutrients, slow bacterial growth [23].

Table 1.1. Composition of biofilm matrix [22].

Component	Per cent of matrix
Water	up to 97
Microbial Cells	2-5
Polysaccharides	1-2
Proteins	<1-2
Nucleic Acids	<1-2 (From lysed cells)

EPS can show variety in chemical and physical characteristics, however its major component is polysaccharides [21, 24]. EPS contains homopolysaccharides and mostly heteropolysaccharides [20, 21, 23, 25]. Many common exopolysaccharides are polyanionic such as alginate, colanic acid and xanthan. As an example, even though alginate is not an obligatory molecule for biofilm formation, the presence of alginate has a notable effect on biofilm structure [22]. Overall, exopolysaccharides has an important role in adhesive and cohesive properties of EPS and the structure of biofilms.

EPS can contain significant amounts of proteins including enzymes, cellular appendages and structural proteins. Proteins play a role in cell-to-cell interconnection during the biofilm formation [26, 27]. Cellular appendages (flagella, fimbriae, and pili) are structural protein components which play crucial role during the initiation and in the later stages of biofilm formation and also provide interaction with other EPS components in biofilm [18, 28]. Furthermore, extracellular enzymes are involved in the biopolymer degradation process. While they degrade EPS components, breaks down biopolymers to low molecular mass products which then can be used as carbon and energy sources [22, 29, 30]. Extracellular enzymes (such as hydrolases and lyases) in biofilm also can be involves in the degradation of EPS structure which promotes the detachment of bacteria to another surface [31].

EPS incorporate large amounts of water [20, 23, 32]; therefore it’s highly hydrated environment and can be both hydrophilic and hydrophobic. Because extracellular polysaccharides, proteins and DNA are highly hydrated hydrophilic molecules, other EPS have hydrophobic features. Water channels in biofilm provide to exchange of nutrients and metabolites, enhancing nutrient availability and removing toxic metabolites [14, 33].

EPS also contain extracellular DNA (eDNA) which is mostly originated by lysed cells in biofilm; however, it can differ between species [22, 24, 34]. eDNA has a function as an intercellular connector, stabilization of lipopolysaccharide and the bacterial outer membrane in some biofilms [22]. Although eDNA is derived from whole genomic DNA, it has some differences. Biofilms provide an ideal environment to exchange genetic material and to access a large gene pool. For instance, conjugation in bacterial biofilms is higher than in planktonic cells [35]. Horizontal gene transfer play an important role for the genetic diversity and also evolution which gives chances to the cells to transcribe the crucial genes to become the active member of the biofilm [14, 36]. Consequently, bacteria in biofilm display different phenotypic characteristics from planktonic counterparts.

The formation and maintenance of a biofilm are crucially related to the presence and quantity of EPS which protects the bacteria in biofilm from adverse environmental factors [19]. For example, the EPS provides to physically inhibit accession of antimicrobials through the biofilm and protects from ultraviolet radiation and host immune defenses [36, 37]. EPS has also supplied the intercellular cavity within microbial aggregates and create a structure and hold the biofilm together and is responsible for cohesion in the biofilm [4, 38, 39]. Moreover, EPS has a role as recycling center to keep the components of lysed cells available which can serve as a nutrient source and also symbolize a container of genes for horizontal gene transfer in biofilm [22, 39, 40]. For example, water channels in biofilm allow diffusion of nutrients, oxygen and efflux of waste products [23, 41].

The biofilm development is sophisticated process (physical, chemical and biological) and requires collective multicellular behaviors. The most important mechanisms are aggregation and attachment. Aggregation improves cell to cell communication and the sedimentation percentage of cells [42, 43]. Attachment on a surface is an important characteristic for persistence of the bacteria. This provides to form communities and benefit of the phenotypic versatility of their neighbors [4, 21, 24]. Nonetheless, development of a biofilm is effected by a set of various parameters such as ambient and system temperatures, hydrodynamic conditions, nutrient availability, surface roughness, hydrophobicity and electrochemical characteristics of the surface, pH, and the effectiveness of biofilm control measures [5, 32, 44].

Scientific studies show that development of a biofilm requires multiple steps [18, 25, 36]. Biofilm is a well-organized and complex, cooperating community of microorganisms. When a microorganism irreversibly attaches to a surface, they develop a biofilm. A biofilm can originate from a single cell, however, different environmental factors can potentiate the development of distinct subpopulations [34]. A cell in a biofilm is differentiated by up and down regulation of the gene from suspended counterparts, reduced growth rate and production of EPS [4]. Moreover, the metabolic properties of bacteria within biofilm shows the difference from their planktonic counterparts. For instance, cell division rate in biofilm is 5-15 times slower than planktonic conditions because of the limited nutrient availability [23, 45]. Biofilm provides an ideal environment for syntrophic relationships and metabolic cooperation.

The biofilm formation process can be summarized in several consecutive stages (Figure 1.1): (i) initial reversible attachment of bacteria on a surface via weak interactions, (ii) irreversible adhesion to the surface via hydrophilic/hydrophobic interactions by flagella, fimbriae or other adhesive proteins, (iii) proliferation of cells and production of EPS, (iv) development of mature biofilm which includes water channels, (v) detachment of biofilm cells, and (vi) cells dispersion and colonization on other surfaces [4, 44, 46–48].

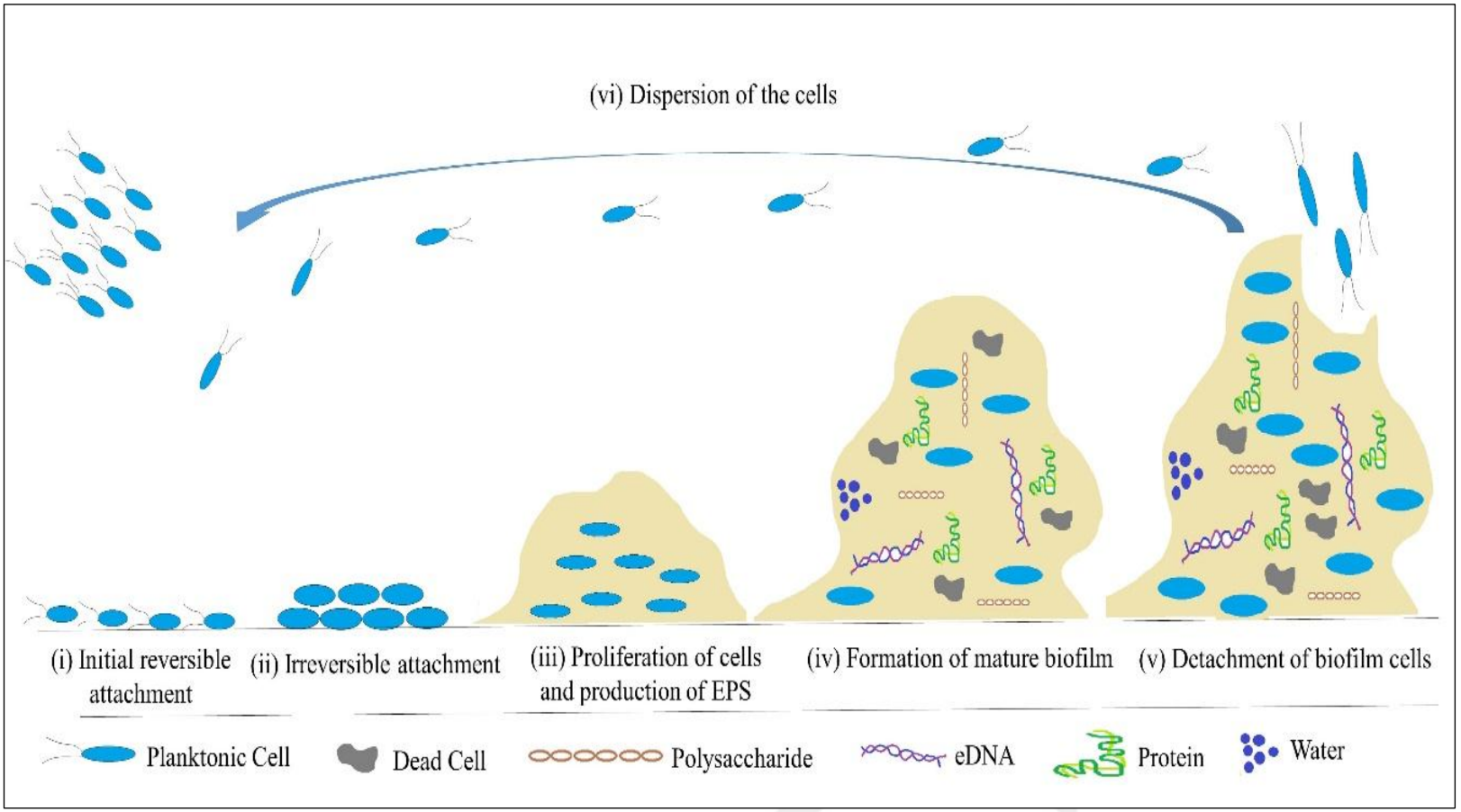

Figure 1.1. Schematic representation of biofilm formation.

1.2.1. Initial Attachment of Bacteria

Initial attachment is the first and major stage for bacterial biofilm formation. Microorganisms attach to a surface reversibly which is a crucial and a complex process for biofilm formation. Bacterial biofilm begins to form by the initially attachment of planktonic cells to a biotic/abiotic surface [15, 48]. This attachment is effected by variable factors including the amount of nutrient, temperature, pH, and surface characteristics such as roughness and hydrophilicity/hydrophobicity [18, 41, 48, 49]. For instance, the surface area are higher on rougher surfaces and microbial attachment to a surface increases as the surface roughness increases as a result of decrease of the shear forces [14, 21]. Also, studies show that microorganisms mostly prefer to attach hydrophobic and nonpolar surfaces [14].

Cell surface properties such as flagella, fimbriae, pili, and surface related polysaccharides or proteins, has also important roles during initial attachment to a surface (adhesion) and among bacteria (cohesion) [4, 22, 48]. These surface appendages dominate attachment to hydrophobic surfaces, however, EPS and lipopolysaccharides mostly prefer to attach on hydrophilic materials. Bacteria swim using flagella(called as flagellar-mediated motility) which are important for initial attachment, according to previous studies nonswimming bacteria shows reduce the ability for biofilm formation [48].

After initial attachment, plenty of the reversibly attached cells become irreversibly adhere to the surface. The surface appendages of bacteria consolidate the interactions between bacteria and the surface [47]. Then, cells proliferate and spread as a monolayer on the attached surface to form microcolonies. Although at the beginning of the initial attachment cellular metabolic activity is high, following colonization and formation of microcolonies the activity decreases at the beginning in the central parts of the microcolony and then at the surface, and developmental changes start [37].

1.2.2. Maturation of Biofilm

Afterward bacterial cells have irreversibly attach to a surface, they form aggregates and start to secrete extracellular polysaccharides which is one of the crucial steps of a mature biofilm and reinforce the biofilm structure [50]. While the synthesis of exopolysaccharides and

alginate are increased, flagellar synthesis decreases. Thus, the cells become an effective member of biofilm.

During biofilm development, another important step is maturation. In this step, microcolonies start to communicate among each other and produce extracellular polysaccharide substances (EPS) to stabilize the biofilm [24, 37, 45]. EPS creates a protective environment for biofilm, increase genetic exchange and secondary metabolite production [41, 42, 51]. During this step, extracellular components such as polysaccharides, lipids, proteins and DNA are secreted which are primarily hold the bacteria together to maintain the characteristic biofilm architecture [31]. Biofilm manipulates its structure, physiology and metabolism which become adapted. Formed biofilm provides an excellent environment for the construction of syntrophic association which is a type of symbiosis [41].

On this stage, microbial community become multilayer and thickness of biofilm is increased. Nutrient availability is important during increase of biofilm thickness. For instance, limited nutrient availability cause to halt of bacterial growth in the inner core of biofilm [31].

During maturation stage, the bacteria exchange genetic material by releasing DNA and also to form and stabilize biofilm. This stage establishes long term relationship among microcolonies in biofilm which provides optimal environment for gene transfer [19, 22, 38].

1.2.3. Biofilm Detachment and Dispersion

Detachment is another important and complex stage during biofilm life cycle. During detachment, bacteria in biofilm reduce adhesiveness and to break down the biofilm structure. Although the mechanism underlying is not well understood, detachment may be affected by various parameters including temperature, pH, nutrients, presence of organic molecules [46]. The reasons behind bacterial detachment are species specific, maybe the most prevalent effect is starvation. Physical forces also have importance in the detachment that stating in detail three major processes: erosion or shearing, sloughing and abrasion. Erosion or shearing is continuously small, sloughing is rapidly massive and abrasion is due to collision removal of the biofilm portion [52].

During the detachment, microcolonies secrete saccharolytic enzymes. These enzymes break polysaccharides which cause to breakdown biofilm stabilization. As an example, the

overexpression of alginate lyase in *Pseudomonas aeruginosa* speed detachment and cell dodging from biofilm [53]. Microcolonies also start to overexpress surface appendage proteins like flagella so that the microorganisms become motile.

The last part of biofilm life cycle is dispersion which means the shedding of the biofilm and returning of cells from sessile to the motile form [54]. The mechanisms underlying on dispersion are diverse and are affected by diverse intrinsic and extrinsic factors including nutrient starvation, molecular signals in biofilm and flow effects of environment. Before active dispersion, death cells localize and lysis in the center of mature biofilm. These lysed cells supply nutrients for the cells in the biofilm that will transform the dispersal cells. Genetic changes occur to regulate the bacteria in biofilm from sessile to motile lifestyle in the dispersal cells. While the secrete of exopolysaccharide and fimbria are downregulated, flagellar and chemotaxis genes are upregulated [47, 53]. Bacteria start to actively swim to gain an access to nutrients and new surfaces. Biofilm spreads and cells colonize on the new surfaces and form a new biofilm.

1.3. ECOLOGICAL ADVANTAGES OF MAKE A BIOFILM

A microorganism in biofilm has a number of advantages compared to its planktonic counterparts. Biofilm provides to increase expression of beneficial genes and horizontal gene transfer, enhances access to nutrients, and also it is suitable environment for mutualistic or synergistic associations between microorganisms [12, 13, 52]. Additionally, bacteria embedded in biofilm matrix is protected against wide range of environmental and host stresses. Therefore, the bacterial aggregation into biofilms demonstrate a survival strategy.

Bacteria can improve biofilms on various surfaces including living tissues, acidic, aquatic and soil environments, medical devices and many other various conditions. It also maintains a sufficient environment for complex interactions between the cells providing homoeostasis in the fluctuating and harsh condition such as extreme temperature, pH, and ultraviolet (UV) light [44]. In addition to facilitating cellular interaction and chemotactic motility, biofilm structure also concentrate nutrients [29]. Beside to provide structure for biofilm, extracellular polymeric substance (EPS) also takes a crucial part in physical prevention against metals, toxins, osmotic shock, and desiccation. For example, EPS acts as an ion exchanger, thereby

prevents the access of many antimicrobial agents into the biofilm [4, 55]. EPS restricts the diffusion of foreigner compounds into the biofilm.

The high permeable water channels surrounded the microcolonies in biofilm maintain a circulatory system to exchange nutrients and metabolites. Hereby, they enhance nutrient availability while to remove toxic metabolites [15]. Microcolonies within biofilm show different characteristic from their planktonic counterparts and the structure of biofilm ensure an excellent opportunity for metabolic cooperation and syntrophic relationships.

1.4. BIOFILM RELATED INFECTIONS

Biofilm can form on various type of surfaces including plastic, metal, wood, glass, medical materials, portable/natural water system piping, food products and living tissues. Therefore, biofilm formation causes problems for different areas. Infectious biofilms can be separated into tissue and/or surface associated (biotic/abiotic surfaces) [31]. The number of biofilm-related infections and conditions are increasing each year, thus biofilm-mediated infections are recognised as important in public health challenge. The presence of biofilms increases the pathogenicity of bacteria due to its protection against external conditions [56]. Moreover, the biofilm related infections become more problematic when biofilm colonises on medical devices and biomaterials.

Extracellular polymeric substance increases the resistance of biofilm against antimicrobial and prevents the microorganisms in biofilm from environmental hazards, and thus make the biofilm extremely difficult to eradicate from living hosts [19]. Previously, it was reported that 65 per cent of all hospital infections are biofilm originated in humans [16, 50, 57]. The presence of biofilm can be detected in different kinds of diseases, including endocarditis, cystic fibrosis, periodontitis, rhinosinusitis, middle ear infections, osteomyelitis, chronic prostatitis and native valve endocarditis [16].

Bacteria that adhere to indwelling medical devices and then form biofilm structure. Biofilms cause problems due to colonizing on indwelling medical devices such as contact lenses, orthopedic prostheses, urinary, venous and arterial catheters [57]. Bacteria embedded in biofilm shows high resistance to antibiotic, and also immune killing and clearance systems, therefore bacterial attachment on implanted medical devices contributes to the chronicity of infections [58]. By the way of shedding sloughed pieces of biofilm and individual bacteria

inside bloodstream system and surrounding tissues increase the rate of bacterial infection and may causes acute illness [37].

1.4.1. Biofilms and Pathogenesis

In the literature, chronic *Pseudomonas aeruginosa* infection in patients with cystic fibrosis was the first report about chronic infection and aggregation of bacteria [8].

Formation of biofilm is an important protective mechanism against host defenses and antimicrobial agents. Because, biofilm matrix limits to diffusion of antimicrobial agents. This frequently causes to unsuccess of antimicrobial treatment. Also, bacteria within the biofilm are better able to survive attacks against biocides, surfactants, bacteriophages, amoebae, mechanical trauma, and white blood cells. Therefore, biofilm-associated organisms take a crucial part in many infectious diseases including urinary tract infection, cystic fibrosis (severe lung infection), otitis media (acute ear infection), bacterial endocarditis, and Legionnaire's disease. Microorganisms commonly associated with biofilm on non-devices related infections are shown in Table 1.2.

Table 1.2. Microorganisms commonly associated with biofilm on non-devices related infections.

Infectious Disease	Microorganisms
Cystic Fibrosis	*P. aeruginosa, Burkholderia cepacia,*
Chronic Ear Infection (otitis media)	*S. pneumoniae, H. influenzae, M. catarrhalis, group A beta-hemolytic streptococci, Enteric bacteria, S. aureus, S. pneumoniae, M. catarrhalis, S. epidermidis, P. aeruginosa,*
Periodontitis	*P. gingivalis, S. gordonii, S. sanguis, F. nucleatum, P. micros, E. timidum, E. brachy, Lactobacillus spp., A. naeslundii, P. anaerobius, Eubacterium sp. strain D8, B. intermedius, Fusobacterium sp., S. sputigena, Eubacterium sp. strain D6, B. pneumosintes, H. aphrophilus*
Chronic Sinusitis	*S. pneumoniae, H. İnfluenza, S. aureus, S. pneumoniae, H. influenza, M. catarrhalis*
Native Valve Endocarditis	*Candida spp., Aspergillus spp., Streptococcus bovis*
Chronic Bacterial Prostatitis	*E. coli (most common isolate), Klebsiella, enterobacteria, Proteus, Serratia, P. aeruginosa, coryneforms, E. faecalis, P. aeruginosa, Bacteroides spp., Gardnerella spp., Corynebacterium spp.*
Endocarditis	*S. aureus, C. albicans, Pneumococci, Streptococcus ssp., Enterococcus ssp.,*
Osteomyelitis	*S. aureus*
Pneumonia	*methicillin-resistant S., Group B Streptococcus, S. pneumoniae, P. aeruginosa, Acinetobacter,*

Biofilms are often responsible for hospital-related (nosocomial) infections and chronic illness. Biofilms are involved in more than 65 per cent bacterial chronic inflammatory and infectious diseases. The most recent health care associated infection (HIA) progress report includes 2014 date, published in 2016 by The Centers for Disease Control (CDC). This report estimated that 75,000 patients with HIAs died during their hospitalisations each year. Annual financial losses due to HIAs are estimated about US$ 6.5 billion in the USA [59].

According to World Health Organization (WHO) report in 2015 that nosocomial infections related annually for more than 37,000 deaths in Europe, and an economic cost of up to €7 billion [60].

Although research on bacterial pathogenesis has interested in acute infections, biofilms take a crucial part in non-device related chronic bacterial infections. Because they show resistance to conventional antimicrobials as well as innate and adaptive immune systems [31]. The role of biofilm in implant-based infections is clearly identified, their act in non-device related diseases is not well understood.

1.4.2. Implant-Based Infections

Bacterial biofilm formation is detected on much different environments including water, industry, food, and human diseases, especially implanted biomaterials and indwelling devices being placed into patients. In the early 1980s, biofilm formation on medical devices was first reported via electron microscopy onto intravenous catheters [61] and cardiac pacemakers [62]. It is obviously known that the formation of biofilm on medical surfaces is the major reason for the pathogenesis of associated infections, and hereby for nosocomial infections. Adhesion of bacteria onto medical indwelling device increases the level of infections, adversely affects device functionality, and also limits its lifetime [40].

In recent years, medical devices and/or artificial organs are increasingly applied in the treatment of human diseases with the progress of medical science. Also, the increased resistance of biofilm to antimicrobial agents is an important problem during the eradication of biofilms. The rate of medical device-related infections also represent as the most common and complication in the hospitals. Consequently, formation of biofilm on medical devices allow to the characterization of a new infectious disease called chronic polymer-associated infection which they compromise quality of life and even leading to death [40]. There are several biofilm characteristics which can show importance during development of biofilm-associated infectious diseases: (i) aggregates or detachment of biofilm cells may cause bloodstream infections or emboli, (ii) increase horizontal gene transfer in biofilm, hence increase of biofilm resistance, (iii) reduce of antimicrobial susceptibility, (iv) resistance to host immune system clearance, (v) producing of endotoxin [4].

Both Gram-positive and Gram-negative microorganisms can form biofilm on medical devices, and may be composed of single or multiple species. Microorganisms gain access to the indwelling device by migration externally from the microbial sources. These microorganisms may originate from the different sources including health care workers, water, and the skin of patients [14]. Respirators, sigmoidoscopes, catheters, dentures, contact lenses, urinary prostheses, voice prostheses, orthopedic prostheses (such as artificial joints and pins), and artificial implants including, heart valves, pacemakers, ventricular assist devices, synthetic vascular grafts and biliary tract stents save millions of lives in each year all around the world, but all have a potential risk of surface-associated biofilm infections [57, 63]. Microorganisms commonly associated with biofilm on indwelling devices are shown in Table 1.3.

