IZMIR KATIP CELEBI UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ANTIMICROBIAL EFFECT OF COLD ATMOSPHERIC PLASMA TO PEGDMA HYDROGEL WITH CELL ENCAPSULATION TESTS

M.Sc. Thesis

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Department of Biomedical Technologies

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İZMİR KATİP ÇELEBİ ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

SOĞUK ATMOSFERİK PLAZMA İLE PEGDMA HİDROJELİNE ANTİMİKROBİYAL ETKİ KAZANDIRILMASI VE HÜCRE ÇALIŞMALARININ YAPILMASI

YÜKSEK LİSANS TEZİ

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ŞUBAT 2019

Çağla YILDIRIM, a **M.Sc.** student of **IKCU Graduate School Of Natural And Applied Sciences**, successfully defended the thesis entitled "**ANTIMICROBIAL EFFECT OF COLD ATMOSPHERIC PLASMA TO PEGDMA HYDROGEL WITH CELL ENCAPSULATION TESTS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my lovely family,

FOREWORD

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January 2019 Çağla YILDIRIM

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ABBREVIATIONS

UV : Ultraviolet
CAP : Cold Atmospheric Plasma
ROS : Reactive Oxygen Species
RNS : Reactive Nitrogen Species
DNA : Deoxyribonucleic Acid
DBD : Dielectric Barrier Discharge
FE-DBD : Floating Electrode Dielectric Barrier Discharge
RONS : Reactive Oxygen and Nitrogen Species
RS : Reactive Species
OD : Optical Density
ECM : Extracellular Matrix
PEGDMA : Poly (ethylene glycol) dimethacrylate
RNA : Ribonucleic Acid
EtO : Ethylene Oxide
TSB : Tryptic Soy Broth
ATCC : American Type Culture Collection
PBS :Phosphate Buffered Saline
TSA : Tryptic Soy Agar
PT-PEGDMA: Plasma Treated Poly (ethylene glycol) dimethacrylate
DMEM : Dulbecco's Modified Eagle Medium
MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
DMSO : Dimethyl Sulfoxide
DIW : Distilleted Water
MRD : Maximum Recovery Diluent

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ANTIMICROBIAL EFFECT OF COLD ATMOSPHERIC PLASMA TO PEGDMA HYDROGEL WITH CELL ENCAPSULATION TESTS

ABSTRACT

Sterilization techniques that are used for polymer based 3D scaffolds are uneffective and changes polymer chemical and mechanical properties. Cold atmospheric plasma is well-known antimicrobial agent that is the fourth state of matter. In this study, antimicrobial effect, cold atmospheric plasma effects on cell viability and polymer structure were investigated as an alternative method for sterilization techniques used in polymer sterilization. Firstly, the antimicrobial effect of cold atmospheric plasma on both bacteria *E. coli* and *S. aureus* was determined. Two different antimicrobial tests were performed. First, the plasma-treated PEGDMA solution was mixed with both bacterial species and in another, contaminated PEGDMA solution was treated with plasma. A 5-log decrease in bacterial density was observed in all plasma application periods and complete inactivation was obtained. In the viability tests that were performed with L929 fibroblast cells encapsulated into PEGDMA, increasing plasma application times decreased cell viability and the most suitable time that was determined with MTT and LIVE&DEAD tests was 1 minute for cell viability. It was observed that 1 minute plasma application time increased cell viability by 560,71%. Mechanical properties of control and plasma applied gels were determined by rheometry. It was observed that plasma application did not affect the mechanical properties. In addition, the antimicrobial effect of the plasma PEGDMA solution was investigated with *E. coli* and *S. aureus* bacteria that were inoculated in the cell media. The PT-PEGDMA solution was found to suppress bacterial growth in low concentrations for both bacteria. Thus, it is predicted that plasma treatment may reduce the use of antibiotics. As a result, it has been seen that plasma is a suitable technique for sterilization of scaffold.

SOĞUK ATMOSFERİK PLAZMA İLE PEGDMA HİDROJELİNE ANTİMİKROBİYAL ETKİ KAZANDIRILMASI VE HÜCRE ÇALIŞMALARININ YAPILMASI

ÖZET

Polimer temelli, 3 boyutlu doku iskelelerinin sterilizasyonunda kullanılmakta olan yöntemler yetersiz kalmakta, polimerin kimyasal ve mekanik özelliklerini değiştirmektedir. Soğuk atmosferik plazma antimikrobiyal etkisi bilinen maddenin dördüncü halidir. Bu çalışmada soğuk atmosferik plazmanın polimer sterilizasyonunda halihazırda kullanılan sterilizasyon tekniklerine karşı alternatif bir yöntem olarak antimikrobiyal etkisi, hücre canlılığına ve polimer yapısı üzerine etkileri araştırılmıştır. Soğuk atmosferik plazmanın ilk olarak *E. coli* ve *S. aureus* bakterileri üzerine antimikrobiyal etkisi belirlenmiştir. İki farklı antimikrobiyal test uygulaması yapılmıştır. İlkinde plazma uygulanan PEGDMA solüsyonu her iki bakteri türü ile karıştırılmış, diğerinde ise kontamine edilen PEGDMA solüsyonu plazma uygulamasına tabi tutulmuştur. Bütün plazma uygulama sürelerinde bakteri yoğunluğunda 5-log düşüş görülerek tam inaktivasyon elde edilmiştir. PEGDMA içerisine enkapsüle edilen L929 fibroblast hücreleri ile yapılan canlılık deneylerinde ise artan plazma uygulama sürelerinin hücre canlılığını düşürdüğü, en uygun sürenin 1 dakika olduğu MTT ve LIVE&DEAD testleri ile belirlenmiştir. 1 dakika plazma uygulama süresinin hücre canlılığını %560,71 oranında artırdığı görülmüştür. Kontrol ve plazma uygulanan jellerin mekanik özellikleri reometre yardımı ile belirlenmiştir. Elde edilen verilerde plazma uygulamasının mekanik özellikler üzerinde etki yapmadığı görülmüştür. Ayrıca plazma uygulanan PEGDMA solüsyonunun antimikrobiyal etkisi hücre besi ortamına ekilen *E. coli* ve *S. aureus* bakterileri ile incelenmiştir. PT-PEGDMA solüsyonunun her iki bakteri için düşük konsantrasyonlarda bakteri üremesini baskıladığı görülmüştür. Bu sayede plazma uygulamasının antibiyotik kullanımını azaltabileceği öngürülmüştür. Sonuç olarak doku iskelesi sterilizasyonunda plazmanın uygun bir teknik olduğu görülmüştür.

1. INTRODUCTION

1.1 Cold Atmospheric Plasma

Plasma is described as ionized gas that contains reactive molecules, electrons, ions, uncharged particles such as atoms and molecules, free radicals, electric fields and ultraviole (UV) radiation. The term of plasma was introduced by Irving Langmuir in 1928. Plasma composes 99% of visible universe can be ground or excited state. Plasma can be classified two groups: thermal and non-thermal or cold plasma. Thermal plasma has electrons and heavy particles at same temperature. Non-thermal or cold atmospheric plasma (CAP) has electrons at hotter temperature and heavy particles at room temperature both at non-equilibrium state. Thus, plasma occurs at temperature between 25ºC - 40ºC at atmospheric pressure. The big advantage of CAP is that the effects of bactericidal, fungicidal and virucidal of plasma last long times and does not be lost [1]. CAP is also effective in cell death (especially cancer cells) and cell detachment, it is used in dentistry and oncology [2].

Thermal, light, or electric energy are used for plasma production. There are different types of CAP treatment such as direct plasma treatment and indirect plasma treatment. In direct plasma treatment, plasma generation occurs between CAP device electrode and tissue surface that acts like an electrode in this treatment type. Particles that are contained by CAP contact with tissue directly. In indirect plasma treatment, CAP occurs in a tube that contains a flowing process gas that carries the reactive molecules and free radicals such as argon, helium, or air [3; 4].

Voltage or output power is effective for plasma efficacy. To achieve higher inactivation rate, higher voltage and/or output power is needed. Plasma efficacy is affected by not only increasing power applied but also increasing treatment time. Concentration of reactive species raise with the increase of exposure time and high concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) results damage of cells and cell membranes where cell damage is a plasma dose

dependent factor. In addition, carrier gas type and water content of ambient air affect the inactivation efficacy of CAP. Amount of ozone, hydrogen peroxide, singlet oxygen will increase as a result of humidity and oxygen presence. These reactive species affect deoxyribonucleic acid (DNA) of bacteria and cell membrane, and pH. Ozone, which is a proven sterilization agent, is the one of main antimicrobial ROS and bactericidal effect correlated with ozone generation. But in humidity, as a result of reaction with ozone, hydroxyl radicals, hydrogen peroxide and hydronium ions occur and increase the oxidative stress [5; 6].

1.1.1 Dielectric Barrier Discharge (DBD)

First experiments about DBD was conducted by Siemens in 1857. To generate DBD plasma, there are 2 flat metal electrode and at least one of them covered with dielectric material such as plastic, ceramic, or quartz [1; 2] with a discharge gap. One of the electrodes is high voltage electrode and the other one is grounded electrode. To produce plasma between two electrodes, high voltages are required. Electrical current is limited by dielectric material, so that arcing is inhibited [1;2]. DBD can be used in different applications such as sterilization o living tissue, bacteria inactivation, angiogenesis, surface treatment, and excimer formation [2].

DBD plasma could be directly applied to living tissue and in this case, it is called as floating electrode (FE-DBD). In FE-DBD plasma a grounded electrode is not needed and the living tissue acts as the grounding electrode.

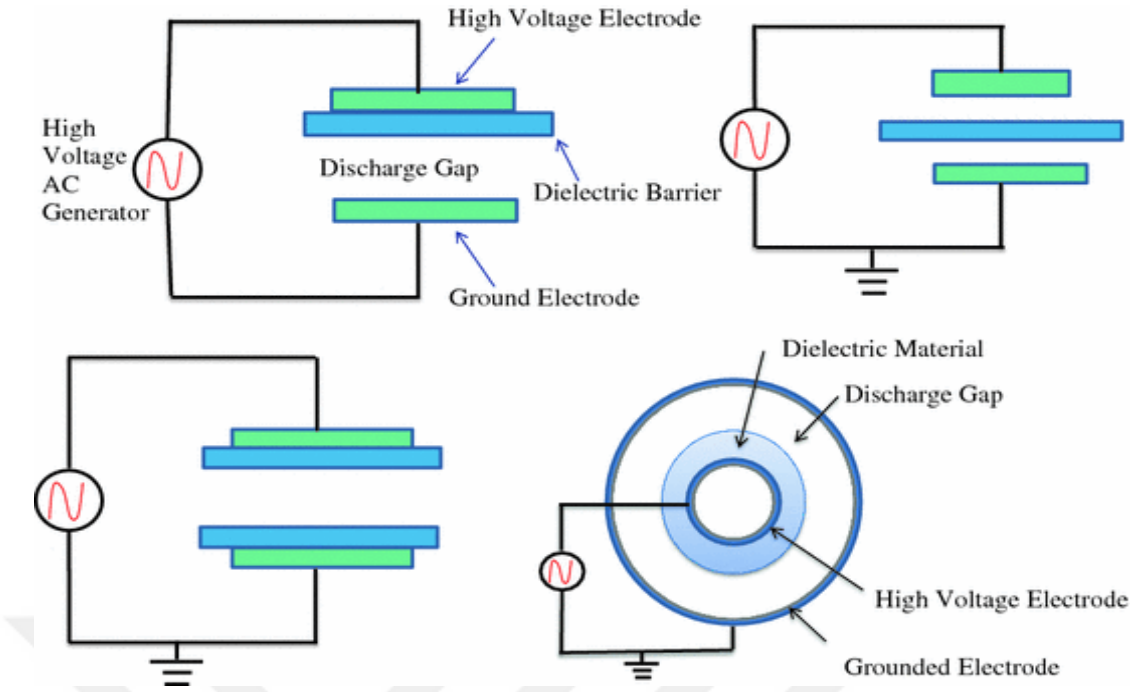


Figure 1.1 : Configuration of common DBD plasma electrode [7].