Table 1.3. Microorganisms commonly associated with biofilms on indwelling medical devices.

Indwelling Medical Devices	Microorganisms
Prosthetic heart valve	*S. epidermidis, S. aureus, S. Sanguis, Coagulase-negative staphylococci, Enterococcus spp., streptococci, enterococci, gram-negative coccobacilli,*
Contact lenses	*P. aeruginosa, S. Epidermidis, S. aureus, Serratia spp., E. coli, Proteus spp., and Candida spp.*
Intravascular catheters	*S. epidermidis, S. aureus*
Central venous catheter	*C. albicans, S. aureus, P. aeruginosa, Coagulase-negative staphylococci, Enterococcus spp., K. pneumoniae, S. epidermis,*
Total artificial heart	*P. aeruginosa, S. epidermidis, S. aureus*
Urinary catheters	*E. coli, P. aeruginosa, E. faecalis, P. mirabilis, Coagulase-negative staphylococci, Enterococcus spp., K. pneumoniae, P. stuartii, M. morganii, A. calcoaceticus, E. aerogenes*
Joint replacement	*S. epidermidis, S. aureus*
Endotracheal tube	*P. aeruginosa, E. coli, S. epidermidis, S. aureus*
Voice prostheses	*S. staphylococci, C. albicans, Coagulase-negative staphylococci*
Intrauterine device	*C. albicans, S. aureus, Coagulase-negative staphylococci, Enterococcus spp. , L. plantarum, S. epidermidis, Corynebacterium spp., group B streptococci, Micrococcus spp., Enterococcus spp., beta-hemolytic streptococci, E. coli*
Artificial hip prosthesis	*Coagulase-negative staphylococci, S. aureus, Enterococcus spp., P. aeruginosa*

Although infection-related data for all devices are not available, contamination of central venous catheters (CVC) is one of the most serious health care-associated infection. In 2011, Center for Disease Control and Prevention (CDC) published that 15 million CVC days occur

infections every year and approximately 80,000 CVC infections related to blood stream infections (5.3 per 1000 catheters days). Meanwhile, in the US, treatment of CVC-related blood stream infection cost is ranged $296 million to $2.3 billion [64].

1.5. RESISTANCE MECHANISMS OF BIOFILM

Formation of biofilm is crucial in the pathogenesis of bacterial infections in humans and eradication of biofilm is difficult with conventional antimicrobial molecules/agents. Because, bacteria embedded in biofilm matrix shows resistance to antibiotics, biocides and many other surrounded threats. Hereby, resistance mechanisms allow biofilm populations to survive. For instance, bacteria in biofilm can tolerate antibiotics or biocides at concentrations of 10-1000 times that needed to kill their planktonic counterparts [19, 36]. This resistant is multifactorial and may act concurrently and/or synergistically. Although the resistance of bacteria in biofilm is still a matter of speculation, there are several potential mechanisms of antimicrobial resistance of biofilm.

Firstly, antimicrobials must penetrate through the biofilm matrix in order to inactivate the cells in the biofilm. However, the presence of EPS limits to diffusion of antimicrobial agents into the biofilm. EPS acts as a diffusion barrier to slow down the infiltration during the entrance of these molecules. This barrier makes the cells embedded in biofilm tolerant to antibiotics and other drugs by the way of reducing the amount of agent into the biofilm to interact with cells [14]. Moreover, EPS also can reduce the efficiency of antimicrobial molecules by the way of reacting with them. Also, enzymes in biofilm matrix may react with antimicrobial agents and modify them to eliminate their activity [65]. For all that, development of tolerance needs time. For example, mature biofilms (3 to 5 days old) are much more resistant to antibiotics than newly formed biofilms (on the first day of formation) [66].

Secondly, cells in biofilm grow more slowly than their planktonic counterparts [18, 37, 65]. The reason behind bacteria’s slow growth rate in biofilm is not only limitation of nutrient availability but also reduced gaseous exchange [4, 18, 67]. These slow growth bacteria have been differentiated into a protected phenotypic state (metabolically inactive) and are mostly placed deep in biofilm structure. Antimicrobials mostly target quickly growing cells, therefore it is clearly estimated that growth rate plays a crucial role in antimicrobial

resistance mechanism [45, 58, 68]. Hereby, reduced metabolic activity of cells in biofilm makes these cells less susceptible to antimicrobials and they can survive the assault. Following discontinuation of antimicrobial therapy, the survived cells would have the ability to re-establish the biofilm. Moreover, it was a review that the initial treatment in killing bacteria is usually effective only at the margins of biofilm at the end of 24 h antimicrobial therapy [37].

Another possible antimicrobial resistance mechanism of biofilm can be related by the gene expression levels of cells in biofilm compared to its planktonic counterparts. Cells in a biofilm can overexpress stress-response and antimicrobial resistance genes, and switch to more resistant phenotypes during exposure to environmental stresses. For example, the growth of biofilm can increase the expression of multidrug efflux pumps as a protective factor. This pump is integrated into the cell envelope of biofilm cells and able to transport antibiotics into and out of the cell [37]. Furthermore, biofilms a suitable area for horizontal gene transfer which facilitate the spread of conventional antimicrobial resistance [36]. As it is well known, antimicrobial resistance mechanisms of planktonic and biofilm cells are different. However, when bacteria in the planktonic state show resistant to an antimicrobial agent, it is also would be resistance to the agent in the biofilm state as a result of horizontal gene transfer within biofilm [65].

In some cases, biofilms are eradicated by the host's immune systems. However, most cells in biofilm also show resistant to these defence mechanisms.

As a conclusion, standard antibiotic therapies are only able to eliminate the planktonic cells. When the therapy is discontinued, the planktonic cells within the biofilm continue to disseminate.

1.6. BIOFILM ERADICATION STRATEGIES

Cells in biofilm are problematic to eradicate by classic antimicrobial drug therapy in previous section the resistance mechanisms were explained in detail. As it was clarified, bacteria attach to a surface, create complex biofilm structure, connect with a host, and cause disease with a variety of complicated mechanisms [7]. Physiology, pathogenesis and complex mechanisms are needed to understand clearly within efficient treatment of biofilm infections. For instance, most of the studies are focusing on physiology and molecular

biology of bacteria in the planktonic state which is the basis for the discovery of currently available antibiotics. Although these antibiotics have successfully treated to acute infections, most of them have failed to cure biofilm-related infections. Because cells in biofilm develop various resistance mechanisms to antimicrobial agents compared to their planktonic counterparts. Therefore, various physical and chemical approaches to remove a biofilm have been developed by researchers in years including flushing, chlorination, heating, and ultraviolet (UV) disinfection (Figure1.2) [33].

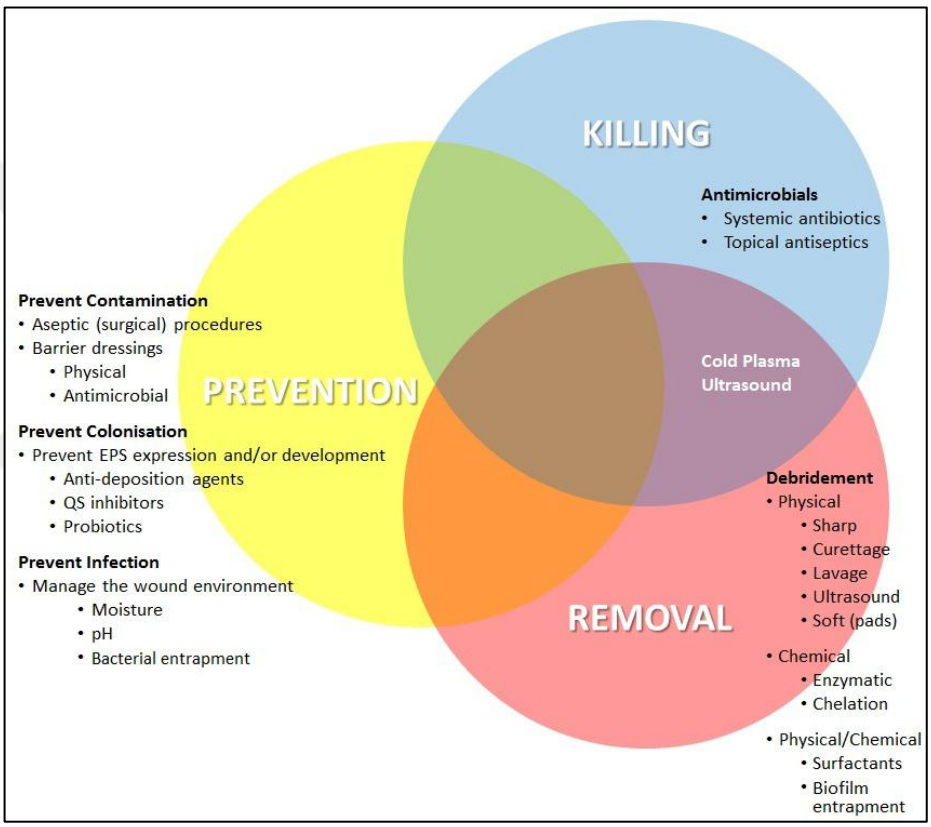

Figure 1.2. Biofilm eradication and prevention strategies [69].

The most frequently used and efficient attempt to treat a biofilm is by surgically or mechanically removing the biofilm from infection area [31]. For instance, tooth brushing is a mechanical removal strategy. However, mechanical removal is only effective on accessible surfaces. Hence, biofilm infections on foreign bodies including implants or artificial joints often need surgical removing and the replacement of the foreign material [31].

Antibiotics only can be used during reduce the biofilm-related infections. However, they cannot be effective to eradicate the biofilm infections because once biofilm matured, it becomes difficult to eradicate via the minimal concentration of antibiotics [31]. Therefore, to the use of antibiotics needs further combinational strategies to eradicate biofilms. For instance, antibiotics may be an alternative for the cure of biofilm infections apart from surgical operation. Moreover, aminoglycosides, fluoroquinolones and β-lactams are used to kill only bacteria which growing part of the biofilm (outer layer). They do not show a function inner layer of biofilm because of the hypoxic conditions [69]. Therefore, antibiotics combinations that are effective at low oxygen concentrations are used to kill the inner part of the biofilm.

There are also several physical approaches for biofilm eradication such as the use of an electromagnetic fields, electrical current, radiofrequency electrical current, and ultrasound, in combination with antimicrobial therapy [65]. However, these strategies are still in preclinical stages under progress.

Consequently, to the absence of effectiveness of used strategies on the eradication of biofilm, novel safety and powerful treatment strategies need to develop to extend scientific knowledge about the biofilm formation and resistance mechanisms for utilize new and effective treatment strategies.

1.7. BIOFILM PREVENTION STRATEGIES

Biofilm formation is a complex mechanism and plays an important role in infection immunity. Structure, composition and other characteristics of biofilm provide complicated survival mechanisms and cause to the failure of eradication strategies. As it was clearly explained in previous sections, cells in biofilm develop various survival mechanisms compared to its planktonic counterparts and they have increased the extent of virulence and pathogenicity. The formation of biofilm also provides protection from external factors such as host defence, and antimicrobial agents. Therefore, to control the biofilm formation and development becomes obligatory. So, prevention of biofilm formation while they are still planktonic could be an optimal strategy. Because bacteria in the planktonic state will be more susceptible to antimicrobial agents, hereby treatment will be more efficient. However, the cells in the planktonic state (the initial stage) creates very little inflammation on the host,

hence it is very difficult to detect the initial bacteria. On the other hand, when the inflammation is detected, the biofilm structure is already formed. Consequently, new strategies for the prevention of biofilm formation are required. These strategies such as coating the implants and catheters with antibacterial products, use of expansive spectrum antibiotics and newer combinations of drugs are only appropriate and effective for sessile bacteria.

The design of alternative/effective surfaces is an emerging strategy to prevent biofilm formation for many different areas such as medical, food, and marine industries. Researchers have focused on to create new surfaces using innovative techniques such as surface materials, surface modifications, new coating, and paint. These newly developed surfaces could immediately kill bacteria upon contact, and/or reduce the initial attachment of bacteria to the surface (Figure 1.3) [40].

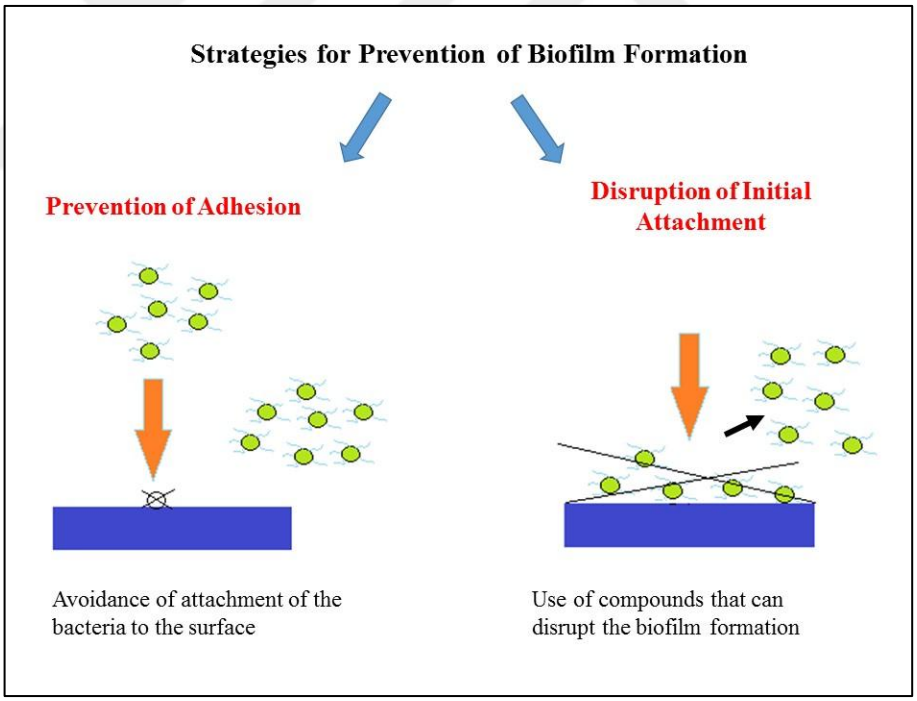

Figure 1.3. Strategies for prevention of biofilm formation on surfaces.

In recent years, researchers have developed different materials to actively or passively preventing adhesion of bacteria and biofilm formation. Cationic polymers, antimicrobial peptides [70], antibiotics [71], silver ions, and nitric oxide can be called as “bacteria-killing” materials. On the other hand, polyethylene glycol (PEG) [72], zwitterionic polymers [73],

and their derivatives also shows successful effect on prevention strategy as "bacteria-resistive" materials. Antimicrobial peptides (AMPs) are natural polymers, part of the innate immune system, called as host defense peptides. These peptides show actively antibiotic effect against a broad range of Gram-negative and Gram-positive bacteria, fungi and viruses via disrupting cellular membranes and the amphipathic structural arrangement of the peptides [70]. Cationic polymers are positively charged and kills the bacteria through various pathways via interacts with negatively charged bacterial cell membrane [40]. Enzymes are also used for prevention via degrading to forming or already formed biofilm matrix components.

These approaches are mostly used on implant and catheter surfaces as a prevention strategy against biofilm infections. As an example, chlorhexidine-silver sulphaziadine coated catheter surfaces use efficiently for the short-term prevention of biofilm formation [64]. There are also many antibiotic integrated orthopedic devices, however they are not routinely used. The disadvantage of antibiotic coating of surface is that release of antibiotics from an implant will end eventually [71].

Modification of surface characteristics is another potential strategy which could be an effective way to reduce adhesion of bacteria on surface. On this method, surface characteristics could be changed such as hydrophobicity, surface free energy, and roughness of surface [14, 40].

Consequently, prevention of biofilm formation is important for in vivo indwelling device-associated infections, therefore various surface materials have been developed to reduce or even suppress biofilm formation. However, alternative strategies still need to be developed.

1.8. *Pseudomonas aeruginosa* as a MODEL SYSTEM FOR BIOFILM DEVELOPMENT

Pseudomonas aeruginosa is a gram-negative, motile, rod shaped pathogenic bacteria that belongs to the family *Pseudomonadaceae* [24]. *P. aeruginosa* is an aerobic and non-fermentative microorganism that is able to grow without oxygen in case of nitrates, nitrites or arginine are available in the environment as alternative electron acceptors [31].

Pseudomonas aeruginosa is the widespread cause of nosocomial infections such as pneumonia, cystic fibrosis, chronic bacterial prostatitis, bacterial keratitis, otitis media and urinary tract infections [8, 45]. These pathogen can also adhere and form a biofilm on various surfaces/environment in nature including water, soil, plants and animals. They can involve any part of body, therefore become an important risk in patients with compromised host defence mechanisms. For instance, *Pseudomonas aeruginosa* forms a biofilm on the lung which is a major factor for development of cystic fibrosis (CF) [8, 50].

Biofilm formation on indwelling medical devices is a highly risk factor for the development of nosocomial infections. *P. aeruginosa* was referred as the second most commonest reason of ventilator-associated pneumonia, the seventh frequent cause of catheter-associated bloodstream infection and the sixth most continuously occurring pathogen [74]. In 2013, the National Healthcare Safety reported that 51,000 healthcare-related *P. aeruginosa* infections occur in the US each year which is roughly 8 per cent of all healthcare-associated infections. The same report informed that these infections cause approximately 400 deaths per year in the US [75].

Pseudomonas aeruginosa is the most common model organism on the in vitro biofilm research. These studies showed that this pathogenic bacteria has ability attach to any available surfaces and/or to each other, and also create biofilms under almost any environment that suitable for growth [31, 47, 76, 77]. The characteristic feature of biofilms like the adaptive and genetic changes improves their resistance against antimicrobial agents which makes the Pseudomonal infections more complicated and life-threatening. For example, multidrug resistant *P. aeruginosa* causes more than 6,000 of healthcare-associated infections (13 per cent in all) only in the US every year [75].

1.9. THE FOURTH STATE OF MATTER: PLASMA

"Plasma" is defined as the non-cellular fluid component of blood in medicine and biology, whereas it ascribes to the fourth state of matter in physical sciences [78]. British physicist Sir William Crookes was first discovered and identified the fourth state of matter in 1879 [79]. Afterwards, in 1929, the name "plasma" was first introduced into physics by an American chemist Irving Langmuir as a result of the characteristics similarities between an ionized gas and blood plasma [80]. Langmuir mentioned [80]:

“Except near the electrodes, where there are sheaths containing very few electrons, the ionized gas contains ions and electrons in about equal numbers so that the resultant space charge is very small. We shall use the name plasma to describe this region containing balanced charges of ions and electrons.”

The material is presented as four states of matter in everyday life: solid, liquid, gas or plasma. When a power source (thermal, electrical or light) is applied to a material, the state of the material is changed. For instance, the solid state of material becomes liquid, and the liquid state of material transforms into a gas. When more energy is applied to gas phase, the gas phase of material ionizes (the electrons separate from the atoms or molecules of gas) which is called the state of plasma (Figure 1.4) [81].

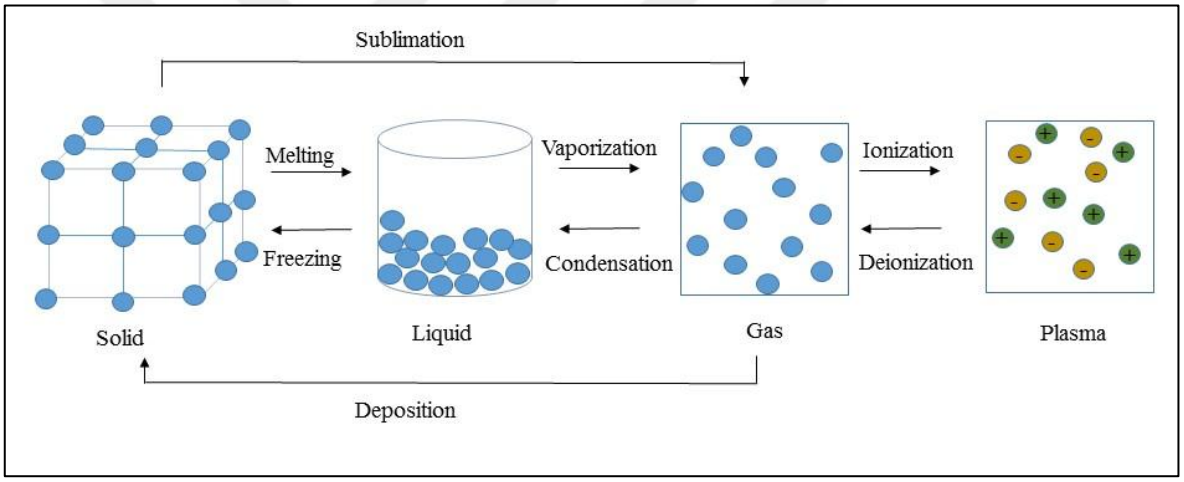

Figure 1.4. Different states of matter.

On the state of plasma, radicals are created by collisions between electrons and molecules, and bond breaks in molecules [82]. Moreover, excited species generates photons. Therefore, plasma is described as ionized gas containing a mixture of free electrons, neutral atoms, molecules, ions, and UV photons [81, 83]. Plasmas are considered to be macroscopically neutral because of the presence of equal amount of charge particles (positive and negative ions), however it is electrically conductive [82].

Plasma can be divided into two categories upon the relative temperatures of the electrons, ions, and neutrals; thermal plasmas (hot/ near-equilibrium plasma) and non-thermal plasmas (cold/ non-equilibrium plasma) (Table 1.4.) [78, 84, 85]. Thermal plasmas are constituted of

very high temperatures of electrons and charged and neutral heavy particles which are close to the greatest degree of ionization. During the plasma operation, entire gas is heated by thermal plasmas. In those plasmas, the neutral species in the gas, ions and electrons are seen in same the temperatures (Table 1.4.) [86]. Non-thermal plasmas consist of relatively high-temperature electrons and low-temperature particles (including charged/neutral molecular and atomic species) which they have a nominal degree of ionization [82, 86]. Non- thermal plasmas are generated at room temperature or a little above. The ions and the neutrals remain relatively cold, therefore do not cause any thermal damage [85].

Table 1.4. Classification of plasma based on temperature [85].