1.1.2 Plasma Jet

Plasma jet is a cold plasma that produced from two-electrode configuration. Cold plasma formed between two electrodes and a carrier gas that is injected in the inner electrode configuration transports the reactive species (RONS), electrons and uncharged particles from nozzle to the target. Noble gases such as helium and argon can be used as a carrier gas. For specific purposes, other reactive gases such as oxygen can be added to increase the amount of RONS.

Plasma jet can be used as a sterilizer in the biomedical application areas. Containing RONS, electrons and uncharged particles are gained to plasma jet sterilization effect. High density of charged particles provides opportunity of local wound and surface treatment to plasma jet. The plasma plume out of the nozzle can be several centimeters and carries even short-lifetime species to the target to involve in reaction. Thus, plasma jet is used for root canal sterilization to tooth bleaching in dentistry and cancer treatments because of its selectivity and toxicity to cancer cells [1; 8].

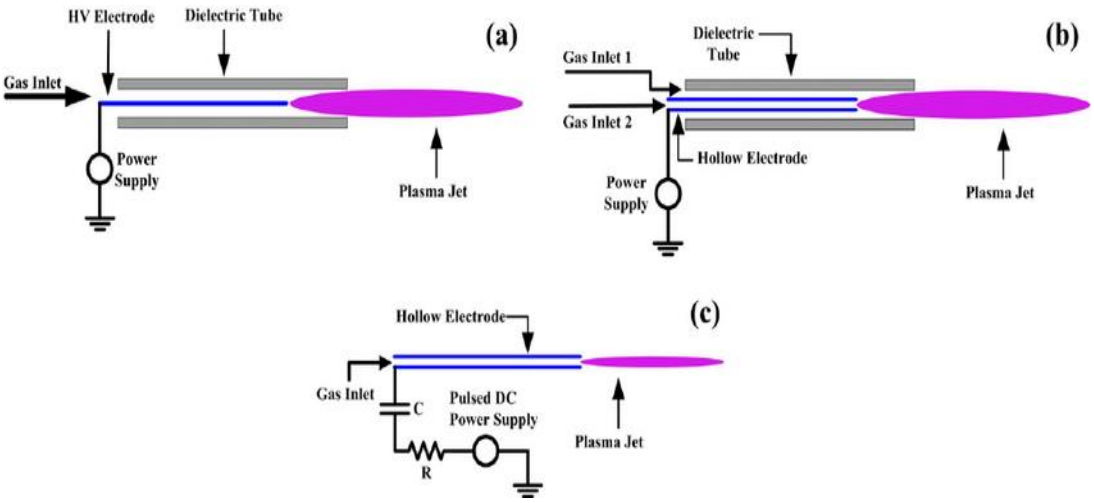


Figure 1.2 : Configuration of plasma jet electrode [9].

1.1.3 Plasma Chemistry

Plasma chemistry is affected by ambient gas that electrons accumulates in it when power is supplied. Electrons from ambient gas interact with other molecules and atoms, reactive species are generated. CAP produces reactive oxygen species (ROS) such as ozone (O_3), atomic oxygen (O), superoxide ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydroxyl (OH), hydrogen peroxide (H_2O_2), reactive nitrogen species (RNS) such as nitric oxide (NO), nitrite (NO_2^-), nitrate (NO_3^-), and peroxynitrite ($ONOO^-$), UV radiation, charged particles and uncharged particles [6; 10]. When energy transfers between electrons and ambient gas, excitation and ionization occur. Chemical reactions takes place in plasma discharge that lead formation of RONS and free radicals are summarized as follow [11; 14]:

$$e^- + H_2O \rightarrow H\cdot + OH\cdot \quad (1.1)$$

$$OH\cdot + OH\cdot \rightarrow H_2O_2 \quad (1.2)$$

$$e^- + O_2 \rightarrow O_2^- \quad (1.3)$$

$$M + O + O_2 \rightarrow O_3\cdot \quad (1.4)$$

$$O_3\cdot + M \rightarrow O_3 + M \quad (1.5)$$

$$N_2 + O_2 \rightarrow 2NO\cdot \quad (1.6)$$

$$NO + O_2\cdot \rightarrow ONOO\cdot \quad (1.7)$$

UV light is absorbed from DNA at 260-265 nm of UV visible spectrum. The effect of UV radiation generated in the CAP is still not shown. Air as a carrier gas is not effective for UV generation. Increasing amount of N_2 in the carrier gas increases UV emission intensity. However, the ratio of N_2/O_2 is equal; UV emission intensity is nearly zero. In addition, gas type changes the UV emission intensity. When argon is

used as a carrier gas at microwave-driven discharges, UV radiation plays an important role for inactivation and sterilization [10]. Operating pressure can affect the UV radiation. Vacuum plasma at very low pressure discharges generates UV radiation at 200-290 nm and can be used for sterilization and inactivation. But at atmospheric pressure does not generate UV radiation in the range of wavelengths of sterilization and inactivation [2].

1.1.4 Plasma Disinfection

1.1.4.1 **Viral inactivation**

The antiviral activity of plasma is reported. The chemical interactions of ROS such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot -}$), and ozone (O_3) and RNS such as peroxynitrous acid (HNO_3) are effective for virucidal activity. The reactive species that previously mentioned react with capsid protein and cause protein peroxidation and capsid destruction [15; 16]. In addition, reactive species can affect the viral genome, reduce gene expression, and eliminate the genome. The primary effect of CAP is chemical modification or denaturation of coat proteins, not genome damage [15; 17].

1.1.4.2 **Fungal inactivation**

The antifungal activity of plasma is weaker when it is compared with antibacterial activity. Because fungi are eukaryotic cells and structure of cell wall is different from prokaryotic cells. Fungi have very thick and chitin based cell wall that provides structural strength and resistance to toxic residues [17]. For inactivation process of fungi, RONS especially ozone is a major inactivation factor in destruction of membrane [18; 19].

1.1.4.3 **Bacterial inactivation**

The antimicrobial activity of CAP is caused by ROS, RNS, UV, and charged particles. Also, the operation parameters and plasma system are effective for antibacterial activity of plasma. Another crucial part of antimicrobial activity of plasma is that application type such as direct and indirect application. In the direct plasma application, ROS, RNS, UV, and charged particles interact with microorganisms, directly. However, in indirect plasma application, there is a carrier

gas to delivery of ROS, RNS, and charged particles and antimicrobial activity is based on long-lived reactive species [2; 10; 17; 20].

Plasma bombard microorganisms with reactive species (RS). Because of RS bombardment and diffusion of RS to the cell membrane, cell membrane proteins can be chemically changed and openings and lesions occur in the cell membrane. Plasma species such as OH, H_2O_2, O, and O_3 break the bounds of both peptidoglycan and lipopolysaccharide (C-O, C-N, or C=C bonds) and destroy the bacterial cell wall [6; 10]. Polyunsaturated fatty acids in the membrane react with ROS and produce a fatty acid radical that is called lipid peroxidase leads to cell leakage. These peroxidases damage DNA and proteins irreversibly [21; 22]. Also ROS can react with DNA and damage in a dose dependent manner [2]. When Gram-negative bacteria is compared with Gram-positive bacteria, their thinner membrane allows inactivation by etching easier than Gram-positive bacteria [20; 23]. In addition, ROS can be effective for inactivation of bacterial spores and biofilm due to breakage of chemical bonds by etching. Reactive species, especially ROS, interact with macromolecules such as membrane lipids, proteins, and DNA and cause increasing oxidative stress. Source of oxidative stress is neutral reactive species such as ozone and atomic oxygen [2; 10]. Mass transport of cell membrane is affected by oxidative stress and reactive species diffuse into the cell, decrease the inner pH. Reduce in the pH cannot maintain by damaged cell and it will be inactivated [20].

Accumulation of reactive species affect the cell membrane and damage the cell surface. Accumulation in the outer side of cell membrane causes the tensile strength and is resulted in rupture [6; 10; 23].

RNS can be form rapidly and damage DNA, protein and lipids. In addition, nitric oxide leads the deamination and convert into other RNS such as $ONOO^-$, HNO_2, and N_2O_3. Guanine, cytosine, and adenine react with RNS and change forms xanthine, uracil, and hypoxantine, respectively, and cause mispairing [6].

Absorbed UV can cause thymine and cytosine in the same strand of DNA into a dimer and inhibit the ability of the bacteria to replicate [10; 21; 24]. In addition, UV photons induce the erosion of the cell due to breakage the chemical bonds.

1.1.5 Plasma-Liquid Interactions

Ions and electrons that are produced by plasma can easily immerse into the bulk liquid and interact with bacteria [25; 26]. Plasma treatment can cause the chemical

changing of physicochemical properties of liquids [27]. As a working gas, air is more effective to inactivate bacteria effectively [26].

Superoxide anion is a radical with bactericidal effect and hydroperoxy radicals can inactivate bacteria directly [25]. The radicals such as $ONOO^-$, HNO_2, and H_2O_2 are necessary for antimicrobial effect [14]. After plasma treatment, acidic solutions that contain hydrogen peroxide (H_2O_2), ozone (O_3), nitrate (NO_3^-), and nitrite (NO_2^-) can be created at pH between 2-3 [28]. The pH of solution treated with plasma decrease because of superoxide anions (O_2^-), hydroperoxy radicals ($HOO\cdot$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) [25-27]. However, antimicrobial effect of plasma correlated with pH decrease of liquids but only acidic conditions can not be effective for antibacterial activity [26]. When pH rises around 3-4, antimicrobial effect decreases.

1.2 Hydrogels

In tissue engineering applications, scaffolds are three-dimensional (3D) structures that mimic the natural extracellular matrices (ECM). The crucial factors of scaffolds encourage cell-biomaterial interactions, cell adhesion promotion, efficient gas, nutrient, and growth factor transport, providing cell survival, proliferation, and differentiation, negligible toxicity, and controllable structure and function of engineered tissue [29].

Hydrogels are insoluble, water-swollen, three-dimensional scaffolds that consist of hydrophilic polymers connected with physical interactions or covalent bonds [29-33]. The hydrophilicity of hydrogels is due to presence of hydrophilic groups such as carboxyl, amino, amide and hydroxyl groups which become hydrated and form hydrogel structure [29; 34]. The advantages of hydrogels are biocompatibility , high water content, availability in wide range of chemistry, low toxicity, and appropriate functionality [30; 33; 34]. Thus, hydrogels can be used in applications such as cell encapsulation, drug delivery, treatment or replacement of tissues/organs, healing of chronic and traumatic wounds, and surface coatings for implants [33; 34]. However, the disadvantages of biomaterials are poor mechanical properties and sterilization difficulties [30]. For sterilization of hydrogels, major sterilization techniques that are ethylene oxide (EtO), radiation, hydrogen peroxide (H_2O_2), or steam are used to elimination of bacterial contamination. However, sterilization techniques might alter

on material structure, chemistry, and molecular weight. The scaffolds made from natural polymers can be denatured with EtO, radiation and steam and lose their function, structure or degradability [35].

Hydrogels can be classified into two categories, the natural and the synthetic hydrogels. The natural hydrogels are chitosan, alginate, collagen, hyaluronic acid, cellulose, agarose, and synthetic hydrogels are polyethylene glycol (PEG), poly(ethylene glycol) dimethacrylate, poly (N-isopropylacrylamide), polycaprolactone (PCL), poly (L-lactic acid) (PLL), poly (lactic-co-glycolic) acid (PLGA) [30; 32; 36-42]. Due to advantages properties such as biocompatibility, inherent biodegradability, similarity to ECM, and critical functions, natural hydrogels can show variety batch-to-batch, poor strength and toughness, do not contain the adequate properties for clinical applications [38; 41]. Besides controllable molecular weight, porosity, producible with uniform quantities, predictable physicochemical and mechanical properties, the main disadvantages of synthetic hydrogels are lack of biocompatibility and biodegradability [36; 39; 43].

1.2.1 PEGDMA

Poly (ethylene glycol) dimethacrylate (PEGDMA) is a PEG-based cross-linkable oligomers and macromers. The cross-linking reaction can trigger with heat or photon [44]. PEGDMA monomers are suspended in a solution then the polymerization reaction starts with exposing UV light. As shown in Figure 1.3, each PEGDMA monomer has two methacrylate groups that react with up to two other methacrylate groups to make covalent bonds [45]. Each monomer link covalently four other PEGDMA monomer, shown in Figure 1.4.

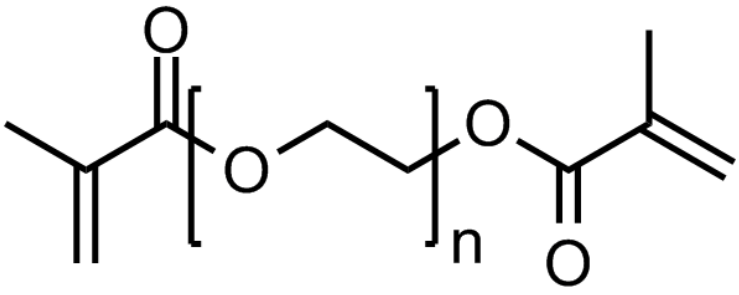


Figure 1.3 : Chemical Structure of PEGDMA.

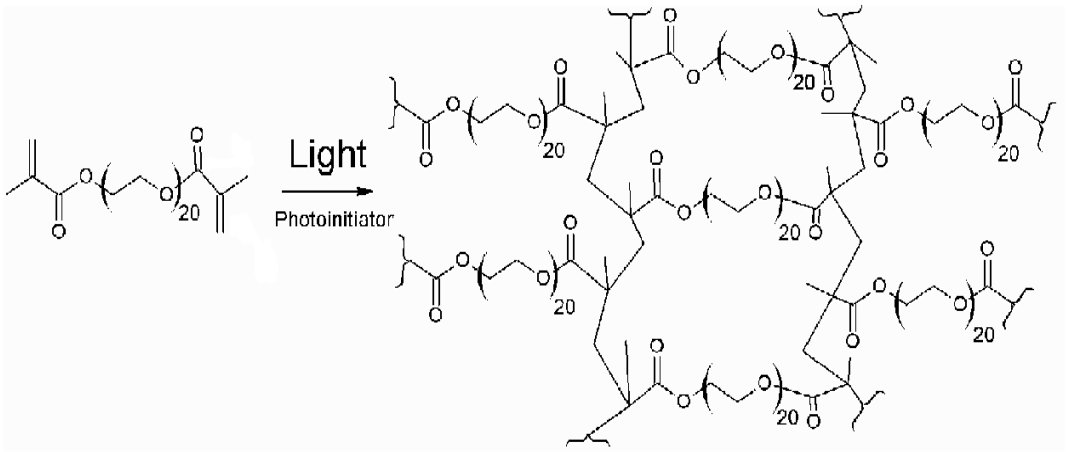


Figure 1.4 : Polymerization of PEGDMA.

PEGDMA is a hydrophilic polymer because of ethylene oxide backbone. In addition, PEGDMA has minimum toxicological response and can be modified to be bio-inert and biocompatible [46; 47]. Higher molecular mass PEGDMA is used to form biocompatible hydrogels for cartilage tissue engineering. Lower molecular mass PEGDMA copolymerize with polymers to use as injectable and cross-linkable composites that have higher biodegradability [44].

1.3 Plasma-Biomaterial Interactions

CAP generally plays role in surface properties, surface activation and functionalization. It is important to design biomaterials and their surfaces for biocompatibility, biofunctionality, biodegradability, cell attachment and proliferation. Plasma treatment of surfaces create unique surface properties to help surface activation and functionalization [48].

Plasma treatment increases surface wettability, roughness, and crosslinking [49]. Plasma treatment can be used for improve the surface properties to immobilize antimicrobial agents such as metal nanoparticles [48; 50]. Thus, biomaterials gain antimicrobial activity with plasma enhanced surface treatment. In addition, immobilization of biomolecules such as peptides, saccharides, provides mimicking extra cellular matrix (ECM) to increase cell attachment and proliferation [49; 51].

Sterilization is a process that eliminates or kills the living organisms, including bacteria, viruses and yeasts. Sterilization techniques of polymers such as dry heat, steam, gamma and UV irradiation, Ethylene oxide (EtO) are used for decades. These

techniques are largely unsuccessful because of sensitive nature and chemical properties of polymers [52; 53].

There are two types of heat treatment: steam sterilization and dry heat sterilization. Steam sterilization occurs at 125-130˚C for 20 minutes and dry heat sterilization occurs at 160˚C for 2 hours. Heat destroys metabolic and structural components. Heat treatment is a sterilization technique that is effective, fast, simple, without toxic residues, and good penetration ability. On the other hand, high temperatures can be effective for polymer chemistry and degrade polymers. It changes the mechanical strength, molecular weight, and structural properties of polymers and scaffolds [52].

Gamma irradiation is a natural phenomenon and obtained from ^{60}Co. For sterilization, gamma rays pass through the polymer or scaffold. Gamma rays breaks DNA and RNA strands and ROS that damages the cells is generated. The advantages of gamma irradiation are simple, rapid, and effective. In addition, this sterilization method can change chemical characteristic of polymer, reduce molecular weight and mechanical strength, and increase degradation rate [52; 54; 55].

UV that is electromagnetic radiation causes DNA damages and prevents DNA replication. There two parameters for the activity of UV irradiation: duration and wavelength- usually between 200-280 nm. The advantages of UV irradiation are low cost and temperature, fast, no toxic residues. However, it can changes structural and biochemical properties of polymer and sometimes is not effective [52; 56; 57].

EtO is chemical agent that is used sterilization processes of rubber and plastic products. EtO causes irreversible alkylation of cellular molecules and suppress cell metabolism and division. Nevertheless, the effect of sterilization of EtO is connected with concentration, temperature, duration, and humidity. EtO sterilization changes the structural and biochemical properties of polymers, decrease mechanical strength, increase stiffness and degradation rate. In addition, residual toxicity of EtO is a post-sterilization problem [52;53].

Plasma as described above is an antimicrobial gas that contains reactive species, charged molecules, electrons, and neutral atoms. Plasma treatment is a sterilization technique that is pyrogen free, no toxic or carcinogenic residues, and does not affect the material properties. Gas choices and flow rate are effective factors for amount of reactive species. To improve the antimicrobial activity, O_2 gas can be added to operating gas to increase amount of ROS. Other important factors include plasma excitation frequency, pressure, and temperature. However, plasma treatment

increases the cross-linking, branching, and degradation that affects the mechanical strength [49; 52; 53; 58]. In addition, presence of RONS after treatment is a post-sterilization problem [52].

1.4 The Aim of The Study

The sterilization techniques such as ethylene oxide, hydrogen peroxide, steam, and irradiation can be used for hydrogels. However these techniques can be ineffective, harmful for hydrogel structure, chemistry, and functionality. Cold atmospheric plasma has strong and broad spectrum antimicrobial effects. PEGDMA is a synthetic polymer that is used for hydrogel production with low toxicity, bio-inert and biocompatible properties.

The purpose of this study is to determine both antimicrobial activity of cold atmospheric plasma as an alternative to currently used sterilization techniques and to investigate the effect on L929 fibroblast cells encapsulated in PEGDMA.

2. MATERIALS-METHODS

2.1 Bacteria Culture Preparation

In the present study, *E. coli* and *S. aureus* were used as gram negative and gram positive model organisms, respectively. Stock cultures of *Escherichia coli* (*E. coli*, ATCC 25922) and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) were removed from -80ºC freezer. The entire contents of the vials were transferred to tube that contains 9 mL Tryptic Soy Broth (TSB, 1.05459, Merck, Germany). Tubes were incubated in shaker incubator at 37 ºC for 24 hours. After incubation, 1 mL of one-night incubated culture was transferred to tube that contains fresh 9 mL TSB and incubated in shaker incubator for overnight at 37 ºC. At the end of second incubation, bacteria were ready to use for antimicrobial tests.

2.2 PEGDMA Solution Preparation

To prepare the photo-initiator Irgacure® 2959 solution, 50 mg Irgacure® 2959 was added to 10 mL sterile phosphate buffered saline (PBS, biowest) and dissolved via sonicator with increasing temperatures. To make 1, 5, 10, and 20% (w/v) PEGDMA (1000, 15178-100, Polysciences, US) solutions, 10, 50, 100, and 200 mg PEGDMA were added to 1 mL of Irgacure® 2959 - PBS mixture, separately, and dissolved with sonicator.

2.3 Dielectric Barrier Discharge (DBD) Electrode

The DBD electrode was constructed by covering a 10-mm thick copper plate (38 mm x 64 mm) with a 1-mm thick glass slide (50 mm x 75 mm). The copper plate was embedded into a polyethylene housing and a high voltage electrical cable connects the electrode to power supply as shown in Figure 2.1. Cold atmospheric plasma was applied at 1.5 kHz and 31 kV at 1 mm discharge gap.