Thermal Plasmas	**Non-Thermal Plasmas**
$T_i \approx T_e > 10^7$ K	$T_g \approx T_i \approx 300$ K; $T_i << T_e \lesssim 10^5$ K

Ti: Temperature of ions, Te: Temperature of electrons, Tg: Temperature of gas molecules.

The electrons present slight mass in the plasma and they have an important role as energy vehicles of plasma [81]. Therefore, the electrical field accelerates to higher velocities to the electrons (than the heavier ions) between the collisions during the non-thermal plasma. Accordingly, the temperature of electrons is higher than the heavy particles in the non-thermal plasmas. Also, the temperature of the gas is detected much lower (around room temperature, <40°C) than an electron temperature [87]. On the other hand, while the pressure is very high ($\geq 10^5$ Pa) or the electric field is really low, the charge particles do not have high velocities; therefore do not move far before the next collision. As a result of slow motion, the energy of electrons may tend towards the heavy particles and they are characterised by a local thermodynamic equilibrium in thermal plasma [82, 88].

Plasma is the most common condition of matter in the universe (more than 99 per cent) [89] and in our planet including the Sun; interplanetary, interstellar, and intergalactic media; in the tails of comets; Earth's ionosphere; the aurora borealis; flashes of lightning [86]. Electromagnetic radiations, thermal, laser, electric current, etc. are different forms of energy which are used during plasma generations. According to the energy type and the amount of

energy, the electron density and temperature are changed (Figure 1.5) [90]. On the other hand, the artificial plasma can be created in a laboratory or industry by the electrical excitation of a gas. During continuous plasma discharge generation, electric current passes though the gas in the plasma system. Different plasma discharges like atmospheric and low-pressure glow, dielectric barrier, magnetron and corona are generated by various frequencies of power sources including alternating current (AC), direct current (DC), low frequency, microwave, radio frequency (RF), etc. [91].

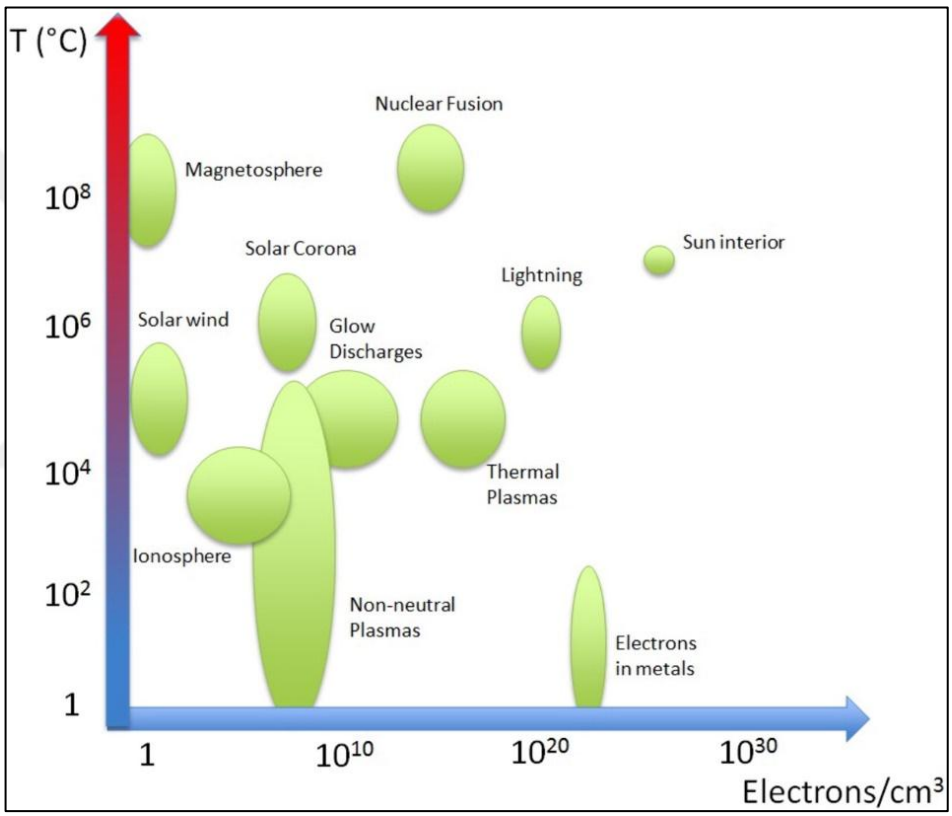

Figure 1.5. Plot of the temperatures and densities of electrons in natural and artificial plasmas [83].

There are some important parameters during plasma generation including a power source, system pressure, gas, presence and flow rate of feed gas, etc. Plasma systems can also be classified or named according to the system pressure. For instance, non-thermal plasmas can be divided into atmospheric pressure and low-pressure cold plasma [90].

In recent years, plasma technologies have an increasing attention because of its increased number of applications in many different areas such as research, industry and technological

domains. For instance, thermal plasma is used in medicine during electrosurgery and coagulation of blood for a long time [90]. However, non-thermal plasmas have an increasing impact due to their important characteristic features such as more reliable, flexible, low cost, a broad range of application area, the absence of residues, and continuous process. Moreover, it is an alternative method for heat sensitive surfaces because non-thermal plasmas do not induce thermal damage to the surfaces (living/non-living)during the plasma application [86, 92]. Non-thermal plasma system is an alternative sterilisation tool compared to conventional methods especially for heat sensitive medical devices [78, 88, 93–96].

1.10. NON-THERMAL ATMOSPHERIC PRESSURE PLASMA

Non-thermal plasmas can be produced at different pressures ranging from low to atmospheric pressure by applying a suitable electrical field (generally in kHz, MHz or MW). Atmospheric-pressure plasmas (APPs) can be operated by a portable power source with a low input power and do not need a complex heavy vacuum system. These are significant advantages for APP compared to low-pressure plasmas [97]. These are also environmental friendly, easy to apply and clean systems with a relatively low cost when compared to other systems. Moreover, APPs are generally hand-held systems which make them useful for clinical applications. APP can be generated by applying a wide range of frequencies including AC, DC, RF, low frequency and microwave [97].

APP system generally contains dielectric barrier discharges (DBD) (Figure 1.6.) and atmospheric-pressure plasma jets (APPJ) (Figure 1.7.) [97, 98]. APPJ has a growing interest in recent years owing to generate non-thermal plasma in an open space area [99].

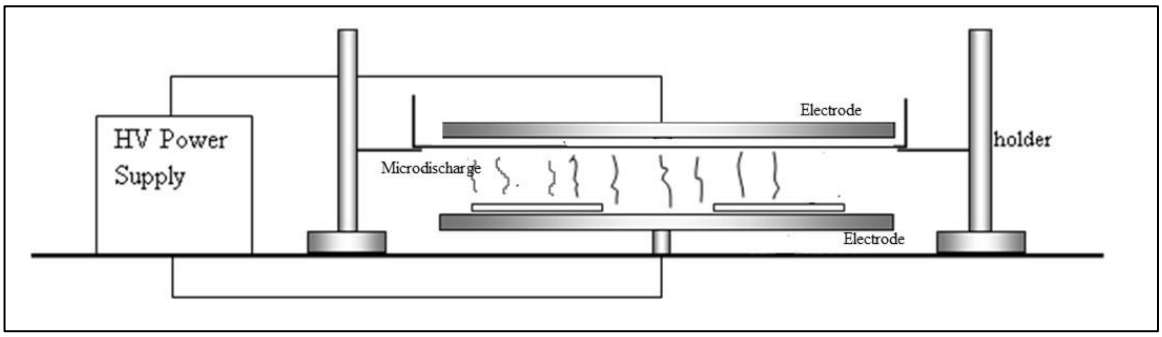

Figure 1.6. Schematic view of dielectric barrier discharge.

Non-thermal APPs can be classified as direct treatment and indirect plasma treatment [100]. As it was previously described in detail plasma is ignited between two electrodes. Therefore, the plasma applied sample assumes one of the electrode function in direct plasma application while the other electrode is the plasma device electrode [78]. Dielectric barrier discharge (DBD) can be given as an example of the direct plasma system. However, in indirect plasma applications, while the gas flows through a cylinder tube, plasma is generated inside the tube between the electrodes as a result of the voltage gradient. Afterwards, the high flow rate of gas transports the discharges through the target [101, 102]. The most common indirect plasma devices are plasma jet, plasma pen, plasma needle etc.

Non-thermal APP is used in many different area including dermatology and surgery to treat bacterial infections, chronic wound healing, and skin infections [100]. The most important characteristic feature is that non-thermal plasmas do not cause a thermal damage to the surrounding tissue which improves its potential use for biomedical applications [78]. Also, non-thermal APPs are more frequently used for surface modifications [99, 103].

1.10.1. Non-Thermal Atmospheric Pressure Plasma Jet

Atmospheric pressure plasma jet system is created in a nozzle and contains two electrodes in different arrangements [93]. APPJ consists of a capillary dielectric tube with a ground ring electrode wrapped around the tube and a metal needle electrode connected to the high voltage power supply (Figure 1.7). The gas flows through the tube and plasma is produced due to the voltage gradient between the electrodes. The high flow rate of gas pushes the discharge out through the nozzle in the form of a jet into the room air.

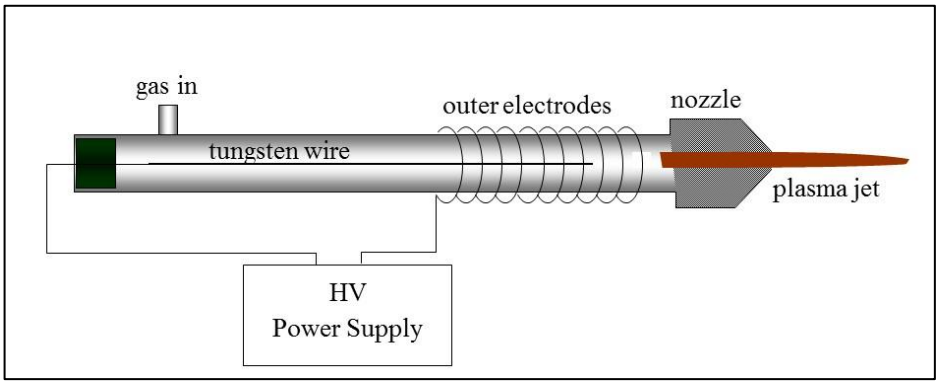

Figure 1.7. Schematic view of atmospheric pressure plasma jet system.

Although the working principle is same, many APPJ systems are available with different properties such as size, working gas, design, frequency, and applied voltage [92, 104–106]. Moreover, APPJ systems have many advantages due to their small sizes and practical uses for small and large scale areas.

APPJ firstly used for bacterial sterilization and then it have gained a growing interest [78]. Moreover, in recent years, APPJ systems have been widely used on biomedical (microbial sterilization, skin and cancer treatments etc.) and surface modification applications [92, 103, 107–109].

1.11. PLASMA CHEMİSTRY

Plasma is an ionised gas containing a mixture of free electrons, free radicals, neutral or excited atoms and molecules, negative and positive ions, and UV photons. The composition and abundance of active species show differences due to the used gas and method during the plasma generation. During plasma generation mostly used gases are synthetic or natural air, oxygen (O_2), nitrogen (N_2), hydrogen (H_2), argon(Ar) or a mixture of these gases. For instance, non-thermal ambient air plasmas are important sources of reactive oxygen species (ROS) and reactive nitrogen species (RNS) species such as ozone (O_3), superoxide (O_2), and hydroxyl radicals ($^{\cdot}OH$) and nitric oxide (NO) and nitrogen dioxide (NO_2) (Table 1.5.) [110, 111].

These reactive oxygen and nitrogen species are used effectively for microbial sterilisation [112]. Moreover, they interact with biomaterials which can be an important tool for surface modification [92, 113, 114].

Table 1.5. Some of possible chemical reactions in the atmospheric plasma discharges.

$e + O_2$	→	$2O + e$
$O + O_2$	→	O_3
$O_3 + H_2O$	→	$O_2 + H_2O_2$
H_2O_2	→	$2\ OH\cdot$
$e + H_2O$	→	$H\cdot + OH\cdot + e$
$e + N_2$	→	$N\cdot + N\cdot + e$
$e + N_2$	→	$N_2\cdot + e$
$e + NO$	→	$N\cdot + O\cdot + e$
$2H_2O$	→	$H_2O_2 + H_2$

1.12. APPLICATIONS OF NON-THERMAL ATMOSPHERIC PRESSURE PLASMA TECHNOLOGY

Non-thermal plasma technology is widely used for many years in many different fields including in gas discharge lamps, removal of gaseous pollutants, chemistry or surface modification [93]. However, at the beginning of 90's, non-thermal gas plasmas were started to use for bio-decontamination [115]. Afterwards, non-thermal plasma technology had significant potential as an alternative method especially for the sterilisation of heat sensitive materials. The use of this novel technology has expanded to new areas including metallurgy, ready-to-eat food industry, microbial inactivation, biofilm degradation, biomedical applications, microsurgery, wound healing, cancer therapy, dermatology, dentistry, automotive industry, electronic devices industry, microelectronics, polymer engineering etc. [109, 110, 116–118]. Moreover, non-thermal atmospheric pressure plasma systems are already available and use for different areas in industry as well.

Non-thermal atmospheric plasma is a widely used system in many technological areas due to its characteristic features and advantages compared to other conventional methods

including low cost, clean, safe, easy to perform, reproducible, no toxic residuals, non-pollutant, etc. [119, 120].

In general, plasma applications in medicine can be subdivided into three main fields: microbial sterilisations, therapeutic applications, and surface modifications. Microbial sterilisations and therapeutic applications are referred as direct plasma applications whereas surface modifications are indirect. Because, plasma technology is applied directly on or in a human/animal/live sources in direct applications, while the plasma is used to treat on the surface for subsequent applications in indirect plasma medicine applications [121].

In recent years, researchers are interested in the effect mechanism of plasma on living cells as a result of plasma technology's wide usage in various fields of medicine.

1.12.1. Plasma Sterilization and Mechanisms of Microbial Inactivation

Bacterial contamination is a serious problem in many different areas. For instance, contaminated medical devices have a high potential risk for health care-associated infections [60, 122]. Especially, sterilisation of medical devices is a crucial step in prevention of hospitalised infections. There are different regimens to eradicate these contaminations such as antibiotic therapies, and conventional sterilisation methods (including dry heat/oven, moist heat/autoclave, ultraviolet (UV), chemicals, etc.) [68, 115]. The features of an ideal sterilization method should be: (i) short sterilization time (approximately 60 min); (ii) low-temperature processing (less or equal to 55 °C), (iii) harmless operation for both operator and patient, (iv) suitable for different materials, (v) do not change the bulk properties [94]. However, treatment of infections is still challenging as a result of increasing microbial resistance [58, 75]. Also, conventional sterilization methods have some drawbacks. As an example, the high temperatures (heat/oven/ autoclave) cannot be used during sterilization of heat sensitive materials. Moreover, sterilization with chemicals needed long times and also can be toxic [94]. Therefore, in recent years, non-thermal atmospheric pressure plasma technology is frequently used as an alternative sterilization method as a result of these limitations.

Non-thermal APP sterilisation has many advantages compared to other conventional sterilisation methods including under low temperature, with non-toxic gases, leaving no residue, short time application, easy to apply, less expensive, safe, and environmental

friendly [94, 110, 123]. Moreover, plasma technologies do not only an effective strategy to kill bacteria and viruses, but also it removes the residues (such as dead microorganisms) from the sterilised surface [123].

Plasma is an ionized gas mixture containing free electrons, neutral atoms, exited molecules, ions, and UV photons. All these active species have an important role during microbial sterilization [124, 125]. ROS and RNS cause to cell death via interact and with cellular macromolecules such as lipids, proteins, and DNA [126]. Although there are some theories about the mode of action of APP, the exact mechanisms are still unclear. It has been reviewed [125, 126] that bacterial killing consist via (Figure 1.8):

(i) permeabilization of the cell membrane/wall (due to leakage of cellular components); The reactive species are getting together (especially in the case of indirect contact with the microorganisms) on the cell membrane/wall and the electrostatic force overcomes the cellular tensile force which causes physical cell disruption [126, 127]. Moreover, ROS and/or RNS interact with cell membrane lipids and cause to the lipid peroxidation. Lipid peroxidation creates the shorter unsaturated fatty acids which damage to the structural integrity of the membrane and cause the cell death.

(ii) irreversible damage to the intracellular critical proteins of cells; When reactive species interact with cellular proteins, functional features of proteins are affected such as peptide fragmentation, modification of amino acids side chains, protein-protein crosslinking, etc..

(iii) direct chemical DNA damage (irreversible); Plasma reactive species interact with bases and sugar moieties and cause to the damage on DNA.

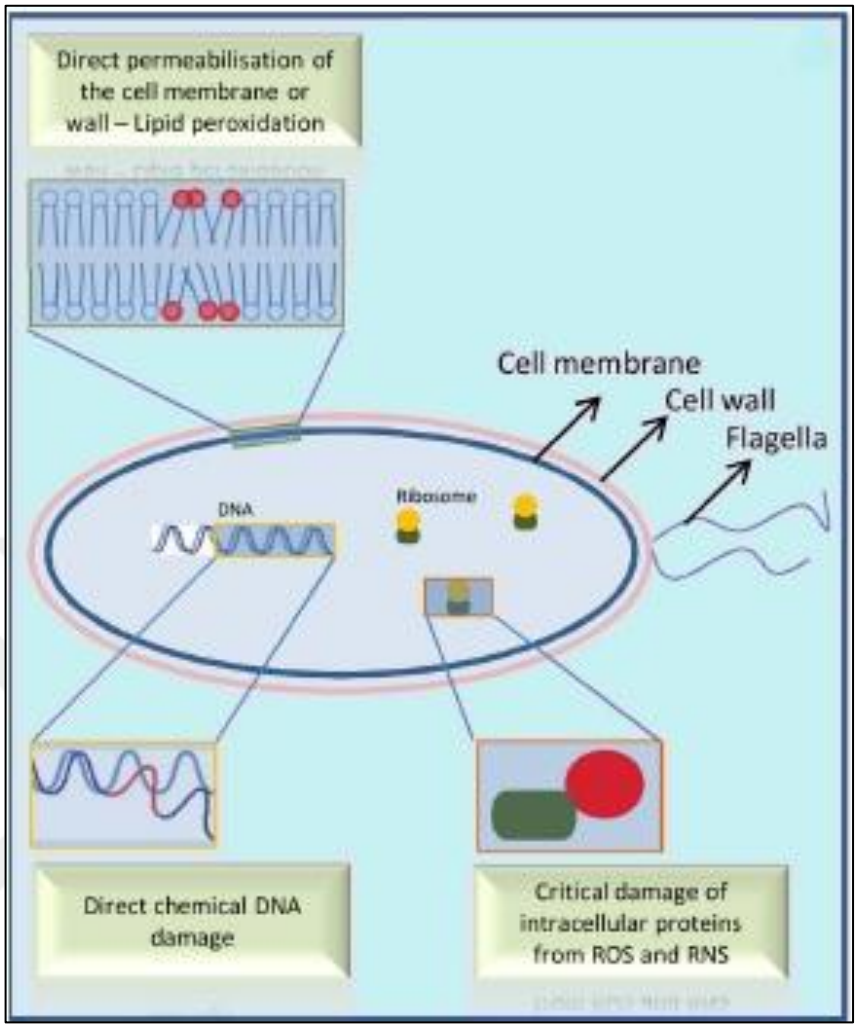

Figure 1.8. Effect of plasma treatment on bacterial cells [132].

A number of reports indicate highly promising results for APP sterilization of both Gram-positive and Gram- negative bacteria strains and also removal of biofilms of pathogenic bacteria and yeast including *Candida albicans* [128], *Pseudomonas aeruginosa* [129], *Porphyromonas gingivalis* [130], *Lactobacillus casei* [107], *Streptococcus mutans*, *Escherichia coli* [107], etc. Eradication of biofilm using APP requires longer treatment times than killing of planktonic bacteria because of the protection mechanisms of biofilms (e.g. biofilm matrix) [131]. And also, biofilm eradication mechanism by APP shows some differences. In mature biofilm, reactive species leads to cell death by localizing in the center of microcolonies [132].

The efficacy of APP is affected by process parameters (including gas composition, exposure time, distance, etc.) and external factors (e.g. type of bacteria, moisture content in the system,

etc.) during microbial sterilisation. For example, different dosage strength and application time of APP can be used to promote healing of mammalian cells [133].

While APP is widely used and more effective novel system for sterilization, it has also some challenges and risks including [134, 135]:

(i) a slow rate of kill to microorganisms,
(ii) large scale-up systems is not always possible with plasma (frequently small scale up systems are used),
(iii) sensitive materials to plasma active species in terms of surface physical and chemical alterations,
(iv) negative effect of UV emissions on the applied material (especially for products that contain proteins)
(v) availability of plasma resistant microorganisms (Gram- positive microorganisms show more resistance to plasma sterilization than Gram-negative ones as a result of the presence of thicker outer membrane (murein layer)).
(vi) use of different plasma systems and diversity of used gases cause challenging to reproducibility and transferability.

1.12.2. Therapeutic Applications

Besides damaging effect in biological systems, reactive species of plasmas have also therapeutic effect. The important characteristics of AAPs have led to their extensive use in various biomedical applications. Therefore, in the early 2000s, non-thermal APP technology has expanded to include work on blood coagulation [136], treatment of mammalian and cancer cells (by inducing apoptosis) [78], wound healing [78], and dentistry [123].

The exact mechanisms of plasma on biological systems are not clearly determined due to the complexity of living systems and plasma. Previous studies showed that reactive species of plasmas play an important role in wound-healing effects and treatment of mammalian and cancer cells [124]. There is a careful balance of reactive oxygen and nitrogen species in living biological systems [124, 131]. APP treatments influence this balance via plasma generated reactive species at the treated site. Reactive species are involved in many different cellular cascades in biological systems [111, 124, 125]. However, these ROS and RNS can cause unwanted damaging against healthy cells. Therefore, to achieve the optimum treatment

effect, APP treatment needs to be applied to the right place at the right time and especially on right concentration [131] (Figure 1.9).

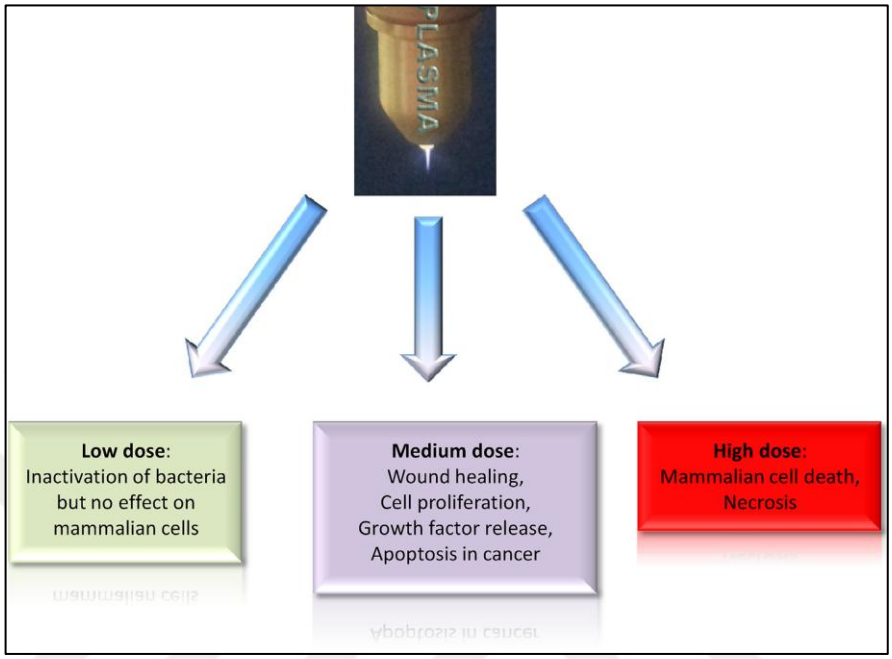

Figure 1.9. Dose-dependent use of atmospheric pressure plasma [133].

AAP application could induce to cell detachment on a surface based on the applied voltage and the treatment time. For example, when the intensity of plasmas is low, the cells stay alive and reattached to the surface, moreover they continue to proliferate after short time incubation [109]. However, high doses (due to increase of exposure time) induce cell apoptosis or necrosis [109] (Figure 1.9.). Moreover, plasma treatment also induces changes in cellular expression of genes. Xu et al. [109] showed that plasma application was up-regulated to the expression of cell differentiation markers of myeloma cells while down-regulated metastasis genes expressions.

APP systems are also used in dentistry due important characteristics features including easy access into the constricted spaces (e.g. dental cavity), high sterilization effect, painless, less destructive, the heating of the pulp is bearable, non-toxic, and do not damage to the mineralized matrix of the tooth [90, 107, 137].

APP has also treatment effect in the chronic skin and wound infections owing to sterilization of bacteria. However, the exact mechanisms of treatment is not clear and long-term side

effects of APP cannot be determined [133]. And also, Lademann et al. [138] show that APP changes the skin barrier properties and drugs are able to pass easily through the skin barrier during the treatment of dermatological diseases.

Consequently, non-thermal APP system is efficient, safe to touch, and fast, therefore it can be used on sensitive materials both living and non-living. However, clearly described interactions between plasma and living cells are needed to research in order to increase the use of plasma systems for many different therapeutic strategies.

1.12.3. Surface Modification and Surface Interactions

Biomaterials and implantable devices have an extensive use in medicine including contact lenses, catheters, artificial heart valves, kidney dialyzers, indwelling devices, and implants. Most of these implantable devices were not originally produced for medical applications, therefore, they have a crucial role in the management of diseases and complications such as infections, inflammations, degradation of the material, and poor biocompatibility [139]. Hence, development of these products is widely interested in the last two decades due to the advancement of health care.

The progress of these materials takes long time and extensive clinical trials. The interaction between the indwelling medical devices/biomaterials and host tissues are determined by advanced surface characterization techniques. Biomaterials and implantable devices need to have several important characteristics including high biocompatibility, low cost, and desired integration with tissues, etc. [140]. Plenty of methods have been developed to improve clinical performance to characterize the surface properties of these materials [90, 141]. Consequently, plasma technology used and developed as a novel technique for surface modification of materials with desirable physical, chemical, and biological characteristics [139, 142–144].

Plasma is an alternative and frequently used surface modification method with significant features including low cost, environmental friendly, non-toxic, solvent free, safe, fast, dry, flexible, and time-efficient [145]. While plasma technology conserve the bulk properties of the surfaces (such as strength, hardness, and inertness), it also gives the opportunity to modify of the surface properties in a controlled manner by choosing the appropriate conditions, treatment time, and suitable gases, etc. [141, 146, 147]. Although plasma

technology does not affect the mechanical and physicochemical properties of surfaces, it facilitates to uniformly change on surfaces. Therefore, it can be used easily various and complex surfaces (such as 3D structures) [141].

Non-thermal plasma applications change to surface properties via activation and functionalization which advance to biocompatibility and functionality of a wide list of materials (e.g. plastics, metals, glasses or polymers) [90, 92]. Most of the unique surface properties cannot be obtained by other conventional methods. The reactive species (ionised and excited atoms/molecules) of plasma does not only modify the surface characteristics of materials but also cause to change in surface energetics [148]. Besides the plasma application alters the surface properties to be highly hydrophilic, and also could establish a hydrophobic stage on the surface. All these modifications based on the gases used during APPJ system [149]. For instance, Kim et al. published that non-thermal APPJ application to stainless steel surface caused to change in surface properties(hydrophobic to hydrophilic, and also increased to surface energies) as a result of reactive etching and oxidation of ions on the surface [92].

Interactions between the plasma applied surface and the excited species of plasma specify difference of the physical and the chemical modifications [139]. Thus, non-thermal plasma technology are used for a number of surface reactions on biomaterials according to various processing conditions (power, gas, etc.). Figure 1.10 shows the different possible surface reactions via non-thermal APP applications.

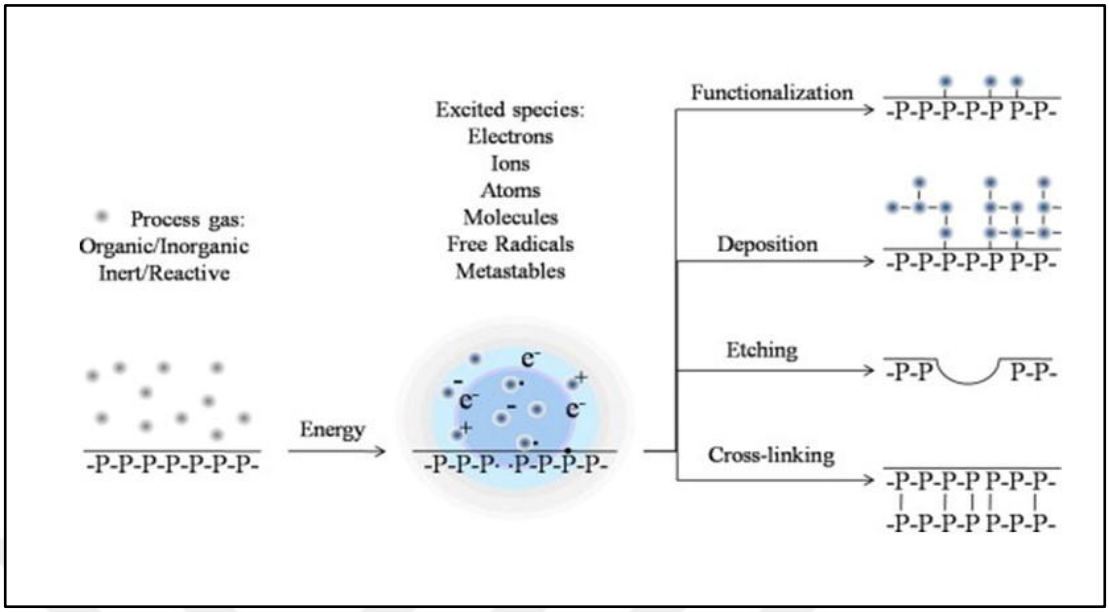

Figure 1.10. Surface modifications exposed by plasma process.

The surface modification of biomaterials is resulted in film deposition, substitution, or ablation by plasma technology. During plasma treatment most frequently used gases are air, nitrogen (N_2), argon (Ar), oxygen (O_2), nitrous oxide (NO), helium (He), carbon dioxide (CO_2), ammonia (NH_3), methane (CH_4), etc. [98, 139, 150]. These gas plasmas can cause to the chemical functionalities, these functionalities show differences depending on the process gas (such as nitration, oxidation, or amination) and surface chemistry of biomaterial (such as hydrophilicity/hydrophobicity). Moreover, free radicals of plasma are also formed on the plasma applied surface as a result of ion bombardment by energetic particles and high energy UV radiation [139]. These free radicals can cause to surface ablation, activation or cross-linking. Ablation (etching) is a process which volatile species and monomers are desorbed. Surface activation occurs when recombination of surface radicals with atoms or chemical groups produced by plasma. Cross-linking is a process that combines two individual polymers with radicals to form a bond (intermolecular bridge) [119, 139]. Plasma polymerization is also mostly used surface modification technique. In this typical synthesis, a monomer is fragmented into reactive species of plasma, and then recombine and be deposited onto the surface [119, 139]. Consequently, all these different surface modifications are relying on a reactive gas used for plasma surface modification treatment. Because different reactive species in plasma produce different free radicals which cause to different surface characteristics.

Due to its listed advantages, non-thermal APP technology is more often used in many different areas: electronic devices, textile, automotive industry, etc. In recent years, plasma treated surfaces are widely researched to prevent bacterial adhesion [151–154]. Because surface properties of materials have an effect on attachment of bacteria to the surface. For instance, surface roughness, surface energy and hydrophobicity, and chemical composition are important characteristics of the surface for bacterial attachment [142, 151, 154–156]. As previously explained in detail, non-thermal APP technology allows designing surface properties according to needed without changing its bulk properties. For instance, O_2 plasma treatment of patterned polydimethylsiloxane film has been demonstrated to reduce bacterial attachment compared to non-plasma treated materials, resulting in *E. coli*, *S. aureus* and *P. aeruginosa* adhesion to O_2 plasma treatment polydimethylsiloxane film [157].

2. AIM OF THE STUDY

This study aims to evaluate plasma activated medical surfaces' antimicrobial and antibiofilm properties. Novel Non-Thermal Atmospheric Pressure Plasma Jet (APPJ) system was used for activation of medical surfaces. Moreover, the possible effects of these plasma applications on medical surfaces were determined.

3. MATERIALS AND METHODS

3.1. SURFACE PREPARATION

The stainless steel grade AISI 316L (SS) were bought from Tan Celik Company (Istanbul, Turkey) and titanium (grade 5, also known as Ti-6Al-4V because of the addition of aluminum and vanadium alloying elements) (Ti) were kindly presented by Timet Company. The sizes of the surfaces were 4 mm by 7 mm by 0.6 mm (for batch assays). Surfaces were sterilised by autoclaving for 15 min at 121 °C.

3.2. NON-THERMAL ATMOSPHERIC PRESSURE PLASMA JET SYSTEM

Non-thermal atmospheric pressure plasma jet (APPJ) systems consist of two coaxial electrodes separated by a quartz tube through which a feed gas is introduced at a flow rate of 1-50 litres per minute. The electrodes are applied an AC high voltage (typically 12-20kV) to ignite a uniform active plasma region in the tube. This plasma is pushed through the nozzle tip of the torch and expands outside from 1 to 5 cm in length depending on power and gas pressure. The excited atoms and molecules as well as free radicals in the plasma exit the nozzle at high velocity and strike on the surface being activated or sterilised. The high gas flow causes cooling so that the surface temperatures can be kept below 70-80°C.

In this study, these novel non-thermal APPJ systems were built by Professor Necdet Aslan, Yeditepe University. The non-thermal APPJ (Figure 1.7) includes an inner tungsten electrode of 1 mm in diameter inserted through a quartz tube and Cr-Ni wire of 0.5 mm was wound around it. The tip of the tube included a specially designed nozzle to allow jet flow. The electrodes are applied a 15 kHz, 12kV AC voltage and gas (separately nitrogen, argon, and oxygen) were introduced into the volume at a flow rate of 7 liters per minute.

3.2.1. Plasma Application

Surfaces of stainless steel L316 and titanium (grade 5) (Ti 6 Al-4V) were treated by non-thermal APPJ system. The electrodes were applied a ~15 kHz, 12 kV AC voltage. The

surfaces were attached on a holder and placed nearly 1 cm against the impinging plasma jet and only one side was activated by plasma. The nitrogen, oxygen, and argon were individually introduced into the volume at a flow rate of 7 liters per minute. The times of exposure to the plasma were 15 min for surfaces.

3.3. OPTICAL EMISSION SPECTROMETRY (OES)

Optical emission spectrometry of non-thermal APPJ system during the application was performed to detect the mechanism behind the plasma effect during different plasma conditions. It is an important tool for plasma diagnostic during the measurement of excited species in the plasma. The light emission intensities of the APPJ system were characterized using a TCD-1304 Toshiba CCD sensor and a UV–visible emission spectrometer (Baki, Istanbul, Turkey), as previously described [158]. The slit resolution was 600 lines per mm and optical resolution of the spectrometer was 1.6 nm. For each spectroscopic data, the integration time of the data collection was chosen to be 10 ms. The spectroscopic data were taken by a light falling on the surface of an optical fibre, where the tip of this fibre was kept at 0.7 cm above the system. To identify the major excited reactive species during plasma application by nitrogen, oxygen and argon gases, OES was employed in the 200–900 nm range. The peaks were identified using the National Institute of Standards and Technology (NIST) atomic spectra database.

Boltzmann plot method was used in order to determine the electron temperature (T_e) of the atmospheric pressure jet plasma by optical emissions spectrometry. NIST atomic spectra database was used to find the upper energy and *gA* values. Then, for nitrogen and argon, wavelengths of first ionisation levels of spectral lines (Ar-I and N-I) were selected. However, for oxygen plasma, O-II wavelengths were chosen as a result of only second ionization levels of oxygen was determined according to spectral peaks. Then, a plot of $\ln\left(\frac{I\lambda}{g\,A}\right)$ versus E_k should present a straight line with a slope of $-1/T_e$, where I is the intensity of the emitting light, λ is the wavelength, g is the statistical weight of the upper level, A is the transition and the energy of the upper level is E_k [149]. Finally, results were converted electron-volt (eV) to kelvin (K).

3.4. SURFACE CHARACTERIZATION

Surface characteristics of materials have an impact on the cell-material interactions. Therefore, physical and chemical characteristics were analyzed using different techniques including atomic force microscopy (AFM), the attenuated total reflection fourier transform infrared (ATR-FTIR) spectra, water contact angle (WCA) and scanning electron microscopy (SEM) in order to evaluate the effect of plasma treatments on the surfaces.

3.4.1. The Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR)

The attenuated total reflection fourier transform infrared (ATR-FTIR) is related to the interaction of an oscillating electromagnetic space with a molecule. FTIR was applied for assignation of functional groups and recognition of pure compounds.

The ATR-FTIR spectra were acquired using a Thermo Scientific Nicolet iS50ATR (USA) equipment in Attenuated Total Reflectance (ATR) mode from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} with a total of 32 scans.

3.4.2. Atomic Force Microscopy (AFM)

Atomic force microscope qualifies the three-dimensional topography and physical characteristics of a surface with a thin probe. Typical AFM resolutions are well below 1 nm. This technique determines the effects of the treatment by calculation of the surface roughness.

The morphology of a surface with submicron features was measured with an atomic force microscope (AFM). The AFM was performed with Park SYSTEMS XE 100 Atomic Force Microscopy (KANC 4F, Lui-Dong, 906-10 Suwon 443-766, Korea). Each sample was analysed in ambient air under non-contact mode using silicone tips; 40 μm × 40 μm AFM fields were analysed and the scan rate was chosen as 0.5, 1 or 1.5 Hz.

Image analysis software (XEI) was used to generate micrographs. Characteristic surface parameters (Ra: average roughness, Rpv: [Rv] maximum peak to valley depth) and height profiles (Rz: mean height of roughness in ten points, Rq: [RMS] root mean square-standard deviation of the surface height) of the plasma applied surfaces were compared to control

surfaces. At least three different spots on each sample were measured for statistical purposes (n=3).

3.4.3. Water Contact Angle Measurement (WCA)

Surface hydrophobicity or hydrophilicity is characterized by surface wettability, which can be measured by the contact angle measurement. The determination of contact angle is a method to detect the hydrophilic/hydrophobic nature of surfaces.

Static WCA measurements were performed at the ambient temperature and air to determine the saturation treatment time for each surfaces by the sessile drop method. These measurements were determined by KSV CAM 101 (KSV Instruments Ltd., Finland) contact angle goniometer. A distilled water droplet with a controlled size of 2.0 µl was placed on plasma treated/ untreated surfaces immediately after the APPJ treatment. The contact angle values calculated in this study were obtained using Laplace-Young curve fitting. An average of contact angle was obtained from at least ten measurement over an extended area of the treated/untreated surfaces. The average values for contact angles and the standard deviation were then calculated. In this study, smaller than 90° water contact angle is considered hydrophilic while larger than 90° value is considered hydrophobic [159].

3.4.4. Scanning Electron Microscopy (SEM)

Differences in surface morphology of APPJ treated and untreated stainless steel and titanium surfaces were examined using scanning electron microscopy (Carl Zeiss, Germany), at different magnifications ranging from 500× to 10,000× at 10 kV. Before this examination, the surfaces did not coated with gold. Therefore, surface difference between plasma treated and non-treated surfaces were observed.

3.5. MICROORGANISM AND BIOFILM FORMATION

In this study, *Pseudomonas aeruginosa* PAO1 (a gram negative, aerobic, coccobacillus bacterium with unipolar motility) supplied from Copenhagen University Department of Immunology and Microbiology was used. Bacterial attachment and biofilm growth

properties of *Pseudomonas aeruginosa* PAO1 on the treated/untreated surfaces were tested using batch assay. The batch assays were performed in 24 well tissue culture plates (Costar, USA). *P. aeruginosa* PAO1 was grown at 37 °C in M9 salt medium supplemented with 20 per cent glucose with shaking at 180 rpm.

3.5.3. Preparation of Bacterial Growth Media

M9 salts were dissolved in dH_2O and autoclaved at 121ºC for 15 min and filter sterilized solutions were added. Chemical listed below were purchased from Sigma-Aldrich (Germany).

Table 3.1. Ingredients of M9 salt medium.

M9 salts		**Filter sterilized solutions**	
$Na_2 HPO_4$	12.8 g	Glucose (20 %)	20 ml
$KH_2 PO_4$	3.0 g	1 M $MgSO_4$ Solution	2 ml
NaCl	0.5 g	1 M $CaCl_2$ Solution	0.1 ml
NH_4 Cl	1 g		
dH_2O	478 g		

3.6. BACTERIAL ATTACHMENT AND BIOFILM FORMATION ASSAY

Each stainless steel and titanium surface was placed in a well of a multiwell dish. 5 ml of *P. aeruginosa* (PAO1) overnight cultures diluted with fresh M9 salt medium to an OD 600 of 0.02 (~10^7 CFU / ml) [160]. Two ml inoculums were transferred into each well. The multiwell plate was sealed with a sterile air-permeable cover foil (no lid) (Greiner bio-one, Germany) and incubated for1 h and 24 h for bacterial attachment and biofilm formation assay, respectively. The multiwell dishes were incubated at 37 °C in an incubator (New Brunswick Scientific, Innova 40 shaker, Germany) with humid atmosphere with shaking at 60 rpm.

3.6.1. Enumeration of Bacteria

After 1 h or 24 h incubation of bacteria on the surfaces, detachment of attached cells from the surfaces was done by the bead vortexing method [161, 162], which is the most convenient technique to remove the attached cells from surfaces. Each surface was removed from the M9 growth medium with a sterile forcep and then was rinsed three times with sterile 0.9 per cent NaCl to remove planktonic cells. Each surface was then transferred to a sterile 2 ml tube including 1 ml of 0.9 per cent NaCl (Merck, Germany) and 3 ea sterile glass beads. Then, the tube was vortexed for 1 min to detach cells from the surface. Afterwards, the suspension was serially diluted with 0.9 per cent NaCl, plated on Luria-Bertani (LB) (Sigma, Germany) agar and incubated at 37 °C for 24 h. After incubation, colonies were counted and CFU calculated.

3.7. STATISTICAL ANALYSIS

All experiments were performed at least triplicate experiments. Data analysis were carried out by way of the Minitab software version 17 (Minitab, Inc., State College, PA). Results were presented as the mean ± standard deviation.

Comparisons of data were evaluated using one-way analysis of variance (ANOVA). Differences between plasma treated and untreated surfaces were determined by two-tailed unpaired Student's t test. A value of $p < 0.05$ among the groups were considered to be statistically significant.

4. RESULTS AND DISCUSSION

The performance of the atmospheric pressure plasma jet with nitrogen, oxygen and argon gases for the activation of the stainless steel and titanium surfaces was analyzed. The corresponding modifications of the surfaces after APPJ treatment were determined. *Pseudomonas aeruginosa* attachment and biofilm formation were linked to the APPJ treated surfaces properties. To further understand the working bases of the APPJ treatment under various process parameters on the activation success was determined.

4.1. OPTICAL EMISSION SPECTROMETRY (OES)

Optical emission spectrometry is an analytical technique and can be used to identify reactive species based on the spectra. Atmospheric pressure plasmas can constitute various reactive species and these reactive species contribute to the surface activation [163, 164]. Therefore, in this study, OES used to identify the major reactive species generated by nitrogen, oxygen and argon atmospheric pressure plasma jets.

Optical emission spectra of APPJ nitrogen gas plasmas was shown in Figure 4.1. Peaks in Fig. 4.1 correspond to the following emissions: O-III (3^{nd} ionization) λ = 453.27; N-II (2^{nd} ionization) λ = 470.42; N-II (2^{nd} ionization) λ = 486.016; N-III (3^{rd} ionization) λ =503.096; N-II (2^{nd} ionization) λ = 517.446; N-II (2^{nd} ionization) λ = 545.42; N-III (3^{rd} ionization) λ = 548.87; O-V (5^{th} ionization) λ = 560.427; O-II (2^{nd} ionization) λ = 581.025; N-I (1^{st} ionization) λ = 623.76; N-II (2^{nd} ionization) λ = 633.08; N-II (2^{nd} ionization) λ =643.34, N-II (2^{nd} ionization) λ = 653.256; Fe-I (1^{st} ionization); Fe-I (1st ionization) λ = 673.31; N-I (1^{st} ionization) λ = 694.52; N-II (1^{st} ionization) λ = 696.68; Ni-II (2^{nd} ionization) λ = 707.80; O-II (2^{nd} ionization) λ = 720.22; N-I (1^{st} ionization) λ = 737.85; O-III (3^{rd} ionization) λ = 745.54; and N-I (1^{st} ionization) λ = 754.62, respectively. According to result of spectral data, the major component was found as nitrogen, however several peaks were also analyzed belonging to oxygen (O) at different energy states as a result of the plasma system was operated in air. Moreover, some nickel (Ni) and iron (Fe) was excited from the nozzle of plasma jet.