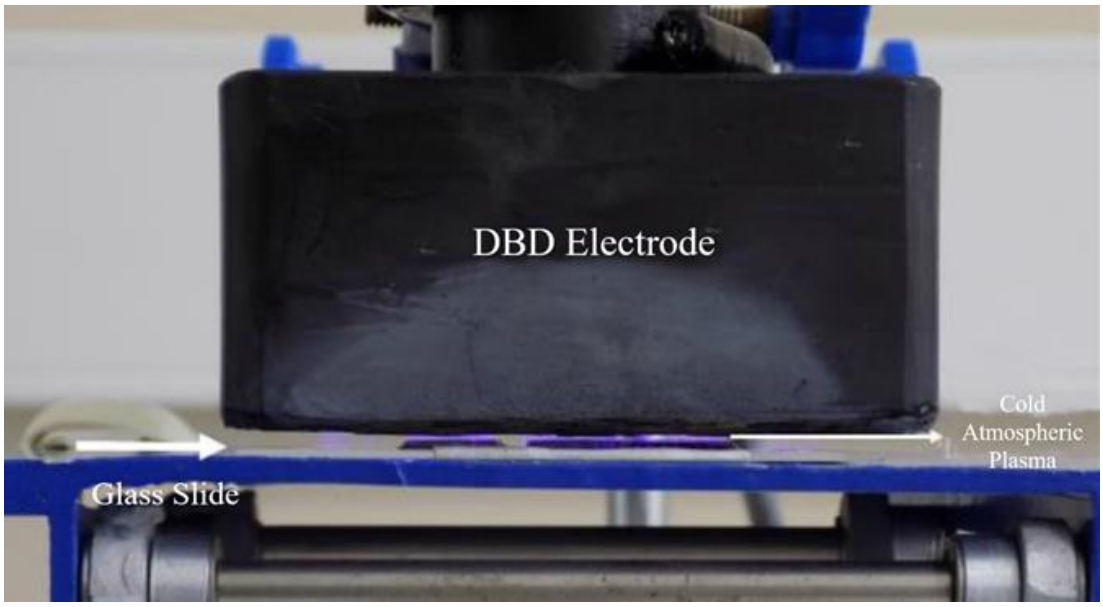


Figure 2. 1 : Dielectric barrier discharge (DBD) electrode.

2.4 Antimicrobial Tests

2.4.1 Determination of Antimicrobial Activity of PEGDMA

Prepared 1, 5, and 10% PEGDMA solutions were taken to the 500 µL glass reservoir for plasma application. Plasma was applied to the 1, 5, and 10% PEGDMA solutions for 1, 2, and 3 minutes with DBD electrode, separately. The 20% PEGDMA solution was treated with cold atmospheric plasma for 1 minute, as describe previously. *E. coli* and *S. aureus* solutions were measured with spectrophotometer to obtain concentration at optical density (OD) 600 nm. Bacterial concentrations were prepared as 10^8 CFU/mL (colony forming units) at 0.2 absorbance (Abs) for *E. coli,* 10^8 CFU/mL at 0.5 Abs for *S. aureus.* The concentrations of bacterial solutions were diluted to 10^5 CFU/mL with PBS by serial dilutions. Plasma treated PEGDMA (PT-PEGDMA) and *E. coli* and *S. aureus* solutions, 100 µL for each solution, were mixed with in tubes, separately, and allowed to stand for 15 minutes. The concentration of PT-PEGDMA-bacteria solutions was then diluted to 10^3 CFU/mL with sterile PBS by serial dilution. Untreated PEGDMA solution as hydrogel control, *E. coli* and *S. aureus* solutions as microorganisms control and PT-PEGDMA solutions were transferred to Tryptic Soy Agar (TSA, Merck, Germany) petri dishes, 100 µL for each solution, and dispersed into the TSA in petri dishes. The petri dishes were waited for 15 minutes to be dried, then incubated for 24 hours at 37ºC. As a result of incubation, the number of the living bacteria cells was counted.

2.4.2 Determination of Antimicrobial Effect of Contaminated PEGDMA

For the other antimicrobial study, 250 µL of 1, 5, and 10% (w/v) PEGDMA solutions were mixed with 10^5 CFU/mL *E. coli* and *S. aureus* solutions, separately, the concentrations were determined at 600 nm as 10^8 CFU/mL with the help of spectrophotometer. Plasma was applied to the PEGDMA-organism solution in a 500 µL glass reservoir with DBD electrode for 1, 2, and 3 minute, 20% PEGDMA, only, was treated with cold atmospheric plasma for 1 minute. Plasma treated PEGDMA-organism solutions were diluted by serial dilution with PBS to 10^3 CFU/mL. Then, plasma treated PEGDMA-organism solutions, untreated PEGDMA-organism solutions as plasma control, and 10^3 CFU / mL *E. coli* and *S. aureus* solutions as microorganism control were transferred to TSA medium in petri dishes and spread on agar. Petri dishes incubated at 37ºC for 24 hours. After incubation, number of living bacteria cell was counted. The steps of antimicrobial tests is shown in Figure 2.2.

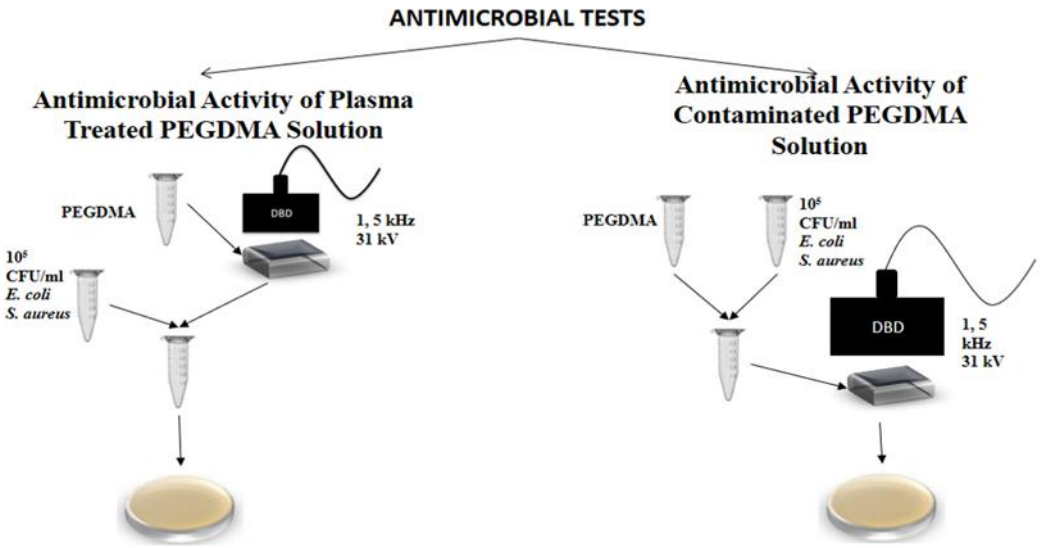


Figure 2. 2 : Steps of antimicrobial tests.

2.5 Determination of pH of PEGDMA Solutions

Plasma treatment was applied for 1, 2, and 3 minutes, separately, on 1, 5, and 10% PEGDMA, as described previously. 20% PEGDMA solution was treated for 1 minute. Solutions were transferred to 500 µL glass reservoir to apply cold atmospheric plasma. After plasma treatment, PT-PEGDMA solutions were

transferred to 24 well plate and for every measurement 2 mL PT-PEGDMA was used.

Plasma treated and untreated samples were measured. 1%, 5%, 10%, and 20% PEGDMA solutions were used for control group. PT-1%, PT-5%, PT-10%, PT-20%, and PT-20/10% PEGDMA solutions were used for plasma treated group. pH probe was used for measurement. pH value of solutions was recorded.

2.6 Determination of Gelation Time

1, 5, and 10% PEGDMA control and cold atmospheric plasma treated solutions were cured with UV lamp in a range of 0 - 9 W/cm^2 for interval of 0-400 seconds to find the lowest power and time that are appropriate for gelation. In addition, concentrations have investigated if gelation was occurred or not. The difference between UV lamp and solution was 7 cm.

2.7 Cell Encapsulation and Viability Test

PT-PEGDMA solutions were applied to different power density and varying durations to determine the appropriate power density and ultraviolet (UV) application (S2000-XLA, Lumen Dynamics, Canada) time for gelation, then optimum PEGDMA density and UV parameters were determined.

In cell encapsulation tests, to promote cell viability, 10% PT-PEGDMA solution was used and 20% PT-PEGDMA solutions was mixed with sterile PBS and the concentration of PEGDMA solutions were reduced by half (PT-20/10% PEGDMA).

L929 fibroblast cells stored in the cryo tube at -80 ºC were thawed immediately using water bath at 37 ºC and cultivated in medium (Dulbecco's Modified Eagle Medium (DMEM), D6429-500mL, SIGMA, Germany) medium containing 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin (1970744, Gibco, US), 100 µg/mL streptomycin (1970744, Gibco, US)). After cultivation at 37 ºC for 24 hours in CO_2 incubator (Forma Steri-Cycle, Thermo Fischer Scientific, US), the cells were removed from the flask surface with using trypsin (03-051-5B, Biological Industries, US). After incubation with trypsin for 5 minutes at 37 ºC, cells were gathered with adding fresh medium in flask and transferred to tube. Tube was centrifuged at 1000 rpm, 4 ºC for 4 minutes with centrifuge (Thermo Fischer Scientific, US). After

removing supernatant, cell solution was mixed with plasma untreated 10% PEGDMA solution, which was control group, 10% PT-PEGDMA, and PT-20/10% PEGDMA solutions with a concentration of $5x10^6$ cells / mL. In the experiments that contains L929 cell line, PEGDMA concentrations reduced by half with cell solution and sterile PBS. UV was applied at 550 mW/cm^2 for 110 seconds to trigger crosslink mechanism. The gels were removed with mold and transferred to 24 well plate. Gel preparation steps are shown in Figure 2.3. Gels which were thickness of 1 mm and diameter of 10 mm were wash with PBS for 3 times. Each gel contains $2.2x10^5$ cells. After removal of PBS, medium was added to them. Gels were incubated at 37 ºC, 5% CO_2 in incubator, MTT and LIVE&DEAD tests were performed at day 1 and day 7, at day 4 only MTT (3- (4,5-Dimethylthiazol-2-yl) - 2,5- Diphenyltetrazolium bromide) test was performed.

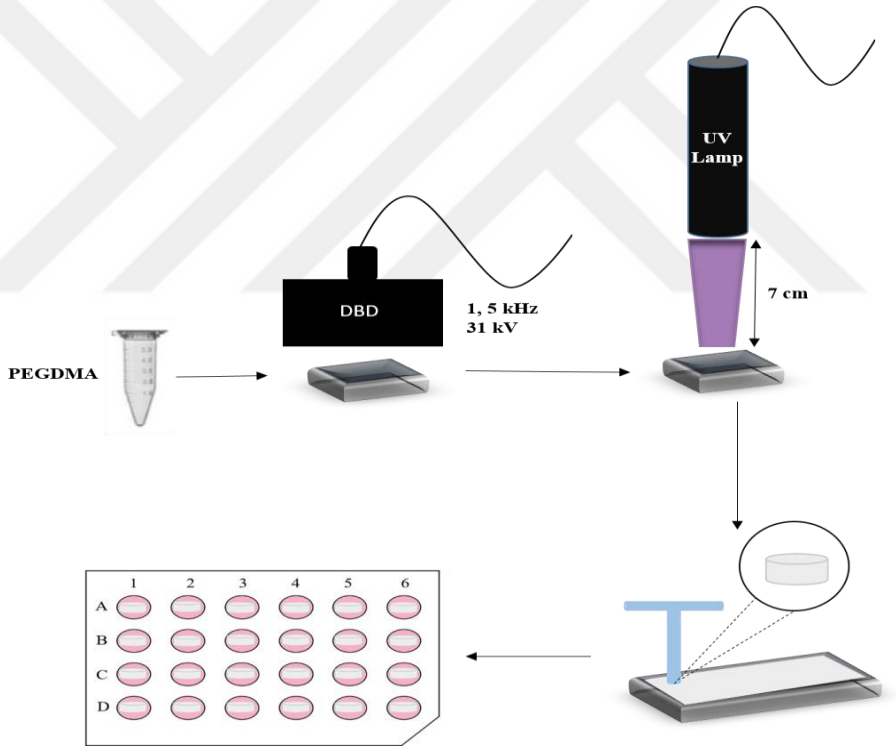


Figure 2. 3 : Gel preparation process.

MTT tests were done at day 1, 4 and 7. MTT solution was prepared with DMEM without supplements and MTT reagent. MTT reagent was mixed with DMEM at 10% (v/v) concentration. Medium that gels were incubated in was removed. MTT solution was added to each well 500 µL and incubated for 2 hours at 37 ºC. MTT reagent was removed and 500 µL dimethyl sulfoxide (DMSO) was added to each well and incubated for 30 minutes at 37 ºC. Then DMSO was collected while using

multiplate reader (BioTek, Winooski, VT, USA), absorbance was recorded at 570 nm.

LIVE&DEAD test was done at day 7. To prepare LIVE&DEAD solution (JV858, Dojindo, Japan), red reagent was added at 0.3% (v/v) concentration, green reagent was added at 0.2% (v/v) concentration to PBS. Medium that gels were incubated in was removed. LIVE&DEAD solution was added to each well and incubated for 30 minutes at 37 ºC. After incubation, gels were washed with PBS for 3 times and some PBS was added to prevent gels from being dried. To view cells inside to gels, inverted fluorescent microscope (Olympus CKX41, Tokyo, Japan) was used.

2.8 Swelling Ratio and Sol Fraction

Untreated 10% PEGDMA gels as control and PT-20/10% PEGDMA gels were prepared as describe above. For each group, 6 gels were used. Gels were removed with mold and dried for 12 hours at ambient conditions and then allowed to dry at 40 ºC for 1 hour under vacuum. After drying, the dried gels weights (W_i) were recorded. After drying, samples were swollen in distillated water (DIW) for 24 hours at 37 ºC and medium was changed at every 6 hours. Swollen weight of gels (W_s) were recorded after medium was removed. Then gel samples were dried again as describe above and weights (W_d) were recoded. The formula of swelling ratio (Q) and sol fraction (S) are:

$$Q = (W_S - W_d) / W_d \times 100 \quad (2.1)$$

$$S = (W_i - W_d) / W_i \times 100 \quad (2.2)$$

The effect of plasma on the amount of water holding capacity and sol fraction of the gels were investigated.

2.9 Rheological Measurements

Untreated 10% PEGDMA and PT-20/10% PEGDMA gels were used for rheological measurements.

Disk shaped hydrogels were prepared with thickness of 4 mm and diameter of 25 mm. To achieve the desired thickness, gels were formed in 2 steps. For the first step, 1 mL of PEGDMA solution was molded and UV applied at 550 mW/cm^2, 400

seconds. The gap between UV lamp and sample was 7 cm. After gelation, for the second step, 1 mL of PEGDMA solution was added to the container on newly prepared gel. Second gelation was done by applying UV lamp at same power and time. Prepared gels were transferred to 60 mm petri dishes that contains DIW and waited in DIW until rheological measurement.

Prepared gels were cut to diameter of 20 mm and loaded on the Peltier plate (Hybrid Rheometer Discovery HR-2, TA Instruments, New Castle, DE). The gap was 2000 μm and applied shear rate was 0,1-1000 1/s. Angular frequency was applied as 0,1-100 rad/s.

2.10 Antimicrobial Effect of Plasma Treated and Gelated PEGDMA

20% PEGDMA was treated with cold atmospheric plasma for 1 minute, as described before. After treatment, 20% PEGDMA solution was diluted at 1:1 ratio with PBS. As PEGDMA solution, there were 2 different solutions: Control (untreated, C) and PT-20/10% PEGDMA solutions. Concentration of *E. coli* and *S. aureus* bacteria solutions were prepared with spectrophotometer. For test, 5 different bacteria concentrations were used: 10^4, 10^3, 10^2, 10^1 CFU/mL and 0. As medium, 2 different DMEM were used: DMEM with Antibiotic (DMEM/A+) and DMEM without Antibiotic (DMEM/A-).

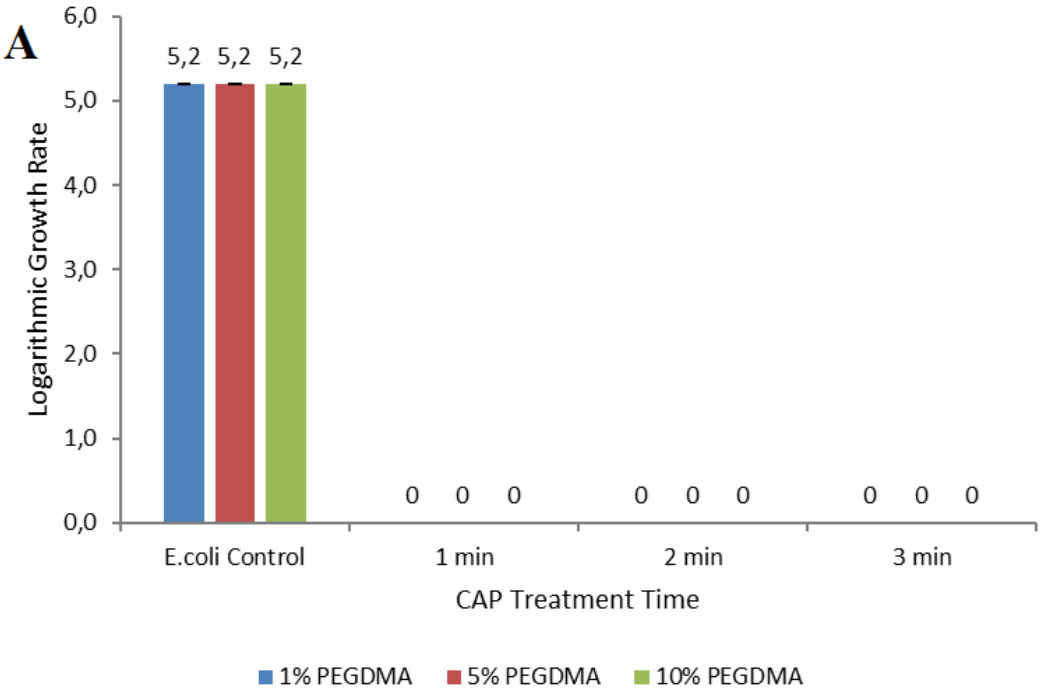
Volume of 1 gel was calculated with using cylinder volume equation as μL. The volume of 1 PEGDMA hydrogel is 78 μL. The volume of gel was mixed with DMEM and bacteria solutions that were different concentrations (10^4, 10^3, 10^2, 10^1 CFU/mL and 0), separately, and the total volume was 500 μL. 300 μL of the mixed solution was transferred to 48 well plate. Well plates were incubated at 37 ºC for 24, 48, and 72 hours. Absorbance of well plates were collected with multiplate reader at 600 nm.

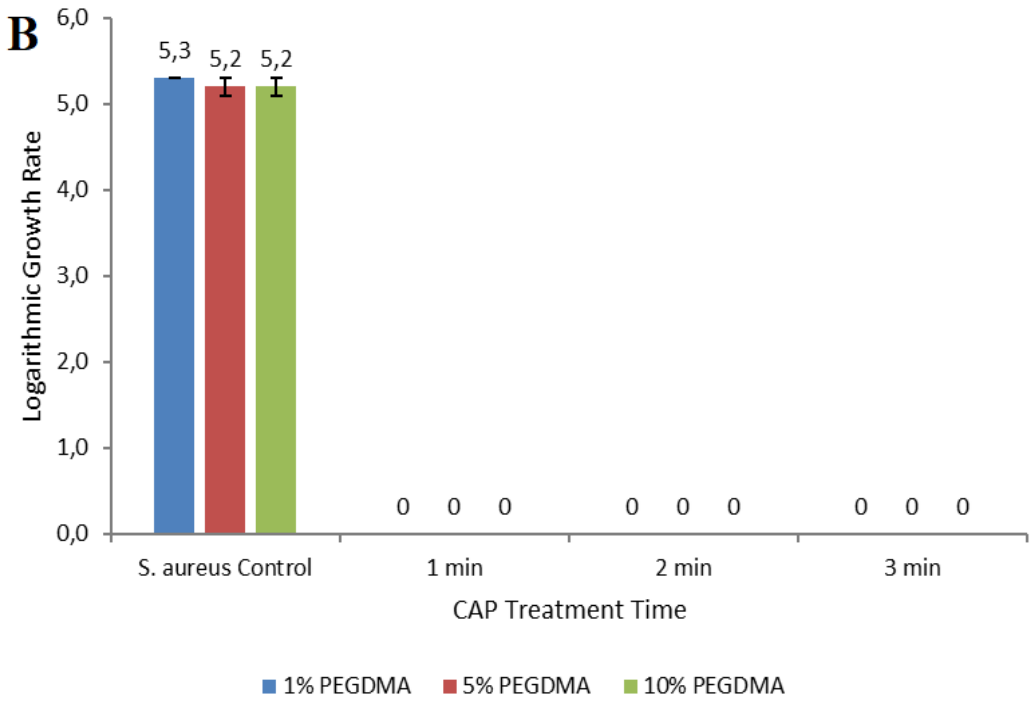
3. RESULTS

3.1 Antimicrobial Tests

3.1.1 Determination of Antimicrobial Activity of PEGDMA

Pre-antimicrobial tests for 1, 5, 10, and 20% PEGDMA were done. Three different concentrations and three different CAP treatment times were tested. PT-PEGDMA solutions were mixed with bacteria solutions and spread on agar then incubated. After incubation, number of colonies were counted. As shown in Figure 3.1-A and 3.1-B, for both *E. coli* and *S. aureus*, bacterial growth was observed only control groups. The colonization of control groups that contained 1, 5, and 10% PEGDMA concentrations, separately, were 5.2-log for *E. coli* and 5.3-log, 5.2-log, and 5.2-log for 1, 5, and 10% PEGDMA, respectively, for *S. aureus*. For PT-PEGDMA groups, for both bacteria, no bacterial growth was observed. For PT-20/10% PEGDMA concentration, the colonization of control group was 5-log for both bacteria, shown in Figure 3.1-C and 3.1-D.