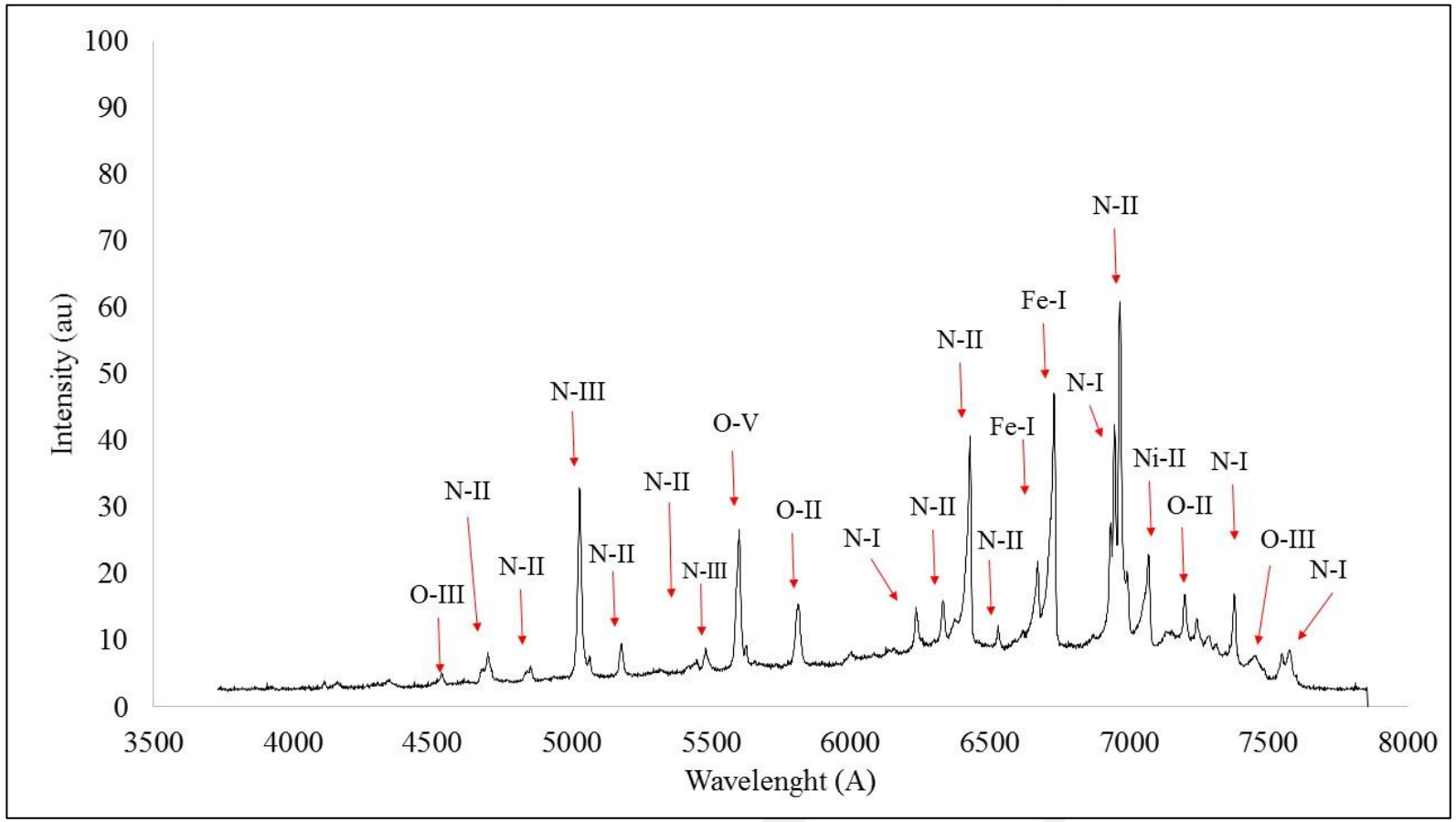

Figure 4.1. Optical emission spectra of APPJ nitrogen gas plasmas.

The optical emission intensity spectrum of oxygen APPJ system used in this study showed peaks indicating the ionization levels of the ions in Figure 4.2. Peaks in Fig. 4.2 correspond to the following emissions: O-III (3^{rd} ionization) λ = 673.616; O-II (2^{nd} ionization) λ = 693.60; N-II (2^{nd} ionization) λ = 697.56; O-II (2^{nd} ionization) λ = 720.22; and O-II (2^{nd} ionization) λ = 734.64, respectively. During oxygen plasma spectrum, having nitrogen emission line is expected since air is the surrounding gas [158].

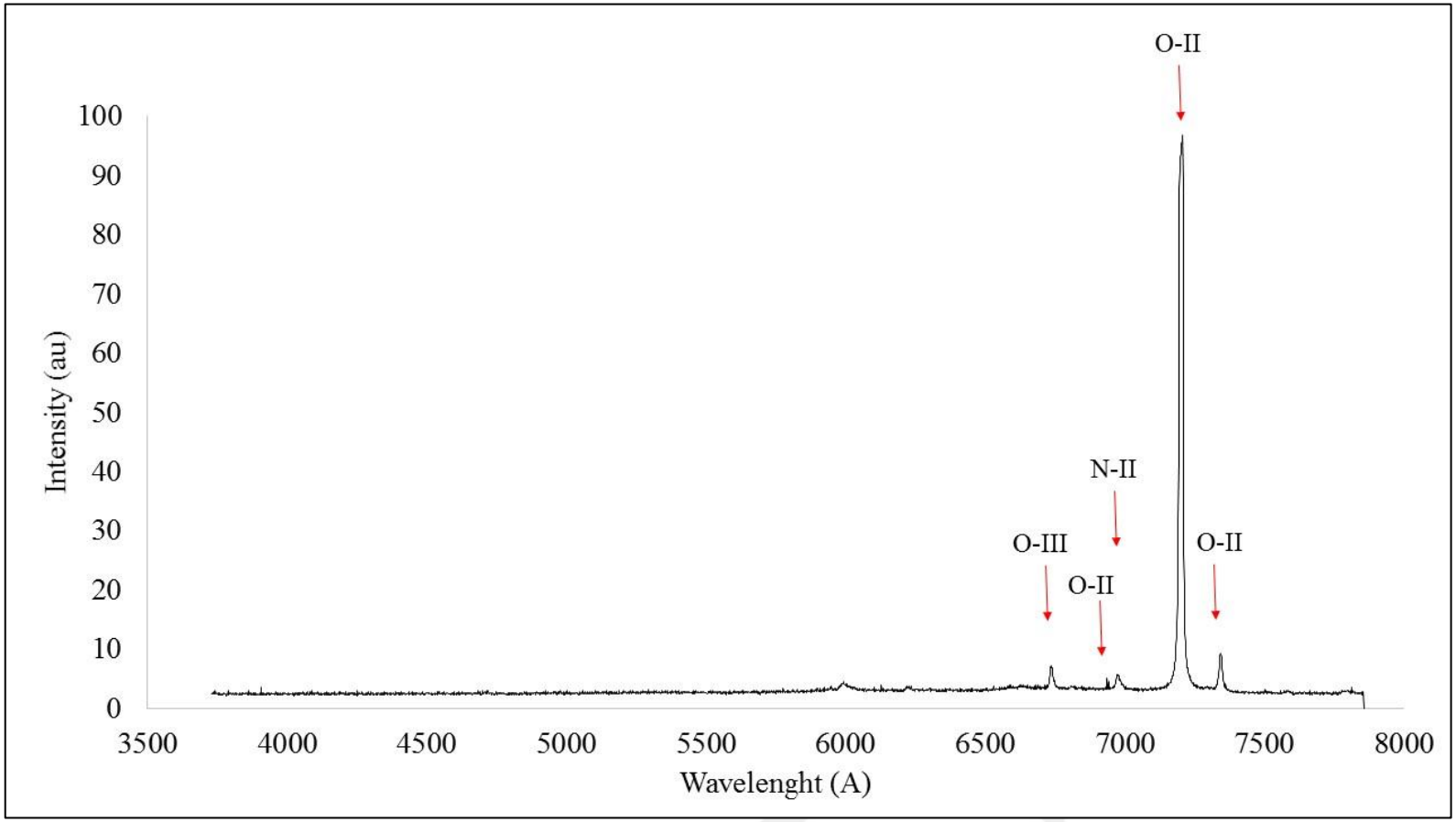

Figure 4.2. Optical emission spectra of APPJ oxygen gas plasmas.

As shown in Fig. 4.3, the emission spectrum was dominated by Ar emission lines, which is expected since Ar is the major component in the gas. Characteristic spectral lines of Ar can be observed in the range between 600 and 750 nm. The argon plasma generated by the APPJ expands to the environmental air and interacts with air molecules, such as O_2, N_2 and humidity, forming reactive species [108, 149]. Peaks in Fig. 4.3 correspond to the following emissions: Ar-I (1^{st} ionization) λ = 659.611; Fe-I (1^{st} ionization) λ = 667.268; N-I (1^{st} ionization) λ = 672.92; Fe-I (1^{st} ionization) λ =682.48; Ar-II (1^{st} ionization) λ = 690.446; Ar-II (2^{nd} ionization) λ = 700.14; Ar-II (1^{st} ionization) λ = 709.056; Ar-I (1^{st} ionization) λ = 716.256; O-II (2^{nd} ionization) λ = 720.22; O-II (2^{nd} ionization) λ = 734.637; N-I (1^{st} ionization) λ = 739.86; and Ar-I (1^{st} ionization) λ = 748.433, respectively. Similar results were also observed in a previous study, the interaction of the Ar plasmas with ambient air was shown Ar emission lines, nitrogen emission lines and oxygen emission lines [162]. The presence of these lines confirms the energy transfer from excited Ar atoms to nitrogen and oxygen molecules leading to formation of reactive oxygen and nitrogen species [108].

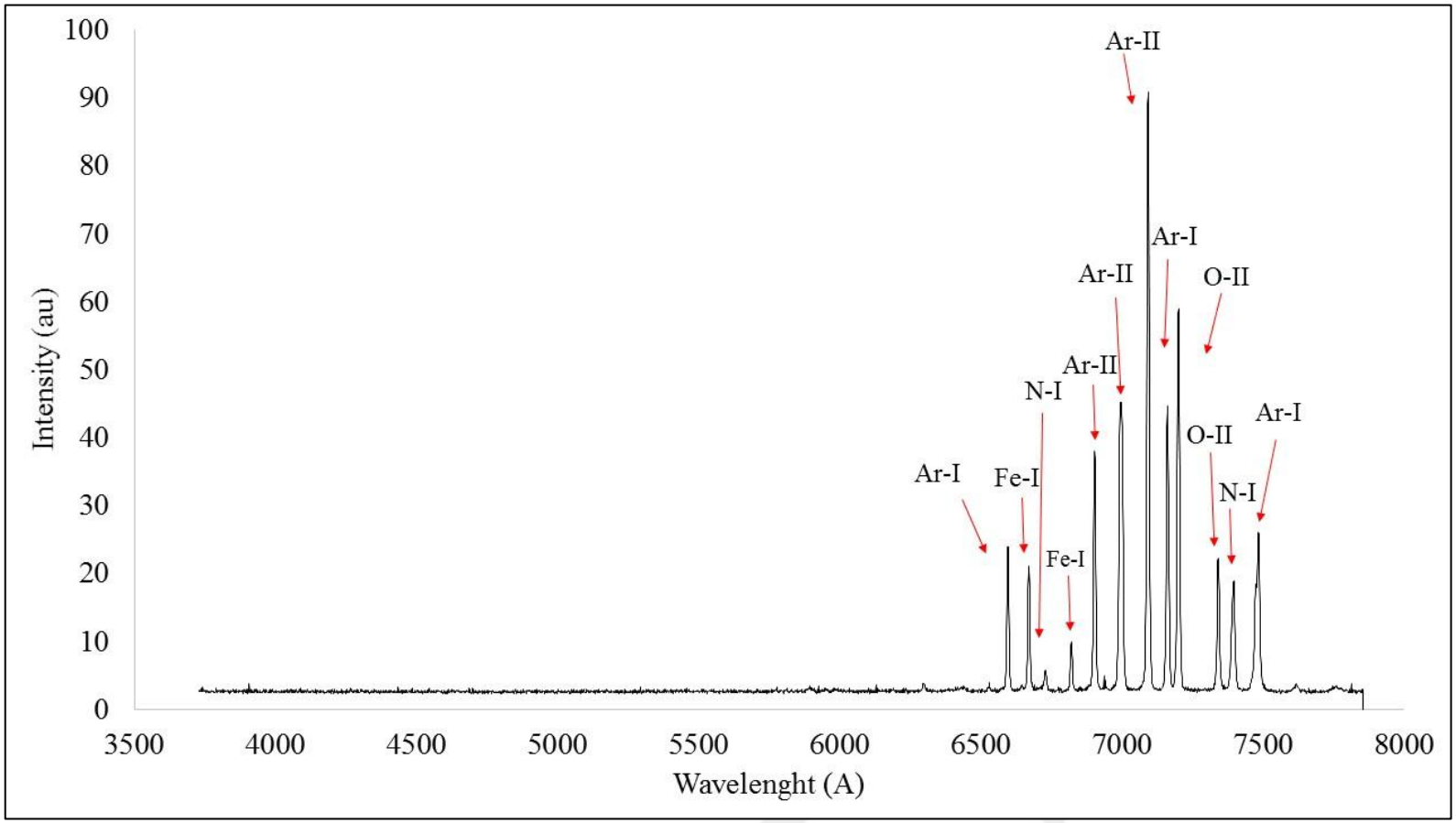

Figure 4.3. Optical emission spectra of APPJ argon gas plasmas.

The reactive and activated species generated by APPJ system took an importance in reactive etching and particle aggregations [92]. Moreover, different plasmas may introduce various functional groups on the surface of materials [165]. However, the most probable limitation of these reactive species is that they have short life time [158].

Boltzmann plot method was used to determine the electron temperature (T_e) of the atmospheric pressure jet plasma by EOS for each gas. Table 4.1 shows the parameters corresponded to Ar-I. Then, the graph of In $\left(I\lambda/gA\right)$ versus E_k for Ar-I was plotted according values in the Table 4.1 (Figure 4.4).

Table 4.1. Spectral lines of Argon I.

Wavelength	**Intensity**	***g A***	**E_k (eV)**	**In $\left(I\lambda/gA\right)$**
659,6	23,096	1,20E+05	14,954	-2,06395
716,26	44,269	1,70E+05	15,003	-1,67923
748,466	26,099	1,70E+06	14,809	-4,46622

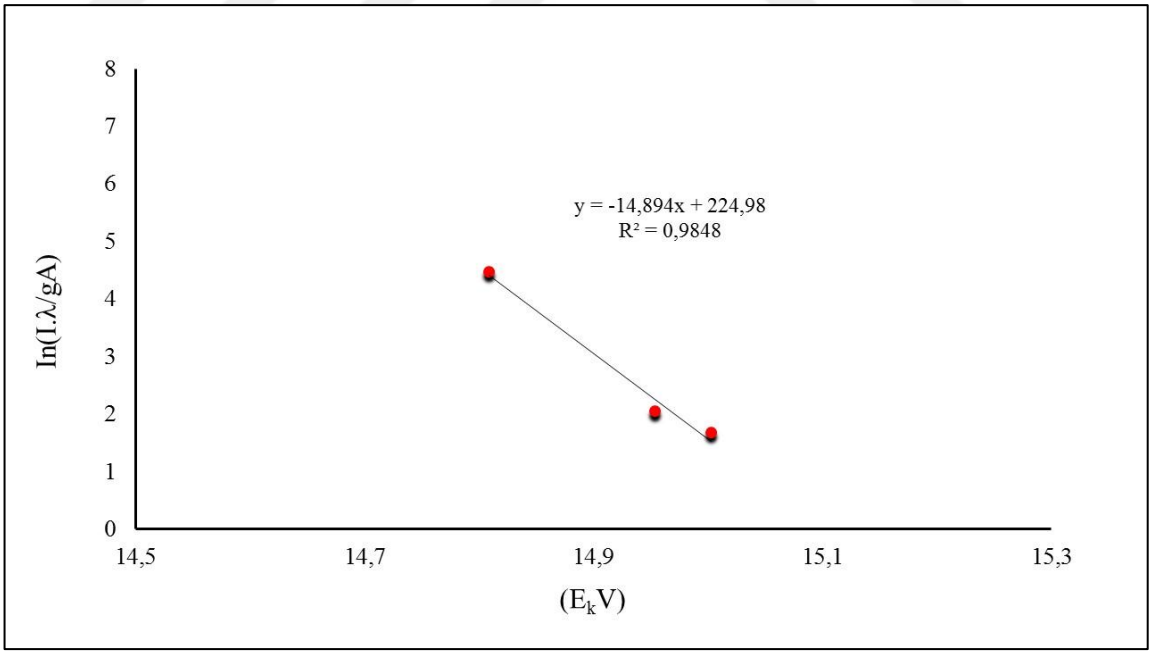

Figure 4.4. Boltzmann plot to estimate the electron temperature.

The slope of the line was found -14. 894 which is equal to $-1/T_e$. Electron temperature was found as 0.067 eV and converted to Kelvin (K) which was equal to 779 K for Argon APPJ.

Same calculations were also done and graphs were drawn to calculate Te values of oxygen and nitrogen APPJ systems (data not shown). The electron temperatures of oxygen and nitrogen APPJ systems were 749 and 4291 K, respectively. It was found that nitrogen plasma had the highest electron temperature compared to the oxygen and argon plasma.

The electron temperature is an important parameter to determine of temperature characteristics of plasmas [82]. In this study, T_e values for each gases were less than 10^5. Moreover, as observed during to the experiment, the gas temperature of the jet keeps below 100 °C because it was safe to touch by hand. While ions and electrons are found in the same temperatures in thermal plasmas, non-thermal plasmas consist of relatively high temperature electrons and low temperature particles (Table 1.4) [82, 86]. Therefore, the APPJ system was used in this study may be called as non-thermal plasma which was produced at room temperature or a little above. The ions and the neutrals remain relatively cold produced by non- thermal plasmas, therefore do not cause any thermal damage [85].

4.2. SURFACE CHARACTERIZATION

Chemical compositions, wettability and roughness are the important surface parameters for the interaction of surface and bacteria [166]. To further understand the effect of APPJ treatments on the stainless steel and titanium surfaces, physical and chemical characteristics of the surfaces were analyzed using various advance techniques, such as: Fourier-transform infrared (FTIR) spectroscopy, atomic force microscopy (AFM), water contact angle measurement (WCA), and scanning electron microscopy (SEM) after APPJ treatment.

4.2.1. The Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR)

Chemical changes induced by nitrogen, oxygen and argon APPJ treatment were characterized by ATR-FTIR spectra. Figure 4.5 shows the ATR-FTIR spectra of the untreated and nitrogen, oxygen and argon APPJ treated stainless steel with processing time of 15 min. It was noted that the signal intensity obtained from the stainless steel samples were very weak and in some wavenumber regions (from 2320 cm^{-1} to 1880 cm^{-1}) it was comparable with the noise generated by the ATR element (diamond crystal). Compared to the untreated SS surface, a noticeable broad band at 758 cm^{-1} generated by the bending

vibration of O-H, which was detected in the spectra of O_2 APPJ treated surface. The other gas plasma treated stainless steel surfaces showed the similar spectral peaks compared to the untreated SS surface (Figure 4.5.).

After oxygen plasma treatment on SS surface, the protective oxide layer may be formed on the surface [167], therefore the bending vibration of O-H was observed after oxygen APPJ treatment on SS. Stainless steel 316L grade surface chemical composition includes; C-0.03 %, Cr-16.82 %, Ni-10.02 %, Mn-1.26 %, Mo-2.07 %, Si-0.46 %, N-0.04 %, P-0.02 %, Fe-base wt % [168, 169]. SS 316L shows higher corrosion resistance than the other type of stainless steel. Therefore, it is the most used alloy in all implants/medical surface [168]. Reactive species of plasmas generally has short life time because of their high ionization degrees [170]. On the other hand, stainless steel is highly stable, therefore, according to these results, plasma reactive species may not be responsible for change on SS 316L surface chemical composition.