A
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Logarithmic Growth Rate
5,2 5,2 5,2
0 0 0 0 0 0 0 0 0
E.coli Control
1 min
2 min
3 min
CAP Treatment Time
1% PEGDMA
5% PEGDMA
10% PEGDMA


B
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5,0
4,0
3,0
2,0
1,0
0,0
Logarithmic Growth Rate
5,3 5,2 5,2
0 0 0 0 0 0 0 0 0
S. aureus Control
1 min
2 min
3 min
CAP Treatment Time
1% PEGDMA
5% PEGDMA
10% PEGDMA

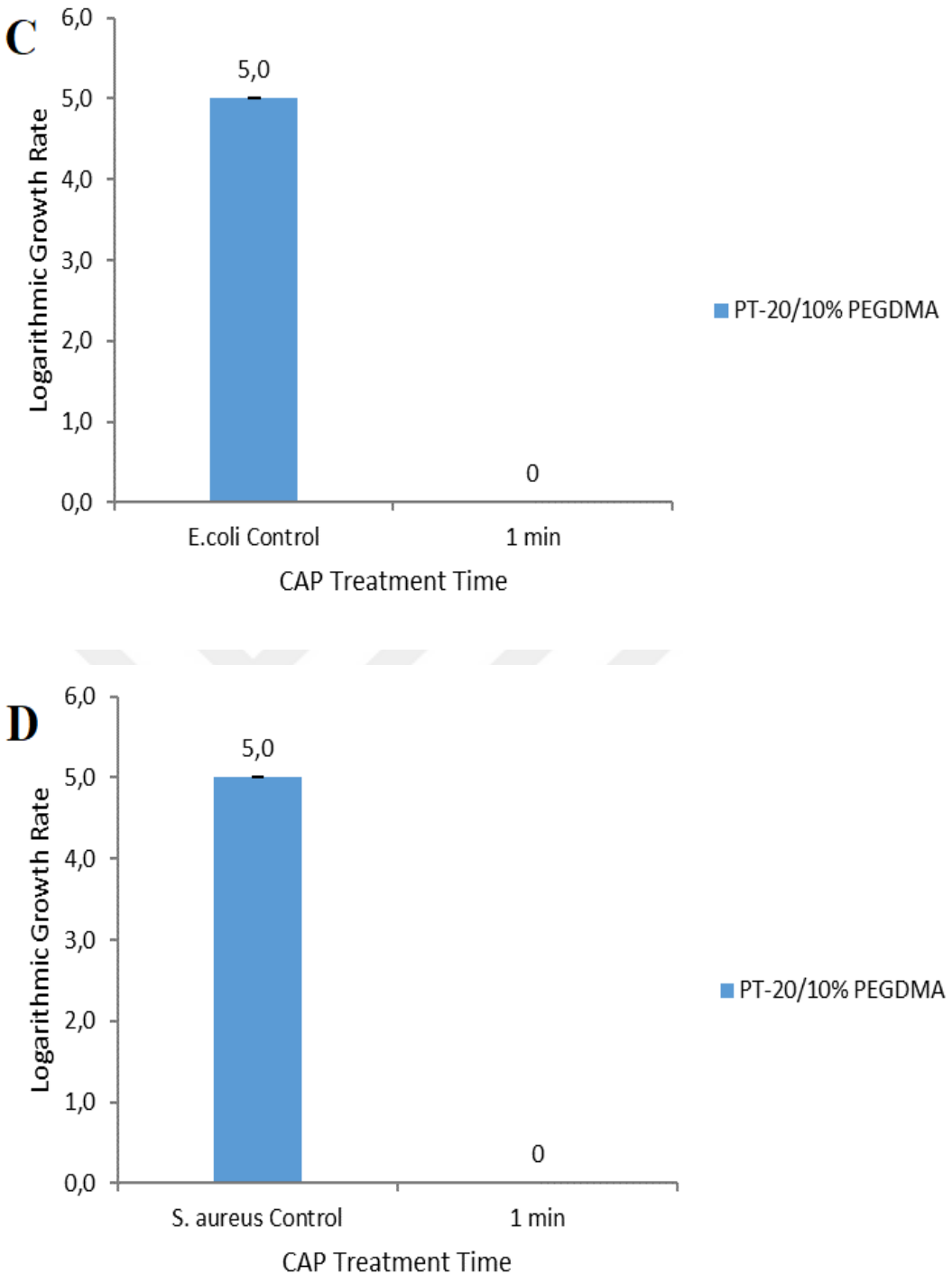
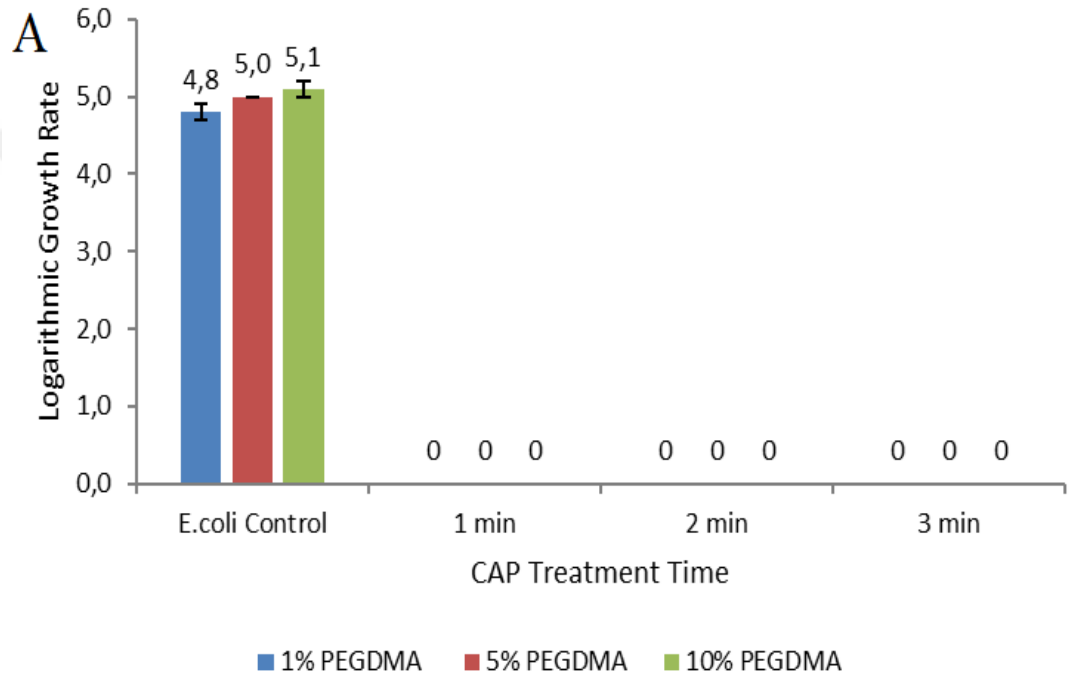


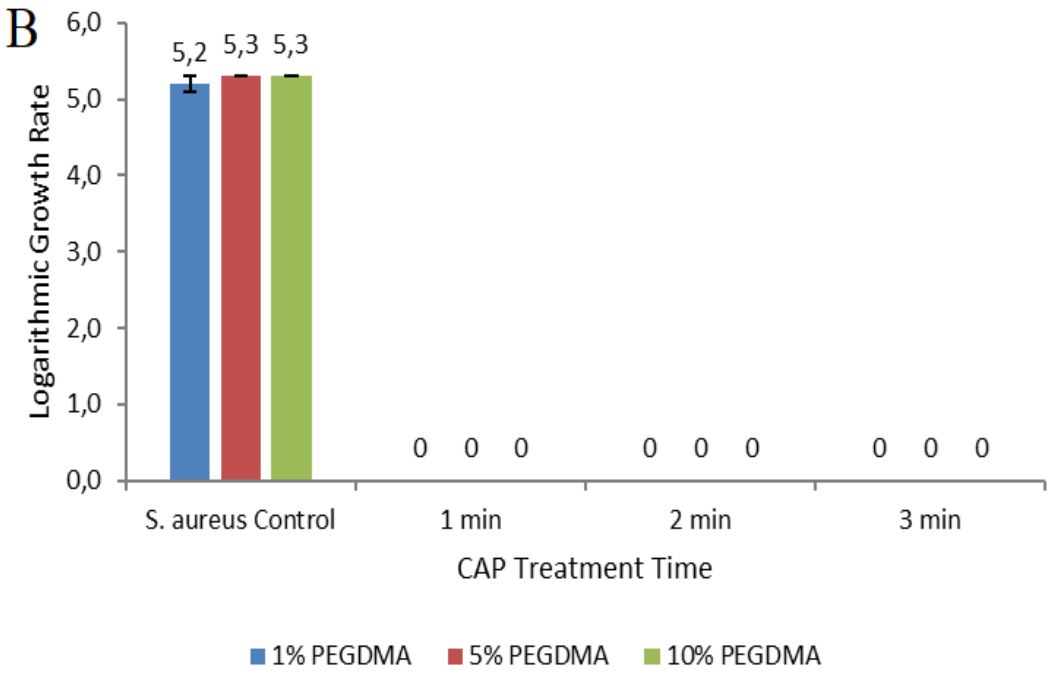
Figure 3. 1 : Antimicrobial activity of PEGDMA on A)1, 5, and 10% PEGDMA concentrations on *E. coli*, B) 1, 5, and 10% PEGDMA concentrations on *S. aureus*, C) PT-20/10% PEGDMA on *E. coli*, D) PT-20/10% PEGDMA on *S. aureus*.

3.1.2 Determination of Antimicrobial Effect of Contaminated PEGDMA

Contaminated PEGDMA solutions of 1, 5, and 10% with both *E. coli* and *S. aureus*, separately, were treated with CAP. After treatment, solutions spread on agar and

incubated. After incubation, colony counting was done. As shown in Figure 3.2, bacterial growth was observed only control groups. For *E. coli*, 4.8-log, 5.0-log, and 5.1-log bacterial growth was observed for 1, 5, and 10% PEGDMA concentrations, respectively. For *S. aureus*, 5.3-log bacterial growth for 1, 5, and 10% PEGDMA concentrations for each treatment time. 5.1-log and 5.2-log bacterial growth for PT-20/10% PEGDMA were observed on *E. coli* and *S. aureus*, respectively. No bacterial growth was observed for PT-PEGDMA groups for each CAP treatment time for both bacteria.