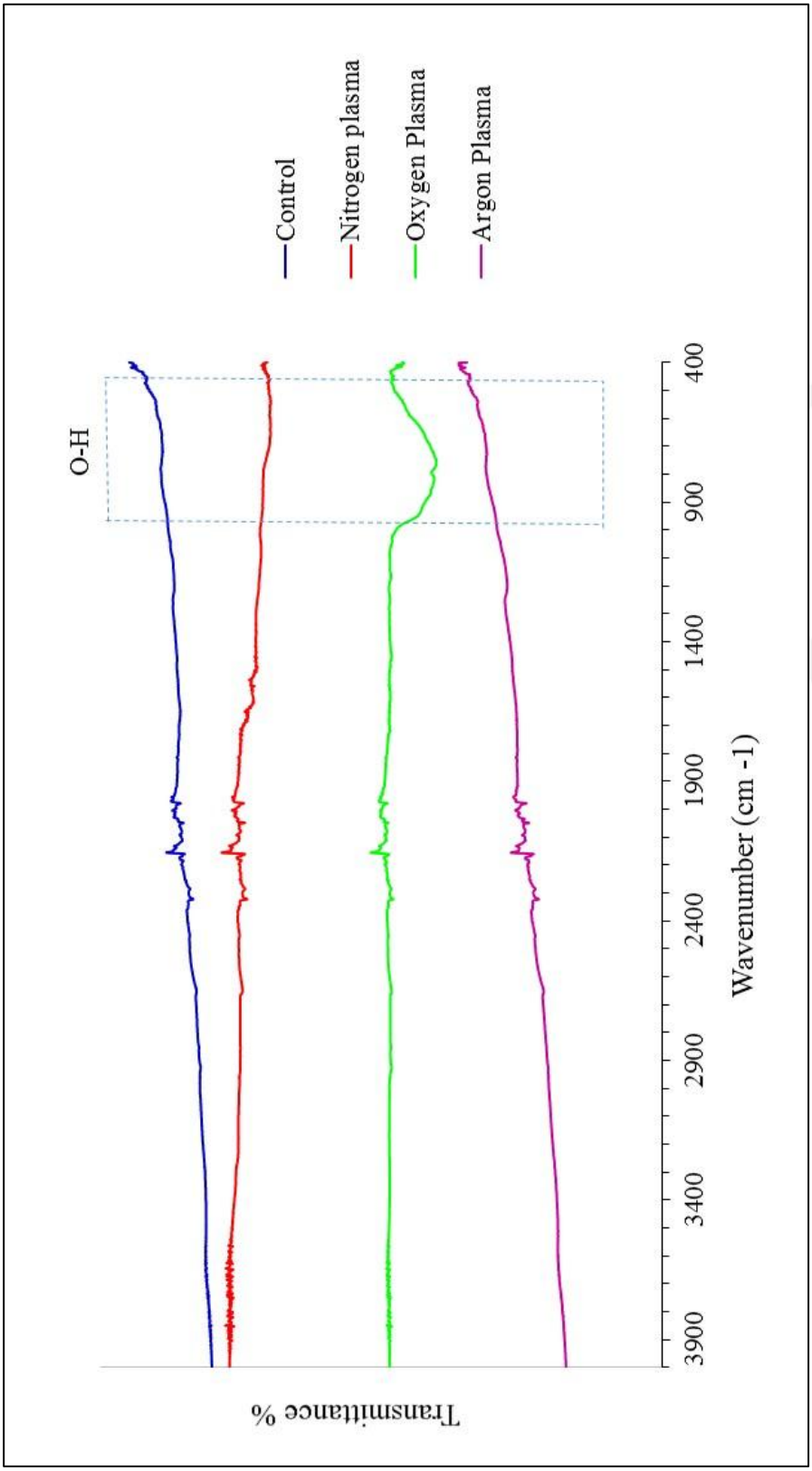

Figure 4.5. ATR-FTIR spectra of non-thermal APPJ treated/untreated SS surface with processing time of 15 min.

Titanium (grade 5) surface chemical composition includes; Ti ~90.0%, Al 5.5–6.75%, V 3.5–4.5%, Fe ≤ 0.3%, O ≤ 0.2%, H ≤ 0.012%, C ≤ 0.08%, N ≤ 0.05%, the rest (max) 0.4% [171]. Titanium and its alloys are quite stable in the environment and widely used in medical surfaces with their corrosion resistance and high biocompatibility [171, 172]. Figure 4.6 displays the ATR-FTIR spectra of untreated and nitrogen, oxygen and argon APPJ treated titanium surfaces with a processing time of 15 min. As similar with SS surfaces, the signal intensity obtained from the titanium samples were also very weak and in some wavenumber regions (from 2320 cm^{-1} to 1880 cm^{-1}) it was comparable with the noise generated by the ATR element (diamond crystal). As can be seen from the figure, the characteristic peak was determined at 1046 cm^{-1} corresponded to the C-O stretching [173]. The peak for Ar APPJ treated surface was sharper than the untreated, O_2 and N_2 APPJ treated surfaces. These change can be explained by introducing reactive free oxygen radicals during Ar APPJ treatment [141].

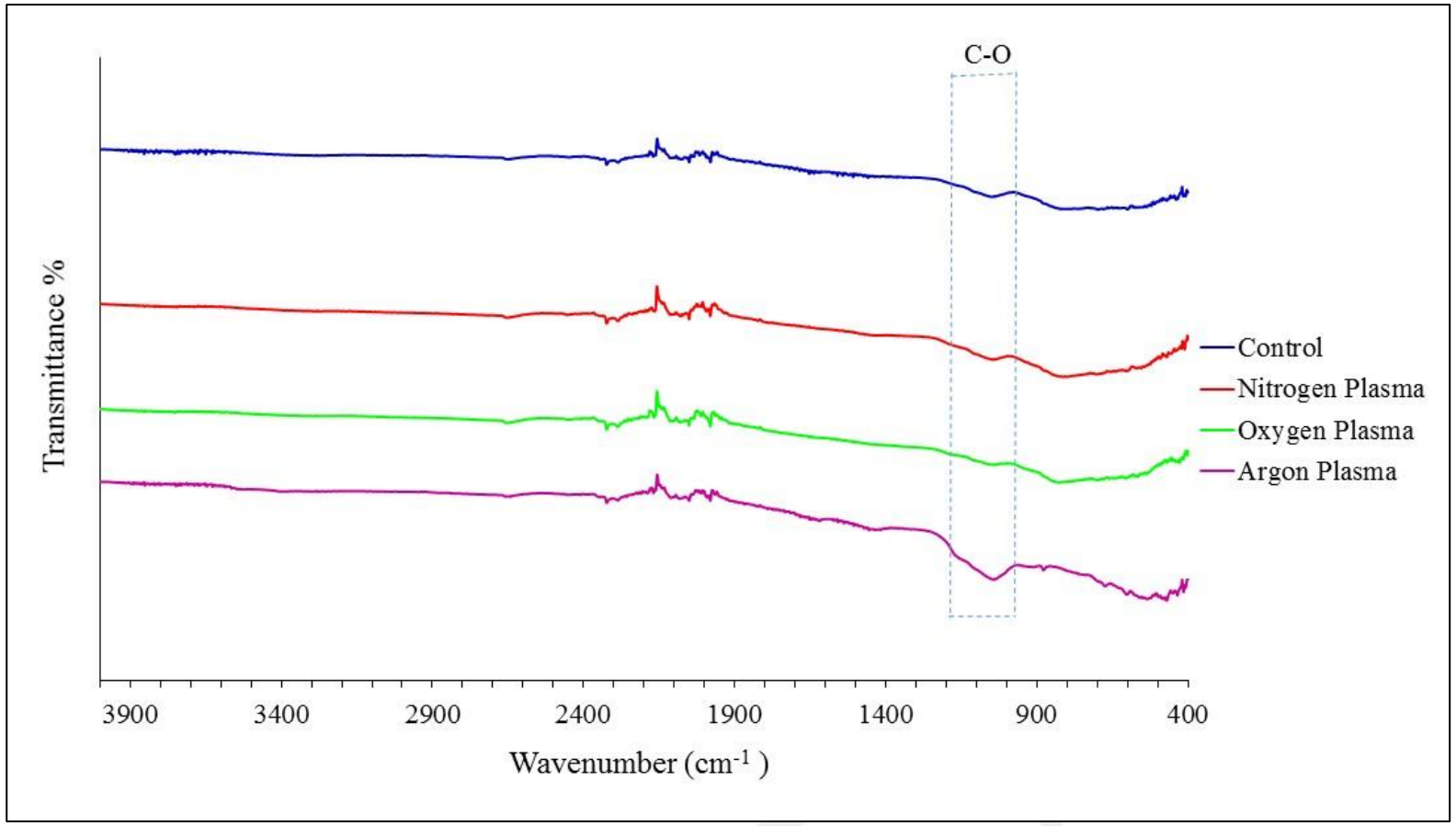

Figure 4.6. ATR-FTIR spectra of non-thermal APPJ treated/untreated Ti surface with processing time of 15 min.

In the present study, stainless steel and titanium surfaces were treated by nitrogen, oxygen and argon APPJ and the results showed that the plasma system only causes some minor changes on the surfaces chemical structures as previously explained in detail. However, plasma technology has been widely and effectively used to change surface properties of polymers in recent years [103, 139, 146, 174, 175].

4.2.2. Atomic Force Microscopy (AFM)

AFM is a microscopic technique which provides information on the surface topography. There dimensional surface characteristics and the surface roughness were determined immediately after plasma treatment for the untreated and the nitrogen, oxygen and argon APPJ treated SS and Ti surfaces. The scan size of all the AFM images was $40 \times 40\ \mu m^2$.

In Figure 4.7 represents the AFM images in a 3 D view of untreated and APPJ treated SS surfaces with various gases with 15 min constant processing time. From the results, as expected, the surface of untreated SS was homogeneous. Clearly, the morphology of the SS surface was changed after 15 in nitrogen, oxygen and argon APPJ treatment. According to the 3D images, the nitrogen APPJ treatment made the SS surfaces smoother, while SS became rougher after the oxygen and argon APPJ treatment. A similar topographical morphology was observed for oxygen and argon APPJ treated SS surfaces (Figure 4.7 C and D).

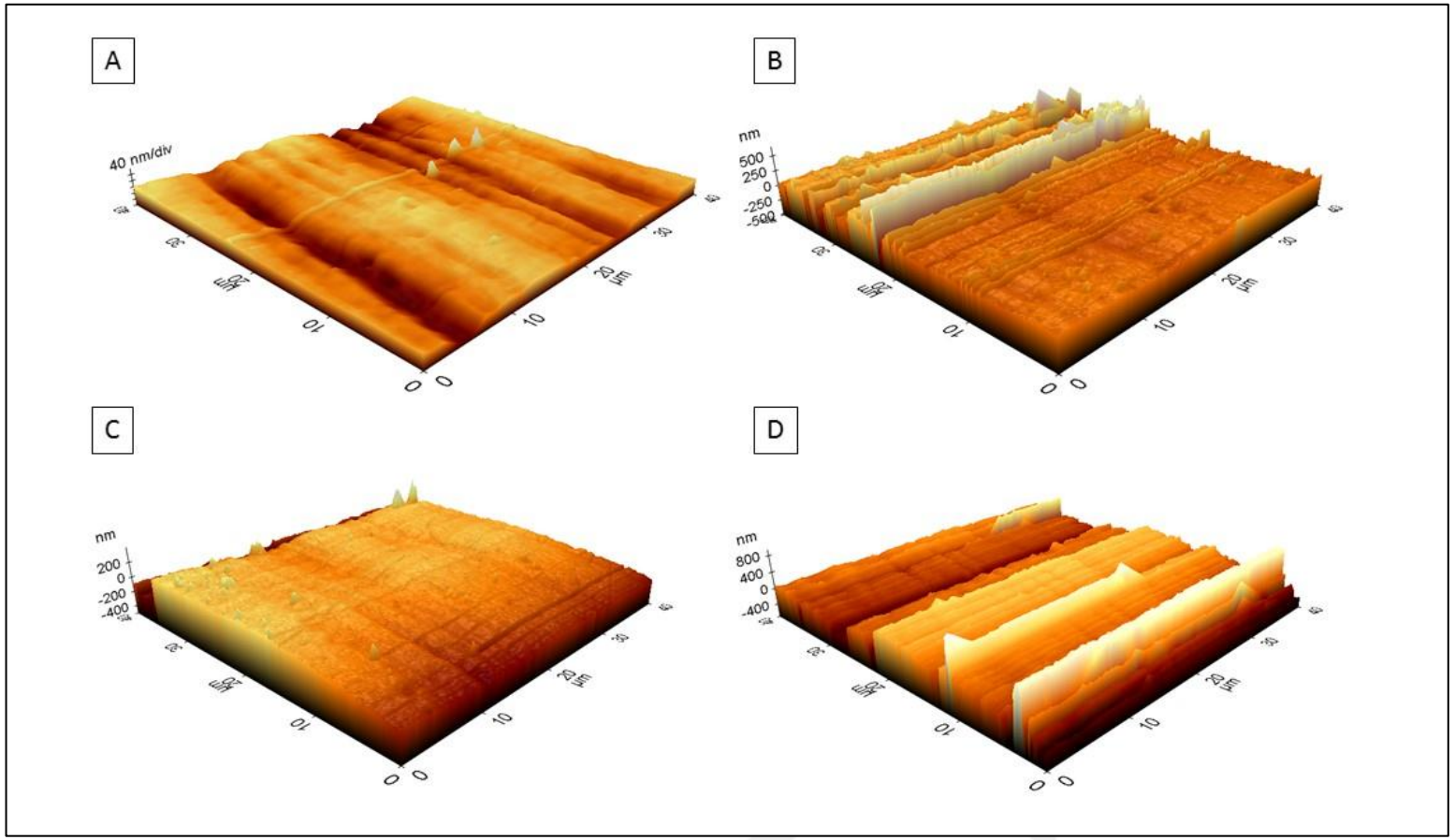

Figure 4.7. AFM images of stainless steel surfaces before and after APPJ treatment with each gases with an analyzed area of 40x40 µm. (A) Untreated; (B) Nitrogen plasma treated; (C) Oxygen plasma treated; (D) Argon plasma treated.

Table 4.2 shows the roughness parameters of SS before and after APPJ treatment using different gases with 15 min constant processing time. The untreated SS surface was relatively rough with Ra surface roughness 15.5 ± 3 nm. After nitrogen APPJ treatment, the average surface roughness compared to control significantly decreased to 0.06 ± 0.01 nm ($p< 0.001$). In contrast to nitrogen APPJ treatment, the surface average roughness of SS was significantly increased upon the oxygen and argon APPJ treatment to 22.03 ± 3 and 21.86 ± 3.5 nm, respectively ($p< 0.001$). Moreover, the oxygen and argon APPJ treatment caused the statistically similar effect on the average roughness (Ra) of SS surfaces ($p> 0.05$), whereas the oxygen and argon APPJ treated SS surfaces were the rougher than untreated SS surface ($p< 0.001$).

These comparisons showed that plasma treatment in the presence of different gases caused the hardly change in surface morphology by increase or decrease of surface roughness. Furthermore, the 3 D AFM images depicted were found in accordance with surface roughness parameters of SS surfaces.

Table 4.2. Roughness parameter values of stainless steel surfaces before and after APPJ treatment with different gases.

Sample	Roughness parameters (nm) Rz			
	Rpv (nm)	**Rq (nm)**	**Ra (nm)**	**Rz (nm)**
Control	94.26 ± 10^{a}	19.6 ± 3.7^{a}	15.5 ± 3^{a}	61.3 ± 5^{a}
Nitrogen Plasma	0.35 ± 0.11^{b}	0.06 ± 0.03^{b}	0.06 ± 0.01^{b}	0.21 ± 0.07^{b}
Oxygen Plasma	144.34 ± 21^{c}	31.2 ± 5.7^{c}	22.03 ± 3^{c}	108.27 ± 8.4^{c}
Argon Plasma	121.78 ± 10^{d}	24.33 ± 2.9^{d}	21.86 ± 3.5^{c}	98.01 ± 9^{d}

Rpv: (Rv) peak to valley; Rq: (Rms) root mean square-standard deviation of the height; Ra: average roughness; Rz: mean height of roughness in ten points. The mean values ± standard deviation are listed. The different superscript letter within the same row are shown significantly difference.

Similar to these results, decrease in surface roughness was observed after mixed gas (including high percentage nitrogen) APP treatment on stainless steel and silicon wafer [92]. Compared to these results, the increase of surface roughness was also observed after oxygen and argon plasma treatments for different polymer surfaces [175–177]. The increase of the roughness after plasma treatment could be because of the impact of heavy oxygen and argon

ions on surface [175]. Based on the literature the similar results could be observed and explained as different plasma forming gases induce the different reactive mechanism on surfaces which resulted in offering surface roughness [177].

In figure 4.8, AFM images revealed that untreated titanium surfaces were rough and after APPJ treatments they became quite uniform and smooth surfaces. Surface roughness parameters were decreased after nitrogen, oxygen and argon APPJ treatments with 15 min constant processing time (Table 4.3). Moreover, 3D structures of untreated Ti showed remarkable differences compared to APPJ treated surfaces, whereas APPJ treated surfaces with different gases exhibited similar topographical morphology (Figure 4.8).

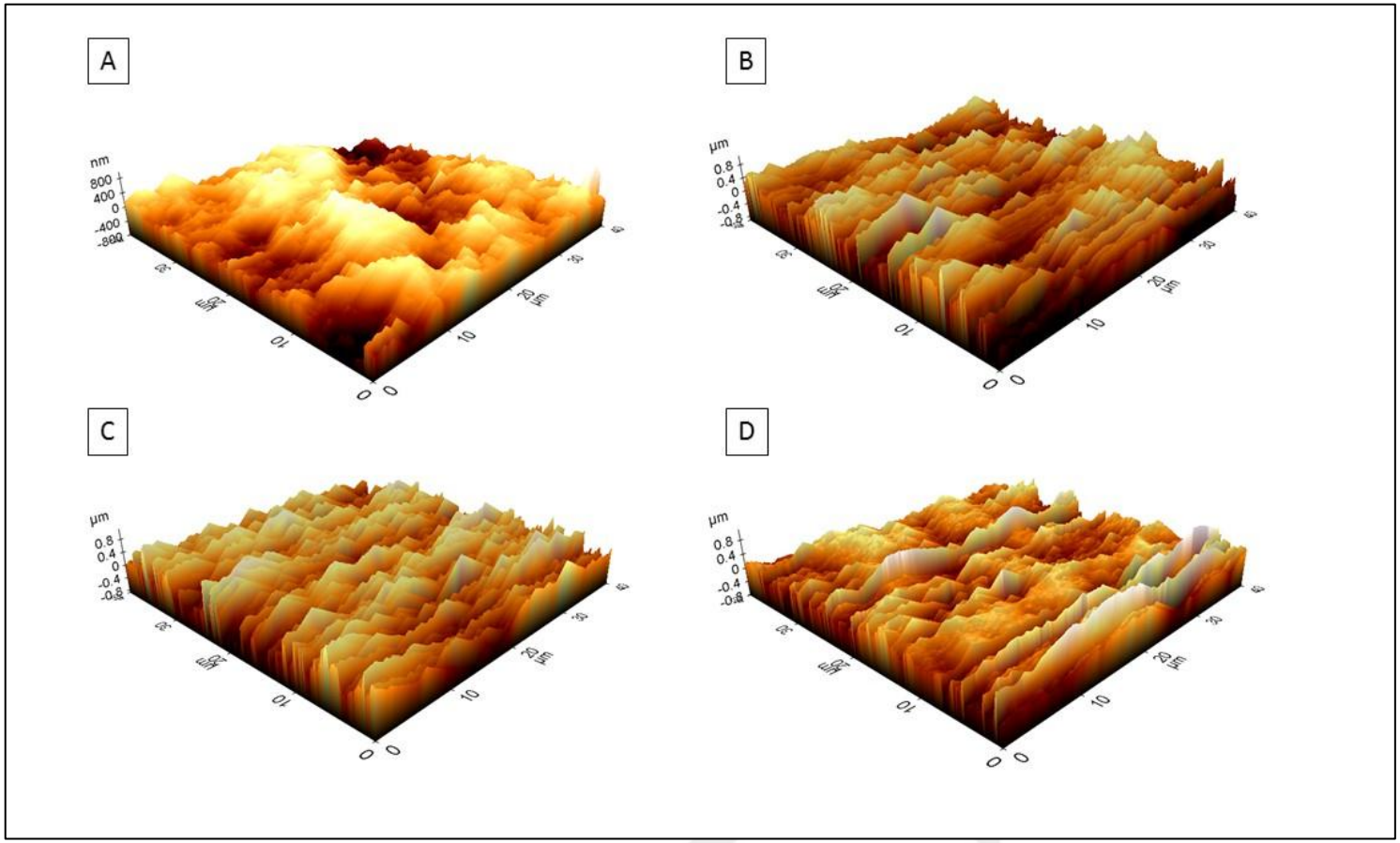

Figure 4.8. AFM images of titanium surfaces before and after APPJ treatment with each gas with an analyzed area of 40x40 µm. (A) Untreated; (B) Nitrogen plasma treated; (C) Oxygen plasma treated; (D) Argon plasma treated.

The treatment of titanium surfaces with APPJ resulted in highly decreased of surface roughness compared to untreated surfaces ($p< 0.001$) (Table 4.3). It was clear that untreated Ti surface exhibited high surface roughness and showed Ra about 155.9 ± 12 nm, while after the nitrogen plasma treatment Ra was 0.15 ± 0.02 nm. When the Ti surfaces were treated with the oxygen APPJ, Ra was found to be 0.18 ± 0.03 nm, whereas argon APPJ treatment exhibited (Ra) to be 0.127 ± 0.24 nm. The recent results have shown that the nitrogen, oxygen and argon APPJ treatment induced significantly low surface roughness of Ti compared to untreated surfaces ($p< 0.001$). Moreover, there were not observed significant differences in surface roughness values among the nitrogen, oxygen and argon APPJ treated Ti surfaces ($p> 0.05$). Surface roughness parameters of Ti surfaces were in agreement with the 3 D AFM images depicted. A previous study was showed similar result as titanium surface became more smother after 10 min air plasma treatment [171].

Table 4.3. Roughness parameter values of titanium surfaces before and after APP treatment with each gas.

Sample	Roughness parameters (nm) Rz			
	Rpv (nm)	**Rq (nm)**	**Ra (nm)**	**Rz (nm)**
Control	839.46 ± 95^{a}	193.11 ± 14.4^{a}	155.9 ± 12^{a}	652.4 ± 95^{a}
Nitrogen Plasma	0.84 ± 0.07^{b}	0.19 ± 0.02^{b}	0.15 ± 0.02^{b}	0.63 ± 0.07^{b}
Oxygen Plasma	0.95 ± 0.13^{b}	0.22 ± 0.03^{b}	0.18 ± 0.03^{b}	0.70 ± 0.05^{b}
Argon Plasma	0.65 ± 0.1^{b}	0.16 ± 0.03^{b}	0.127 ± 0.24^{b}	0.51 ± 0.08^{b}

Rpv: (Rv) peak to valley; Rq: (Rms) root mean square-standard deviation of the height; Ra: average roughness; Rz: mean height of roughness in ten points. The mean values ± standard deviation are listed. The different superscript letter within the same row are shown significantly difference.

It is worth mentioning, that the nitrogen, oxygen and argon APPJ treatment did not influence the bulk properties of stainless steel 316L and titanium (grade 5). Plasma systems were frequently used to modify polymer surfaces without changing to the bulk properties [81, 155]. The bulk properties of surfaces (especially used in medical area) are important for maintaining the anti-corrosive properties [154]. Also, there are some studies reported the effect of plasma ion implantation to improve corrosion resistance of materials without affecting the bulk characteristics [90, 178].