B
6,0
5,0
4,0
3,0
2,0
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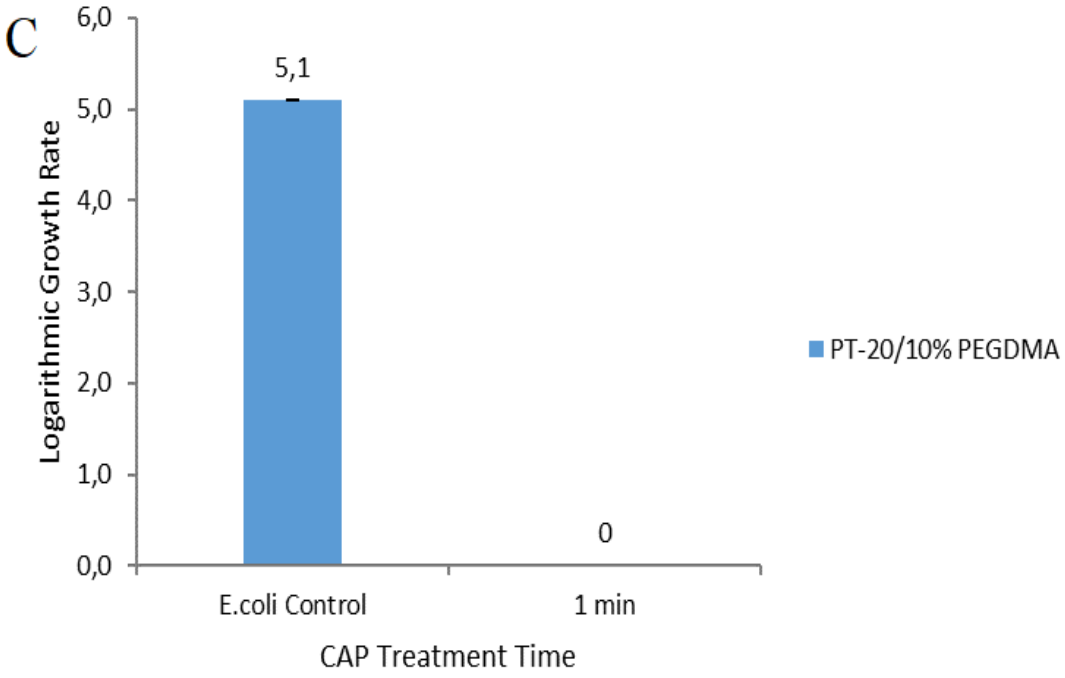
Logarithmic Growth Rate
5,2
5,3
5,3
0 0 0
0 0 0
0 0 0
S. aureus Control
1 min
2 min
3 min
CAP Treatment Time
1% PEGDMA
5% PEGDMA
10% PEGDMA


C
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4,0
3,0
2,0
1,0
0,0
Logarithmic Growth Rate
5,1
0
E.coli Control
1 min
CAP Treatment Time
PT-20/10% PEGDMA

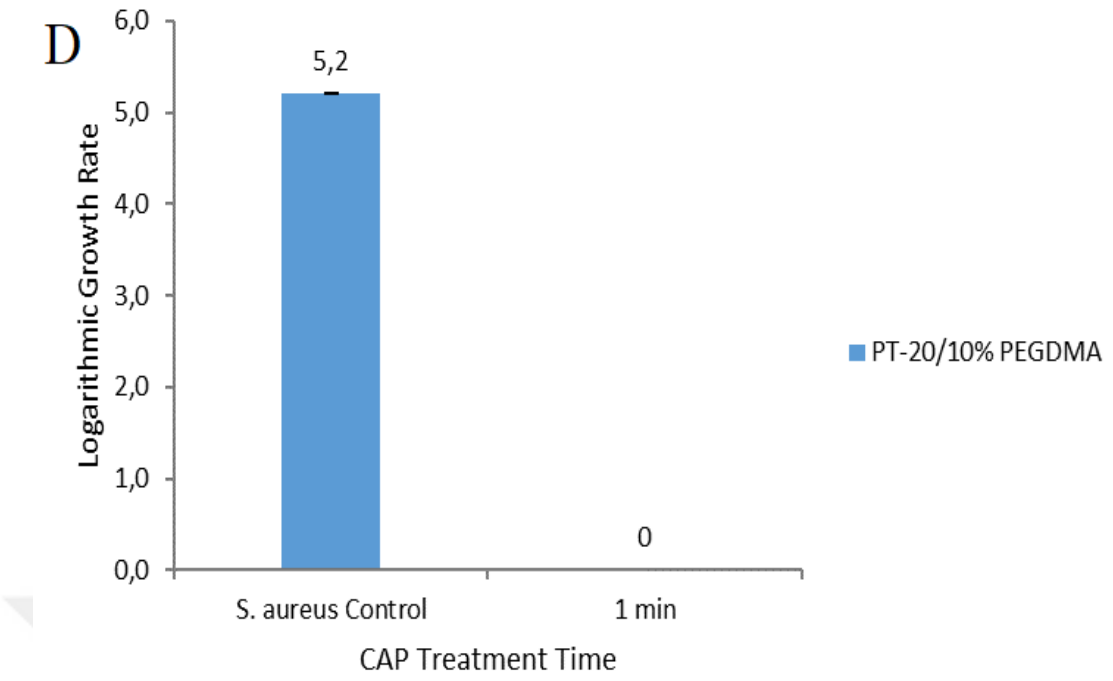


Figure 3. 2 : Antimicrobial effect of contaminated PEGDMA, A) 1, 5, and 10% PEGDMA on *E. coli*, B) 1, 5, and 10% PEGDMA on *S. aureus*, C) PT-20/10% PEGDMA on *E. coli*, D) PT-20/10% PEGDMA on *S. aureus*.

3.2 Determination of pH of PEGDMA Solutions

As shown in Table 3.1, pH values of 1% PEGDMA were 3.95, 2.80, 2.32, and 2.00 for control, PT-1 min, PT-2 min, and PT-3 min, respectively. For 5% PEGDMA solutions, pH values were 3.80, 3.19, 2.64 and 2,16 for control, PT-1 min, PT-2 min, and PT-3 min, respectively. The pH values of 10% PEGDMA were 3.92, 3.30, 2.81, and 2.43 for control, PT-1 min, PT-2 min, and PT-3 min, respectively.

Table 3. 1 : pH values of 1, 5, and 10% PEGDMA concentrations.

	Control	PT-1 min	PT-2 min	PT-3 min
1 % PEGDMA	3.95	2.80	2.32	2.00
5 % PEGDMA	3.80	3.19	2.64	2.16
10 % PEGDMA	3.92	3.30	2.81	2.43

As shown in Figure 3.3, the pH values were 3.26, and 3.60 for control 20% PT-PEGDMA and PT-20/10% PEGDMA, respectively. The pH value of groups clearly showed that cold atmospheric plasma treatment affected the pH of PEGDMA solutions. The pH of control group was higher than cold atmospheric plasma treated groups ($p<0.05$).

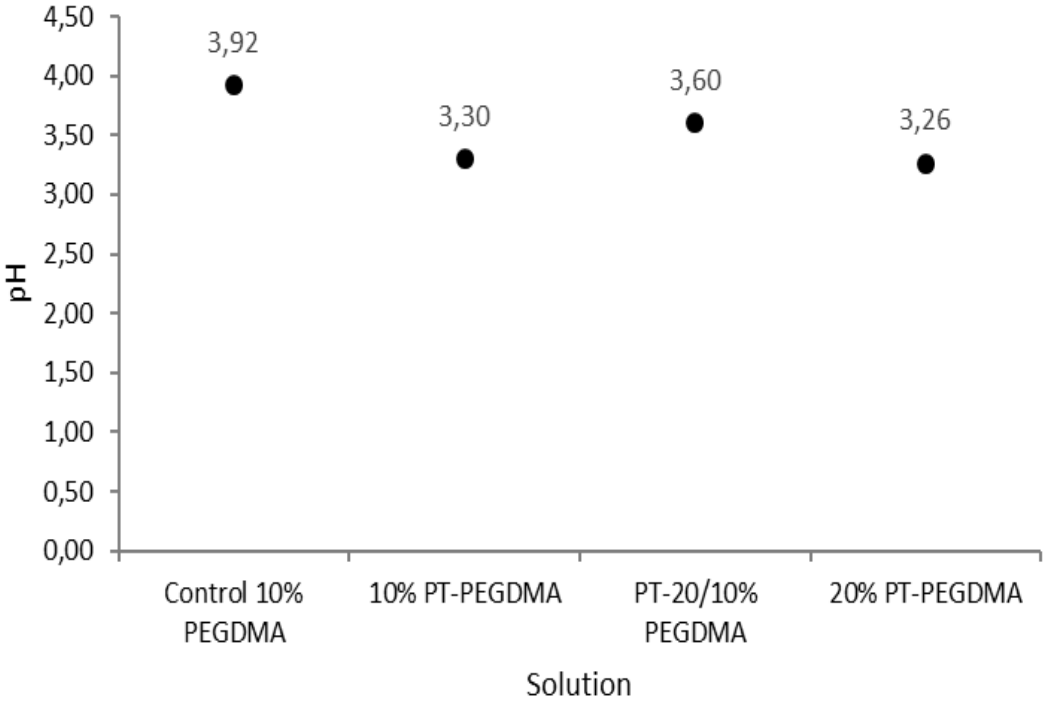


Figure 3. 3 : pH values of plasma treated and untreated 10% PEGDMA, PT-20/10% PEGDMA and 20% PT-PEGDMA.

3.3 Determination of Gelation Time

Gelation time tests were done for each concentration, time and power. No gel formation occurred for both control and CAP treated 1% and 5% PEGDMA solutions at each power and time. For 10% PEGDMA solutions, both control and CAP treated solutions formed gel. Lowest time and power were selected for cell viability tests: 110 seconds and 550 mW/cm^2.

3.4 Cell Encapsulation and Viability Tests

10% PEGDMA concentration was selected for cell viability tests. Control 10% and 10% PT-PEGDMA solutions that treated for 1, 2, and 3 minutes, separately, were formed gel and to determine the cell viability MTT tests were done. As shown in

Figure 3.4, cell viability values of control group were 94%, 101% and 132% at Day 1, Day, 4 and Day 7, respectively. For 1 min - 10 % - PT-PEGDMA, cell viability values were 185%, 211%, and 288% at Day 1, Day, 4 and Day 7, respectively. Cell viability values of 2 min - 10% - PT-PEGDMA were 58%, 79%, and 55% at Day 1, Day, 4 and Day 7, respectively. For 3 min - 10% - PT-PEGDMA, cell viability values were 33%, 49%, and 41% at Day 1, Day, 4 and Day 7, respectively.