Association between bacterial attachment and surface roughness were previously reported in the literature. Although, it was generally accepted that the smoothness of a surface decreases the probability of bacterial adhesion [179], some studies have suggested that relationship is not linear [146, 154, 180]. For instance, Aires et al [181] reported that the surface roughness was increased after plasma treatment compared to the untreated control samples of Ti (grade II) and also significant reduction of *S. epidermidis* adhesion was observed. Therefore, as previously reviewed surface roughness not to be a major effect in the attachment of bacteria to a surface and many other parameters could also play a role [139, 180, 182].

4.2.3. Water Contact Angle Measurement (WCA)

The WCA measurements were carried out for untreated and the nitrogen, oxygen and argon APPJ treated stainless steel L316 and titanium (grade 5) surfaces. WCAs were measured immediately after plasma treatment in order to assess possible recovery of samples hydrophobicity. Table 4.1 shows the variation of contact angles of untreated and 15 min nitrogen, oxygen and argon APPJ treated SS and Ti surfaces. From the WCA (°) the wettability of the surfaces was analyzed.

Table 4.4. Water contact angle measurements of untreated and APPJ treated SS and Ti surfaces with each gas.

Sample	Water Contact Angle Results (º)	
	Stainless Steel	**Titanium**
Control	92.35 ± 3.1^{a}	92.14 ± 1.8^{a}
Nitrogen Plasma	31.94 ± 3.3^{b}	14.4 ± 2.0^{b}
Oxygen Plasma	37.41 ± 2.8^{c}	12.64 ± 1.76^{c}
Argon Plasma	26.9 ± 2.5^{d}	$13.42 \pm 1.78^{b,c}$

The mean values ± standard deviation are listed. The different superscript letter within the same row are shown significantly difference.

It was observed that untreated SS surface showed contact angle about 92.35± 3.1°. Similar WCA value was detected in the literature for untreated stainless steel L316 and this result

indicates that SS surface exhibits hydrophobic characteristic [183]. The water contact angles for the SS surfaces treated with nitrogen, oxygen and argon APPJ for 15 min were 31.94 ± 3.3°, 37.41 ± 2.8° and 26.9 ± 2.5°, respectively. The results showed that after APPJ treatment WCA values were drastically decreased and it was found statistically significant compared to untreated SS surface ($p < 0.001$). Moreover, the WCAs after 15 min nitrogen, oxygen and argon APPJ treatment for SS were significantly decreased in order to Ar > N_2 > O_2 plasma ($p < 0.001$) (Figure 4.9).

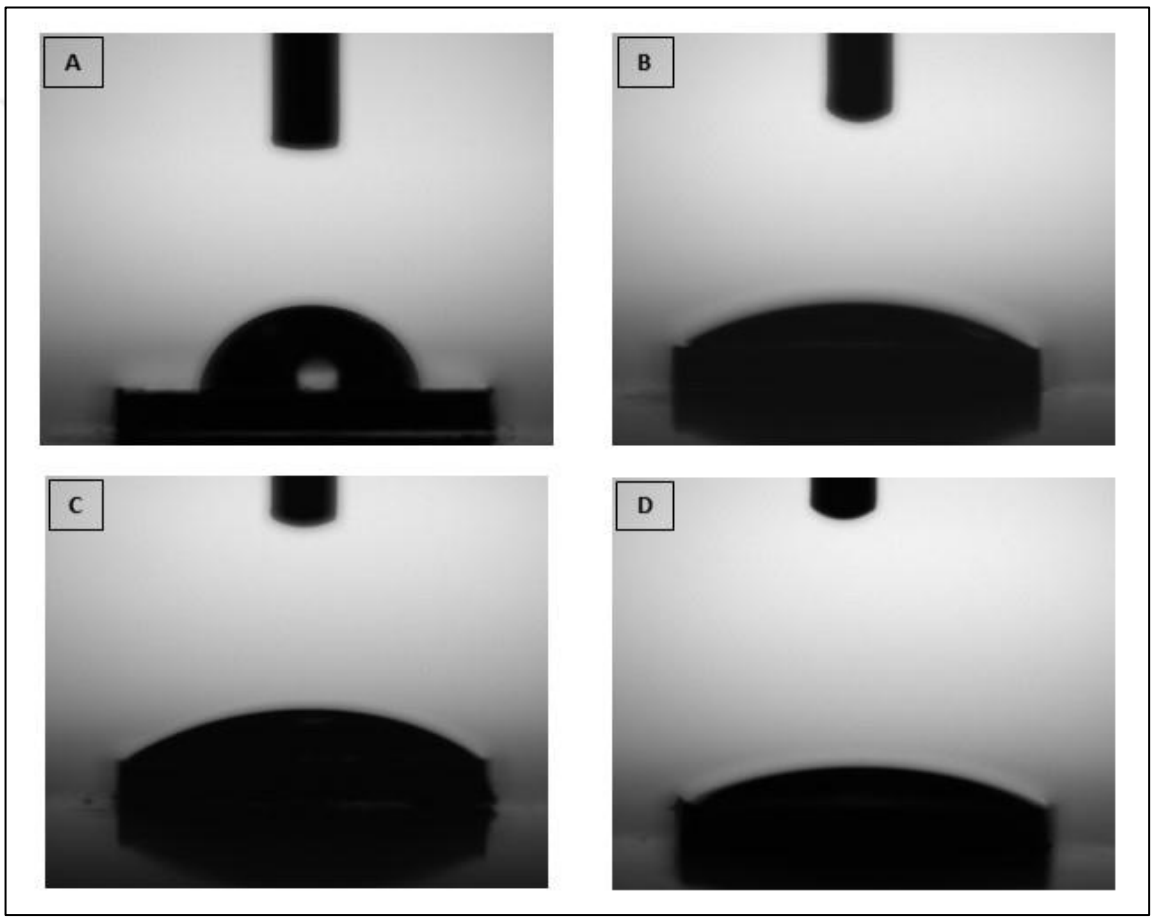

Figure 4.9. Photographs of water drops for contact angle measurements on APPJ untreated/treated stainless steel surfaces. (A) Untreated; (B) Nitrogen plasma treated; (C) Oxygen plasma treated; (D) Argon plasma treated.

Figure 4.10 shows the wettability of the Ti surfaces investigated with measurements of contact angle before and after APPJ treatment. The hydrophilicity was significantly increased for all APPJ treated surfaces (Figure 4.10). The WCA was found to decrease with

nitrogen APPJ treatment from 92.14 ± 1.8° for the untreated surface, down to 14.4 ± 2.0° after 15 min constantly treatment ($p < 0.001$). A similar trend was also found at argon APPJ in contact angle measurement which was reduced to 13.42 ± 1.78°. Furthermore, the lowest WCA value was detected after oxygen APPJ treatment when compared to the nitrogen and argon APPJ treatment, down to 12.64 ± 1.76°. While the WCA value of oxygen APPJ treated surface was not statistically significant than the argon APPJ treated Ti surfaces ($p > 0.05$), however, it was found to be statistically significant than the nitrogen APPJ treated Ti surfaces ($p < 0.05$).

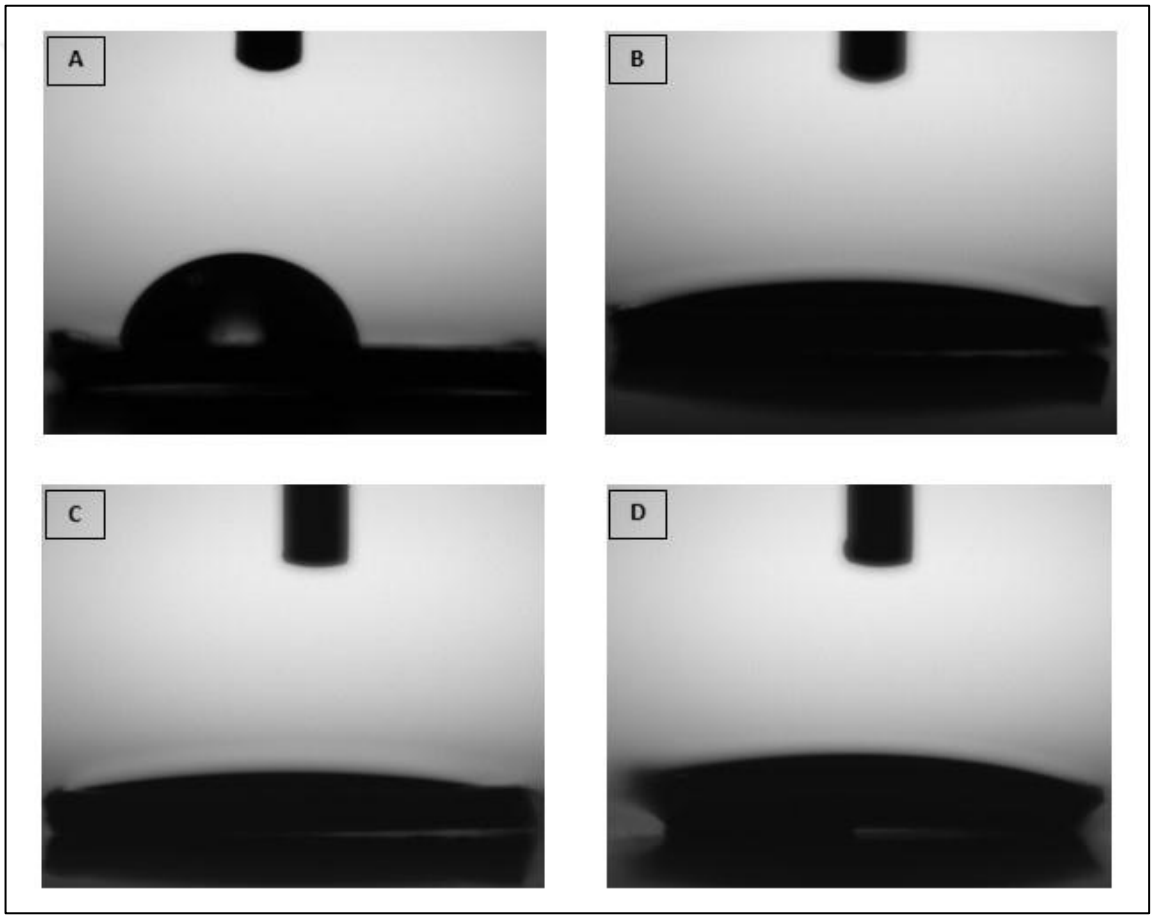

Figure 4.10. Photographs of water drops for contact angle measurements on APPJ untreated/treated titanium surfaces. (A) Untreated; (B) Nitrogen plasma treated; (C) Oxygen plasma treated; (D) Argon plasma treated.

The APPJ treated Ti surfaces were showed the lowest WCA value compared to APPJ treated SS surface. This result was in agreement with surface roughness parameters since contact

angle decreases in smooth and flat surface (Section 4.2.2) [155]. Previously, Yan et al. [171] reported that water adhesion increased with decreasing roughness of the surfaces for plasma treated titanium alloy. The improvement of wettability is related to the surface etching/abrasion during plasma treatment which can induce changes in surface topography [184].

This result is below the contact angle value of untreated surfaces suggesting that there was an important effect by decrease in WCA value of the APPJ on the SS and Ti surfaces, thus confirmed the findings of published investigations [92, 181, 184, 185]. Moreover, previously reported that different polymeric surfaces have also presented the decrease of WCA after plasma treatment [106, 142, 154, 155, 177, 186].

Plasma treatment enhanced wettability and produced hydrophilic surfaces via activating the surfaces at the atomic and molecular levels [187]. In addition, Kim et al. [105] showed that the increase of hydrophilicity was connected to an increase the total surface free energy to clean contaminants and also surface oxidation of plasma treatment. Also, chemically reactive species and free radicals generated by plasma systems could be effectively improve the hydrophilic property of surfaces [177, 184]. However, the literature was shown that the hydrophilic character of plasma treated surfaces without further modifications has only for short life time, as it immediately turn back its original hydrophobic character [105, 186, 188].

Several studies have also reported hydrophilic characteristics of surfaces is important for bacterial attachment since hydrophilic surfaces are more resistant to bacterial attachment than hydrophobic ones [146, 167].

4.2.4. Scanning Electron Microscopy (SEM)

The surface morphology of the stainless steel and titanium coupons before and after the nitrogen, oxygen and argon APPJ treatment were analyzed by SEM with higher magnifications.

The surface morphology of the stainless steel and titanium coupons before and after the nitrogen, oxygen and argon APPJ treatment were analyzed by SEM with higher magnifications.

Figure 4.11 represents the SEM images of untreated and the nitrogen, oxygen and argon APPJ treated stainless steel. It was observed that the untreated stainless steel surface was homogeneous, relatively flat and polished surface. After the nitrogen, oxygen and argon APPJ treatment the new small particles were randomly distributed with a ball shape in different sizes on SS surfaces. The size and number of the particles were increased after the oxygen APPJ treatment compared to the other APPJ treated SS surfaces.

The results obtained from the SEM examination (Figure 4. 11) were in agreement with the 3 D AFM images depicted (Figure 4.7). Furthermore, although the surface average roughness of the oxygen and argon APPJ treated SS surfaces did not show significantly difference ($p > 0.05$), the other surface roughness parameters (except Ra) exhibited statistically difference ($p < 0.05$) (Table 4.2). This difference can be explained by the increase in the number and the size of the particles after the oxygen APPJ treatment compared to the argon APPJ treatment (Figure 4.11 C and D).

The aggregation of particles on APPJ treated SS surfaces were previously determined by the excited atoms, radicals and molecules as a result of reactive oxidation and etching [92]. A previous study showed that rising the oxygen and decreasing the nitrogen amount caused to relatively increase of the aggregation of new particle [92]. These agglomerates could be responsible to the decrease of water contact angle as a result of absorption of water into these cavities [189].

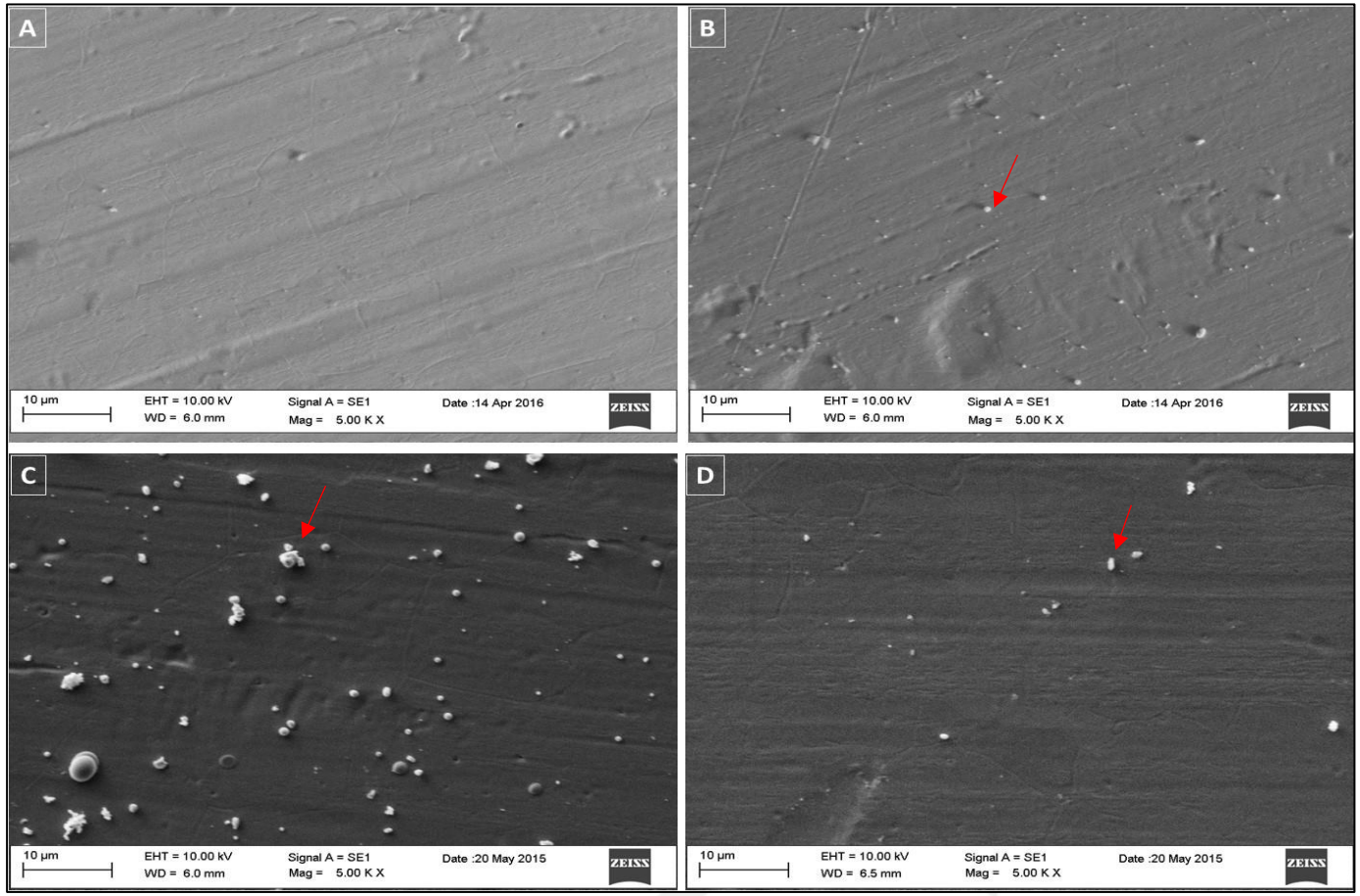

Figure 4.11. SEM images of stainless steel surfaces before and after APP treatment with each gas at 5000 X magnification. (A) Untreated; (B) Nitrogen plasma treated; (C) Oxygen plasma treated; (D) Argon plasma treated. Red arrows showed the aggregated particles induced by APPJ treatment.

Figure 4.12 presents the SEM images of untreated and the nitrogen, oxygen and argon APPJ treated Ti surfaces. SEM image of untreated Ti surface was found to be irregular and rough and also showed defects, such as holes and irregular scratches which were related to the fabrication of surface (Figure 4.12 A).After APPJ treatment, Ti surfaces were exhibited small defects (pits) on the surfaces (Figure 4.12 B, C and D). The appearance of the pits demonstrated the significant effects that the APPJ exposure had on the Ti surface. The SEM images confirmed the surface roughness change after APPJ treatment since the shape structure was etched. Although it was seen that the oxygen APPJ treatment showed the highest number of defects on the Ti surface compared to the nitrogen and argon APPJ treatment, the results could not be significant. Because, AFM roughness results in agreement with this assumption since no significant difference were observed among different gases treatment ($p > 0.05$)(Table 4.3).

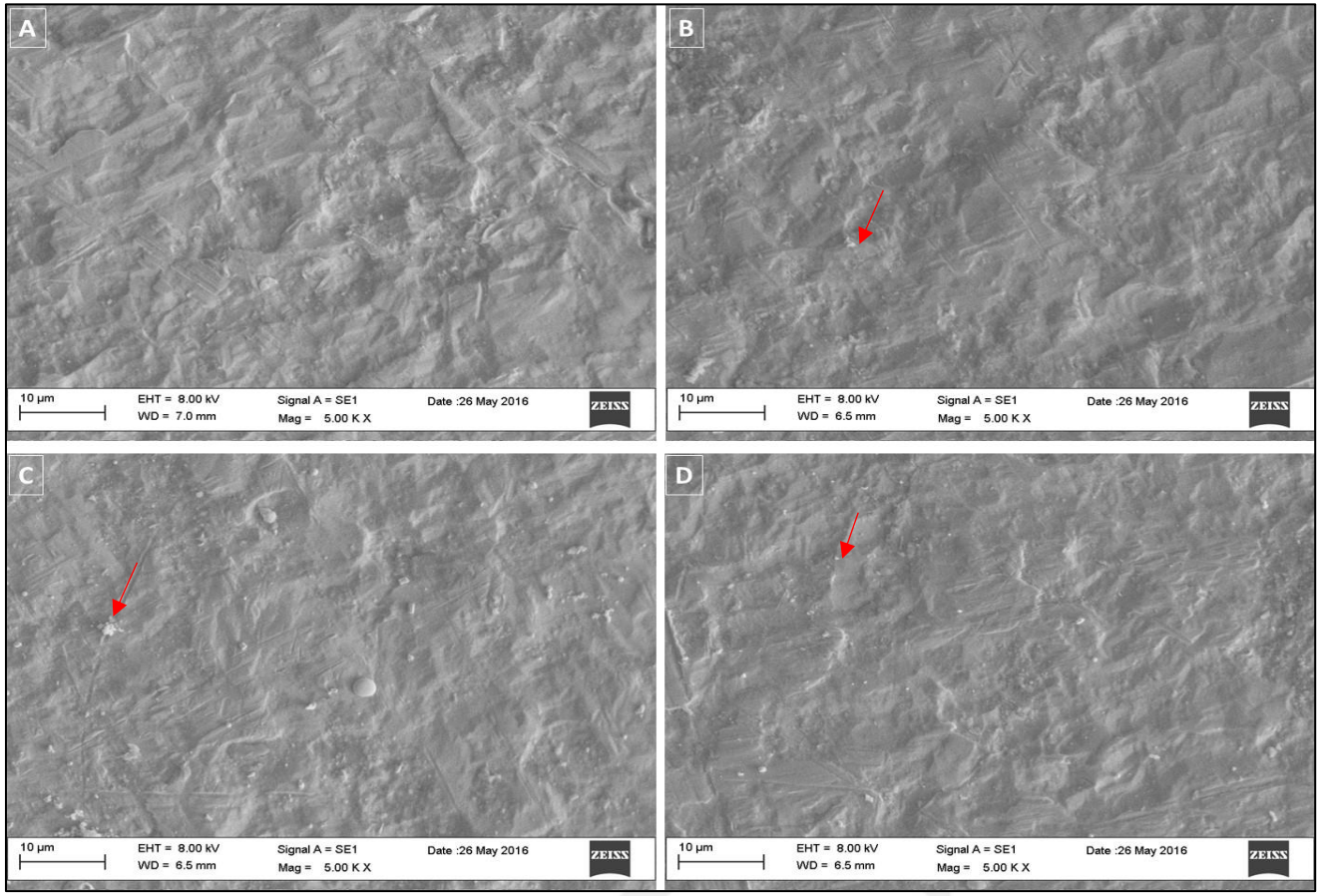

Figure 4.12. SEM images of titanium surfaces before and after APP treatment with each gas at 5000 X magnification. (A) Untreated; (B) Nitrogen plasma treated; (C) Oxygen plasma treated; (D) Argon plasma treated. Red arrows showed the small defects induced by APPJ treatment.

4.3. BACTERIAL ATTACHMENT

In this section, it was examined whether the surface property changes of stainless steel and titanium surfaces induced by the nitrogen, oxygen and argon APPJ treatment had any influence on bacterial attachment. Therefore, the attachment of *Pseudomonas aeruginosa* PAO1to the untreated and APPJ treated stainless steel and titanium surfaces were investigated by the culture plate method. The attachment of bacteria to SS and Ti surfaces that were subjected to before and after APPJ treatment was evaluated after 1 h of incubation of bacterial culture.

The level of bacterial attachment on untreated and APPJ treated SS surfaces was presented in Figure 4.13 which represented the adherence of *P. aeruginosa* by Colony Forming Units (CFU) per ml of the investigated surfaces. The number of attached bacteria to the untreated SS surface was found as $1.4 \times 10^6 \pm 5.36 \times 10^5$ CFU/ml. Following treatment of SS by the nitrogen, oxygen and argon APPJ, the number of attached bacteria was decreased to $1.85 \times 10^5 \pm 6.64 \times 10^4$, $3.60 \times 10^5 \pm 1.85 \times 10^5$and $3.94 \times 10^5 \pm 1.37 \times 10^5$CFU/ml, respectively. The bacterial attachment was significantly decreased as a result of the APPJ treatment of SS ($p < 0.001$). The number of attached cells on the nitrogen APPJ treated SS surface was the lowest compared to other APPJ treated SS surfaces. This result may be explained by the decrease of surface roughness after nitrogen APPJ treatment. However, there was not a significant difference among the APPJ treated SS surfaces for a number of attached cells ($p > 0.05$).

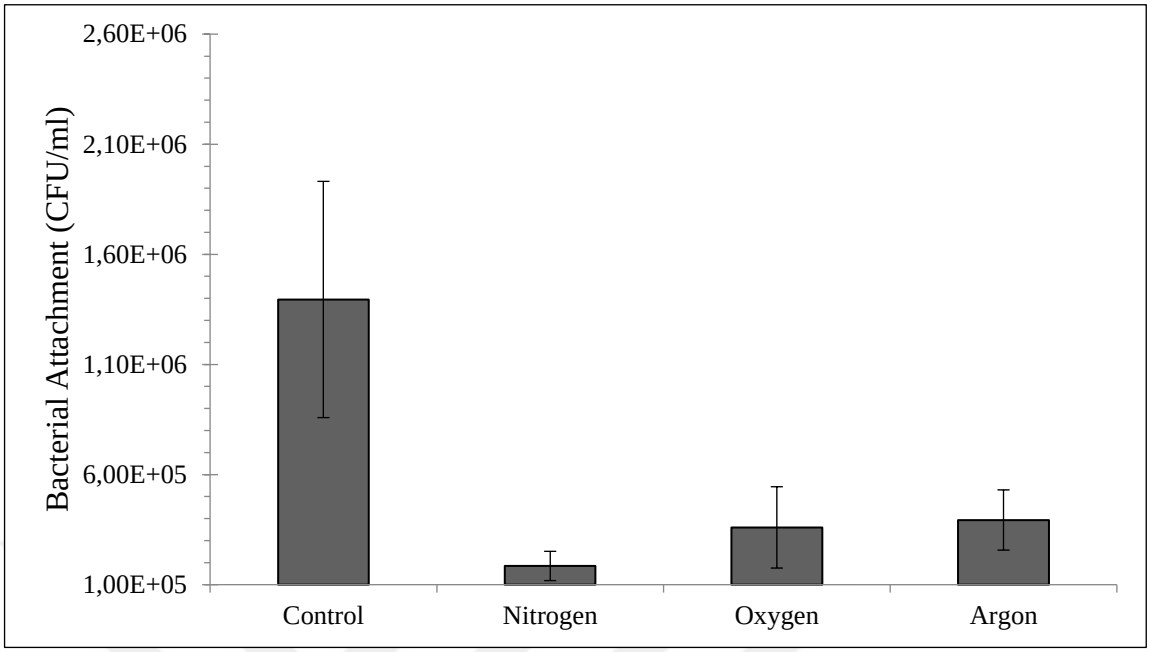

Figure 4.13. Number of bacteria (*P. aeruginosa* PAO1) adhering on different APPJ treated stainless steel L316 with each gas after 1h incubation. Error bars indicate standard deviations.

Figure 4.14 represents the level of bacterial attachment on untreated and APPJ treated Ti surfaces by CFU/ml. The number of attached bacteria to the untreated Ti surface was found as $2.17 \times 10^6 \pm 4.11 \times 10^5$ CFU/ml. Following treatment of Ti by the nitrogen, oxygen and argon APPJ, the number of attached bacteria were decreased to $1.23 \times 10^6 \pm 3.32 \times 10^5$, $8.08 \times 10^5 \pm 2.73 \times 10^5$ and $1.08 \times 10^6 \pm 3.21 \times 10^5$ CFU/ml, respectively. The bacterial attachment was significantly decreased as a result of the APPJ treatment of Ti compared to the untreated surface ($p < 0.001$). Though, the number of cells attached to the oxygen APPJ treated Ti surface was the lowest compared to other APPJ treated Ti surfaces, there was no significant difference was observed among APPJ treated surfaces on the attachment of bacteria ($p > 0.05$).

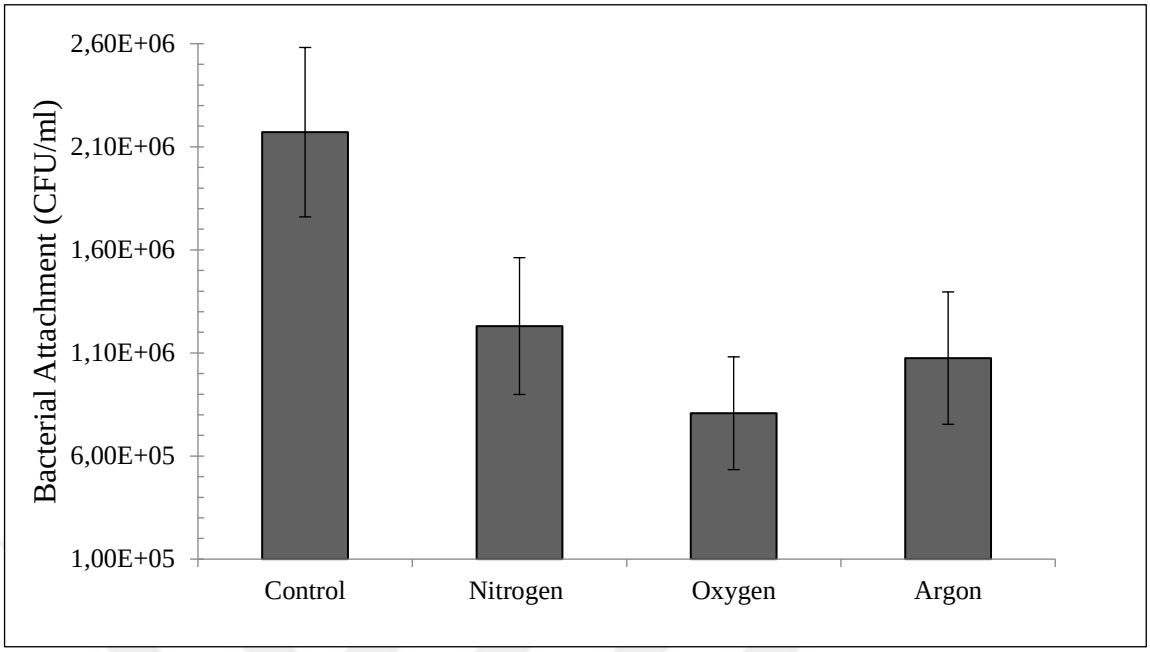

Figure 4.14. Number of bacteria (*P. aeruginosa* PAO1) adhering on APPJ treated titanium (grade 5) (Ti 6 Al-4V) with each gas after 1h incubation. Error bars indicate standard deviations.

The presented results showed that nitrogen, oxygen and argon APPJ treatment limited the early adhesion of *P. aeruginosa* for both surfaces, which exhibited a certain antibacterial property. Many other studies have shown the antibacterial effects of plasma treated various surfaces in agreement with presented results [154, 185]. However, the proper molecular mechanisms for the antibacterial property has not been explained in detail.

The attachment of bacteria on a surface is the most crucial and initial step of biofilm formation (as explained in detail above in the introduction part in section 1.2.1). In recent years, researchers focus on to develop of new surfaces by changing surface properties to decrease initial attachment of bacteria since the properties of the surface and bacteria are important for this initial attachment [161, 185, 190]. Various materials such as metals, polymers, organic and inorganic materials are frequently used to modify by plasma [142, 146, 161, 181, 185].

In this presented research, antimicrobial species generated by the APPJ were determined through Optical Emission Spectrometry. The nitrogen, oxygen and argon APPJ emission spectrums were presented in Figure 4.1, Figure 4.2 and Figure 4.3, respectively. The

emission spectrums demonstrated atomic oxygen species peaks at various wavelengths. These oxygen species have been previously determined to ease etching of bacterial surface resulting in destruction of cell membrane. Moreover, these species cause to disruption of cell metabolism in many different ways which previously described in detail [125, 126, 191].

Furthermore, the reduction in bacterial adhesion may be achieved by reactive species, ions or free radicals produced by plasma, which increased the surface wettability [192]. Surface hydrophilicity/hydrophobicity is important for initial attachment of bacteria [157, 193]. *P. aeruginosa* is hydrophobic bacteria as previously described by Triandafillu et al. [146], and adhere well to hydrophobic materials since hydrophobic interactions generally seem to favour biofilm formation. The APPJ treated SS and Ti surfaces were shown more hydrophilic character (Table 4.4). Thus, plasma treatment could interfere with the adhesion of *P. aeruginosa.*

Bacterial adhesion is also affected by surface topography. Many studies have presented that different bacterial stains prefer to adhere and grow on smoother surfaces [157, 161], however some have suggested that relationship is not linear [181, 190]. For instance, presented results showed that the APPJ treated titanium surface was smoother than the untreated surface with the minimal *P. aeruginous* attachment. However, the nitrogen APPJ treated SS surface had the smoothest surface characteristics, whereas the oxygen and argon APPJ treated SS surfaces were the rougher. Nevertheless, the nitrogen, oxygen and argon APPJ treated SS surfaces showed similarly decreased for *P. aeruginosa* adhesion. Thus, the present results suggested that bacterial adhesion is not limited to the decrease or increase of surface roughness and is presumable dependent on different surface properties including surface charge and free energy which were not studied for in this current work.

4.4. BIOFILM FORMATION

Since APPJ treatment significantly reduced the attachment of *Pseudomonas aeruginosa* PAO1 to stainless steel and titanium surfaces, it was evaluated if this effect was sustained during the subsequent biofilm formation. Biofilm formation of *P. aeruginosa* was determined on untreated and APPJ treated SS and Ti surfaces that after 24 h incubation of bacterial culture by the culture plate method.

Figure 4.15 shows the effect of the nitrogen, oxygen and argon APPJ treatment of SS surfaces on bacterial biofilm formation after 24 h incubation. The biofilm formation of *P. aeruginosa* on untreated SS surface was found with an average of 3.59 x 10^7± 5.12 x 10^6 CFU/ml. The biofilm formation on the nitrogen, oxygen and argon-treated SS surfaces decreased to an average of 1.18 x 10^7± 3.08 x 10^6, 1.47 x 10^7± 4.27 x $10^6$and 3.04 x 10^7± 1.03 x 10^7 CFU/ml, respectively. Biofilm formation on nitrogen APPJ treated SS surface showed a significant decrease compared to the argon APPJ treated ($p< 0.05$) and untreated SS ($p<0.01$), whereas it was significantly similar with oxygen APPJ treated SS surfaces ($p> 0.05$). A significant decrease in biofilm formation was determined for oxygen APPJ treated SS surface compared to the argon APPJ treated ($p< 0.01$) and untreated surfaces ($p< 0.001$). Biofilm formation on the argon APPJ treated and untreated SS surfaces were not detected significantly different in CFU/ml ($p> 0.05$).

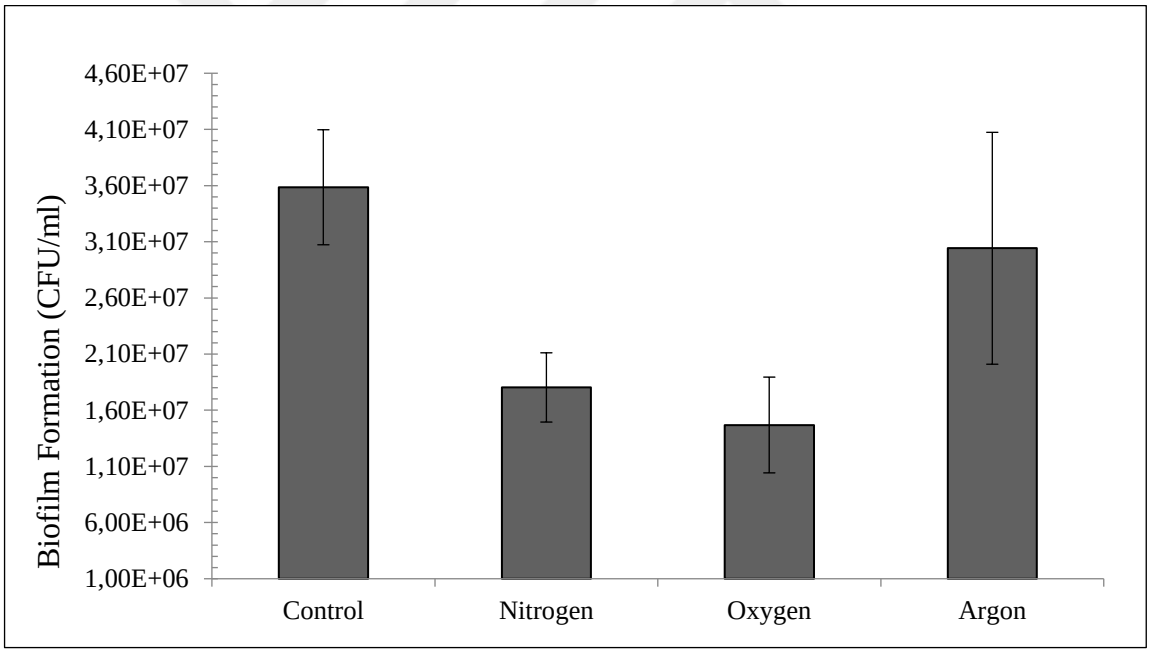

Figure 4.15. Number of bacteria (*P. aeruginosa* PAO1) adhering on APP treated stainless steel L316 with each gas after 24h incubation. Error bars indicate standard deviations.

Figure 4.16 shows the effect of the nitrogen, oxygen and argon APPJ treatment of Ti surfaces on bacterial biofilm formation after 24 h incubation. The biofilm formation of *P. aeruginosa* on untreated Ti surface was found with an average of 2.53 x 10^7 ± 2.29 x 10^6 CFU/ml, while the biofilm formation on nitrogen, oxygen and argon APPJ treated Ti surfaces decreased to

$2.08 \times 10^7 \pm 9.38 \times 10^6$ CFU/ml, $2.11 \times 10^7 \pm 8.32 \times 10^6$ and $1.43 \times 10^7 \pm 3.19 \times 10^6$ CFU/ml, respectively. The biofilm formation on the APPJ treated and untreated Ti surfaces were not found significantly different ($p > 0.05$).

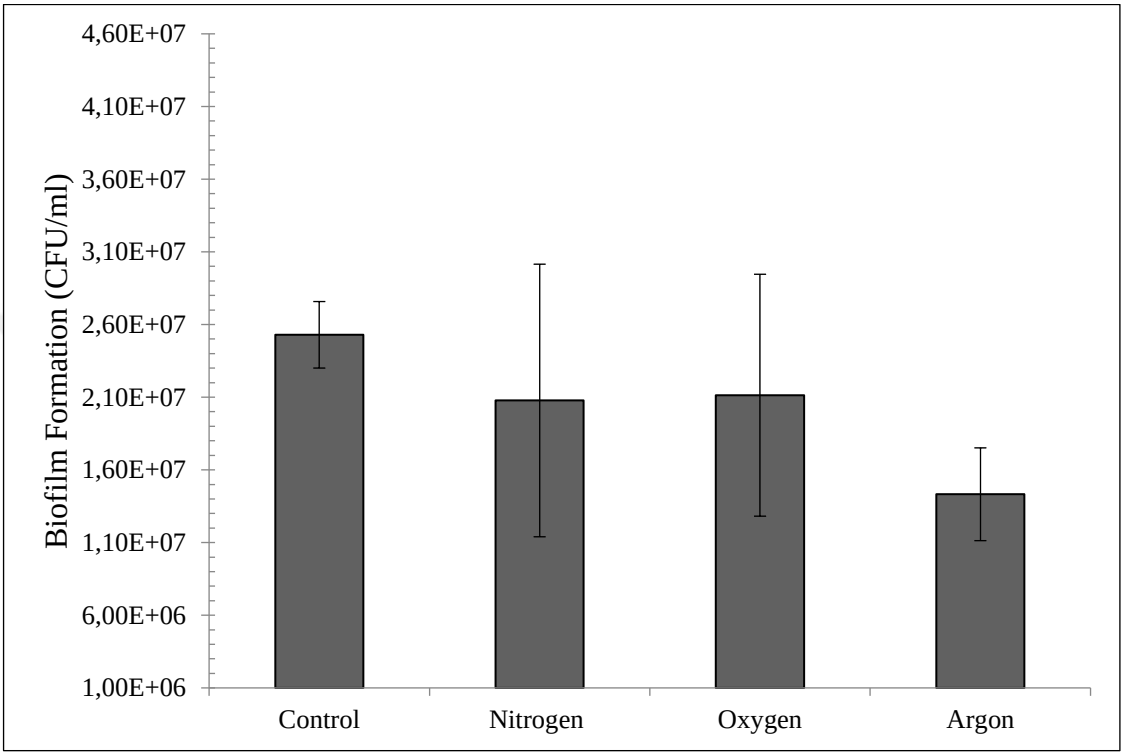

Figure 4.16. Number of bacteria (*P. aeruginosa* PAO1) adhering on APP treated titanium (grade 5) (Ti 6 Al-4V) with each gas after 24h incubation. Error bars indicate standard deviations.

The presented results about the significantly decrease of biofilm formation on the oxygen APPJ treated SS was not found in agreement with previous study. Denes et al. [161] reported to increase of bacterial attachment and also biofilm formation on the oxygen plasma-treated stainless steel. Results were also demonstrated that although the initial attachment of bacteria was decreased on APPJ treated Ti surfaces as a result of changing surface properties, it was not enough to decrease of biofilm formation. To opposite the presented results, previous studies confirmed that biofilm formation was inhibited on plasma treated pure titanium surface compared to untreated ones [181, 194]. These differences may be related to the different parameters during the experiments such as gas, plasma system, microorganism, surface characteristics, etc. However, similar result was also detected for oxygen plasma

treated poly (vinyl chloride) surface. Though the number of adherent bacteria reduced, the reduction was found as not to be sufficient to prevent *P. aeruginosa* biofilm formation [146].

Consequently, the presented results showed that APPJ treatment with different gases on SS and Ti surfaces exhibited diversity for 24 h biofilm formation of *P. aeruginosa.* The factors effecting adhesion of *Pseudomonas aeruginosa* to abiotic surfaces were diverse and physicochemical characteristics of surface and bacteria only were not sufficient to explain the variations in this study.

5. CONCLUSION

Plasma surface activation is an effective and low cost surface treatment technique with a growing interest in many different areas. In this study, stainless steel L316 and titanium (Ti 6-Al 4-V) surfaces were successfully activated using the nitrogen, oxygen and argon gases by non-thermal atmospheric pressure plasma jet system. During plasma treatment, the surface was exposed to a reactive species of a partially ionized gas including free radicals, ions, molecules, and excited species.

The APPJ treatment of surfaces gave the occasion to change the surface properties to obtain better biocompatibility without changing the bulk properties of surfaces. Furthermore, major chemical bond changes on the APPJ treated surfaces were not detected by ATR-FTIR. Surface characterization results showed that the change of the surface properties could be exhibited due to the free radicals created on the surfaces.

The analysis of AFM before and after the APPJ treatment of SS surfaces were exhibited difference depending on the applied gases. To opposite the SS roughness results, the surface roughness of Ti was drastically decreased after APPJ treatment for each gases. Therefore, it may be suggested that surface roughness parameters after plasma treatment may be changed not only depending on the applied gas but also native surface characteristics.

APPJ treatment with different gases significantly enhanced surface wettability and both surfaces displayed a change from hydrophobic to very hydrophilic. As the results of SEM, particle aggregations were influenced by excited molecules and/or reactive species such as O and N atoms, causing bombarding, etching and oxidation, reactively.

Early adhesion and biofilm formation of *P. aeruginosa* PAO1 were investigated on plasma treated and untreated surfaces. The nitrogen, oxygen and argon APPJ treated both surfaces presented lower bacterial attachment compared to the untreated surfaces. The changes in surface properties induced by APPJ treatment affected in the adhesion of bacteria. Hydrophilic property of surfaces after APPJ treatment could prevented bacterial attachment. However, there was not a linear relationship between decrease/increase of surface roughness and bacterial attachment. Different factors may influence the adherence of *P. aeruginosa* to abiotic surfaces, therefore should not be limited to the influence of surface roughness.

Furthermore, though biofilm formation was significantly reduced on the nitrogen and oxygen APPJ treated SS surfaces, the results were not similar for the other APPJ treated surfaces. The decrease biofilm formation was not found significant compared to untreated surfaces.

Within its limitations, the present study highlights potential benefits of APPJ treated SS and Ti surfaces with different gases. These positive effects of APPJ treatment could enhance the biological activity of medical surfaces over time. However, the efficacy of the presented novel plasma system should also be defined for various common hospital-related microorganisms. The results of present study might supply a guidance for the progression of anti-adhesive medical surfaces. Moreover, this surface treatment may be used in medical surfaces before used in surgery.

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