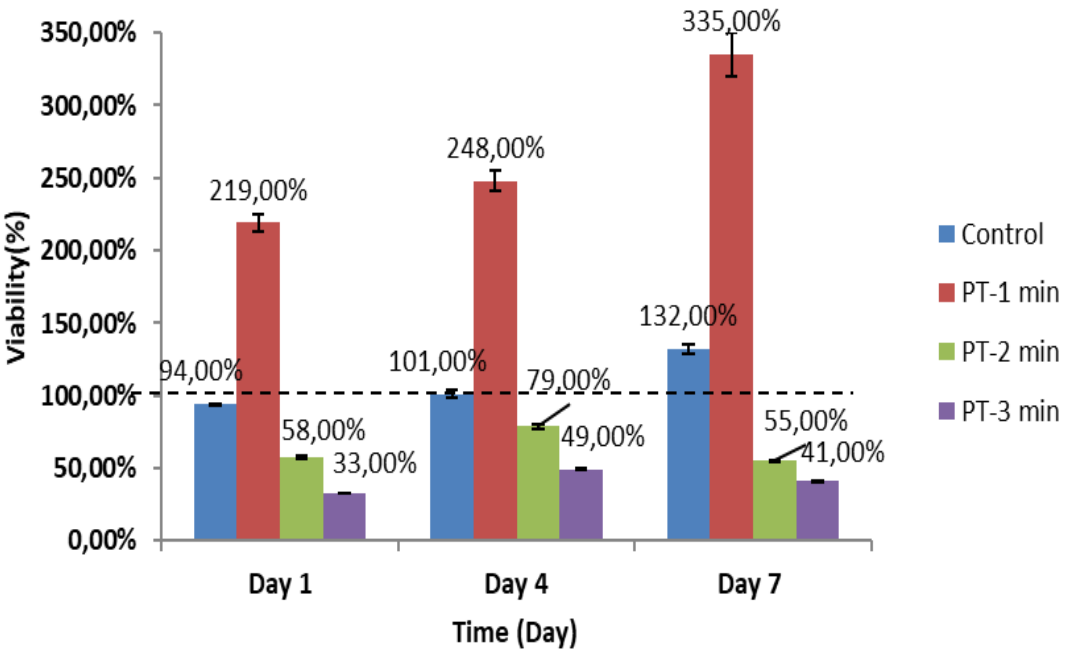


Figure 3. 4 : Cell viabilities of 10 % PEGDMA with changing time.

The cell encapsulation tests were done with 2 groups: Control (untreated PEGDMA) and PT-20/10% PEGDMA. Cell viability of control group at Day 0 was assumed as 100% and other viability percentages were calculated based on Control-Day 0. As shown in Figure 3.5, control groups' cell viability was increased during first 4 days. The viability percentages were 100%, 245.24%, and 292.86% at Day 0, Day 1, and Day 4, respectively, but at Day 7, the viability of L929 cell line was decreased to 122.62%. For plasma treated groups, at Day 0, cell viability (83.93%) was under 100%. In the upcoming days, cell viability of PT-20/10% PEGDMA groups was increased to 161.90% at Day 1 and 560.71% at Day 4. The cell viability was come to a head at Day 4 for both control and plasma treated groups. But at Day 7, percentage of cell viability was decreased to 386.90%.

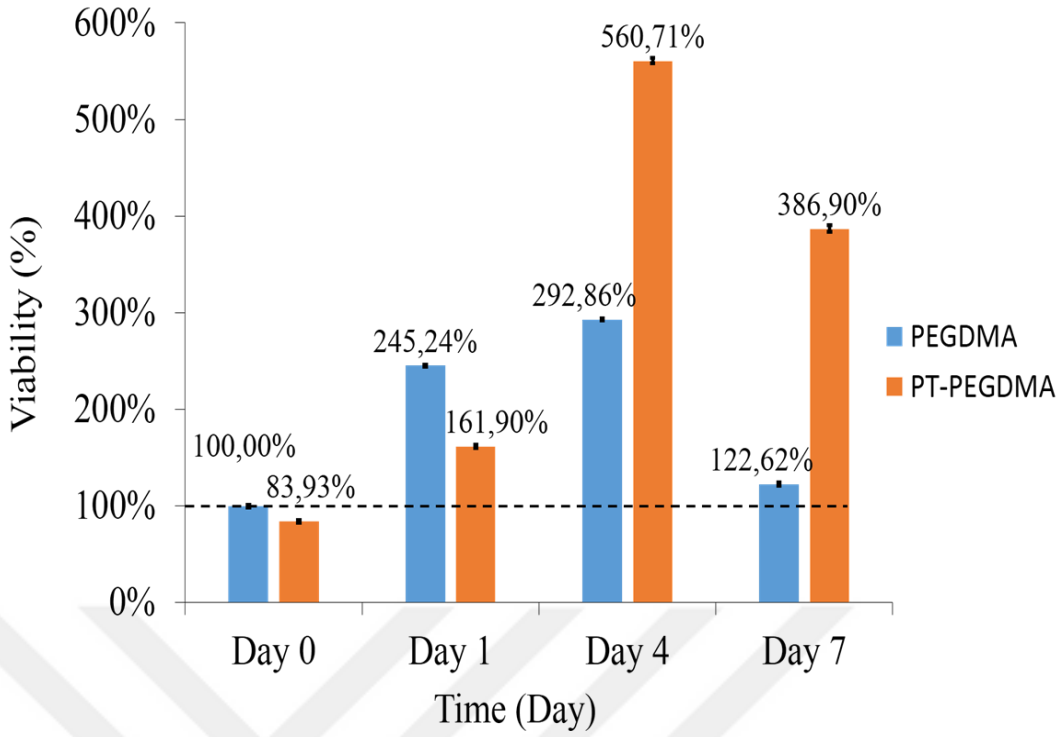


Figure 3. 5 : Cell viabilities of control 10% PEGDMA and PT-20/10% PEGDMA solutions.

Representative images of live (green) and dead (red) L929 cells shown in Figure 3.6. The results showed that cell viability of PT-20/10% PEGDMA gels were higher than control PEGDMA gels.

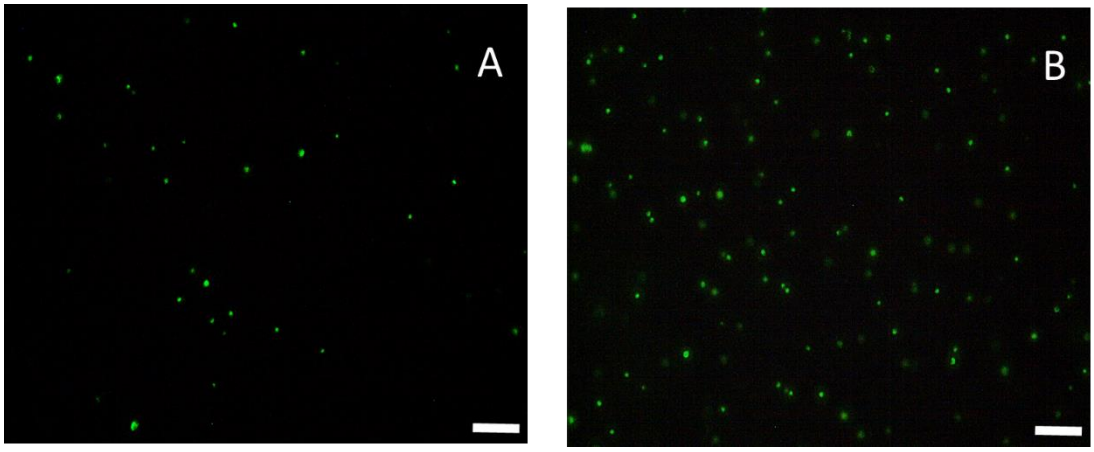


Figure 3. 6 : Day 7 of Control 10% PEGDMA (A) and PT-20/10% PEGDMA (B). The scale bar is 100 µm.

3.5 Swelling Ratio

The swelling of control PEGDMA gels and PT-20/10% PEGDMA gels were investigated to determine the change of swelling properties with cold atmospheric

plasma application. As shown in Figure 3.7, the swelling ratio (Q) of PT-20/10% PEGDMA gels were determined as 555,4% and the swelling ratio of control PEGDMA gels were 612,9%. The decrease of swelling ratio from control gels to PT-20/10% PEGDMA gels is non-significant (p=0,172).

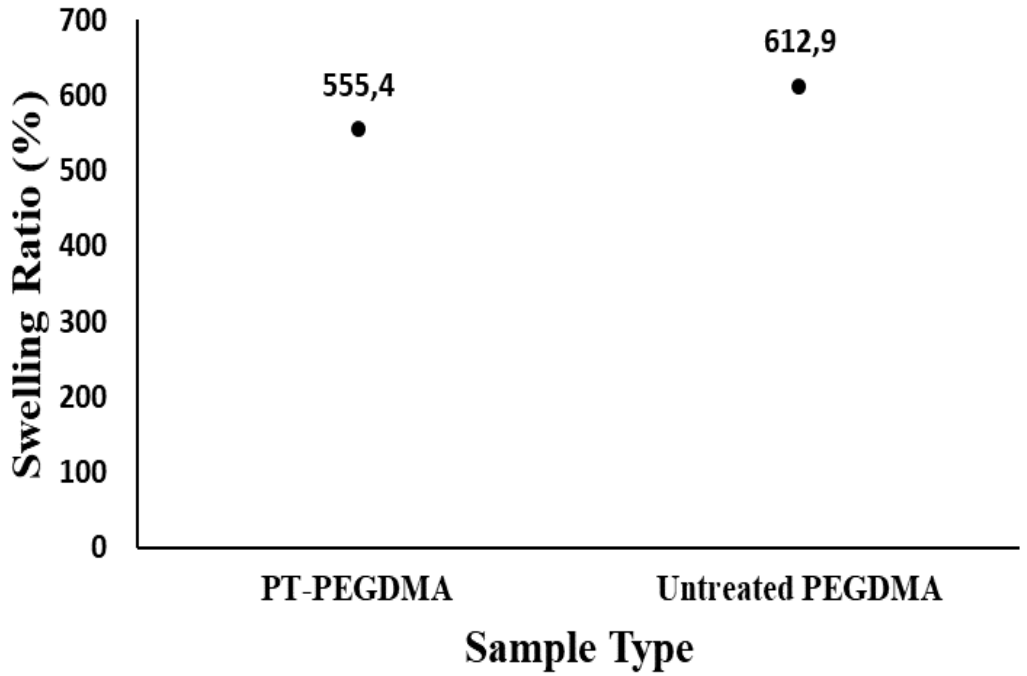


Figure 3. 7 : Swelling ratio of PT-20/10% PEGDMA and Control 10% PEGDMA.

The sol fraction of control PEGDMA gels and PT-20/10% PEGDMA gels were compared and as shown in Figure 3.8, the sol fraction of control PEGMDA gels (30.2%) was lower than PT-20/10% PEGMDA gels (30.7%). No significant difference in sol fraction was found between control and plasma treated gels (p=0,824).

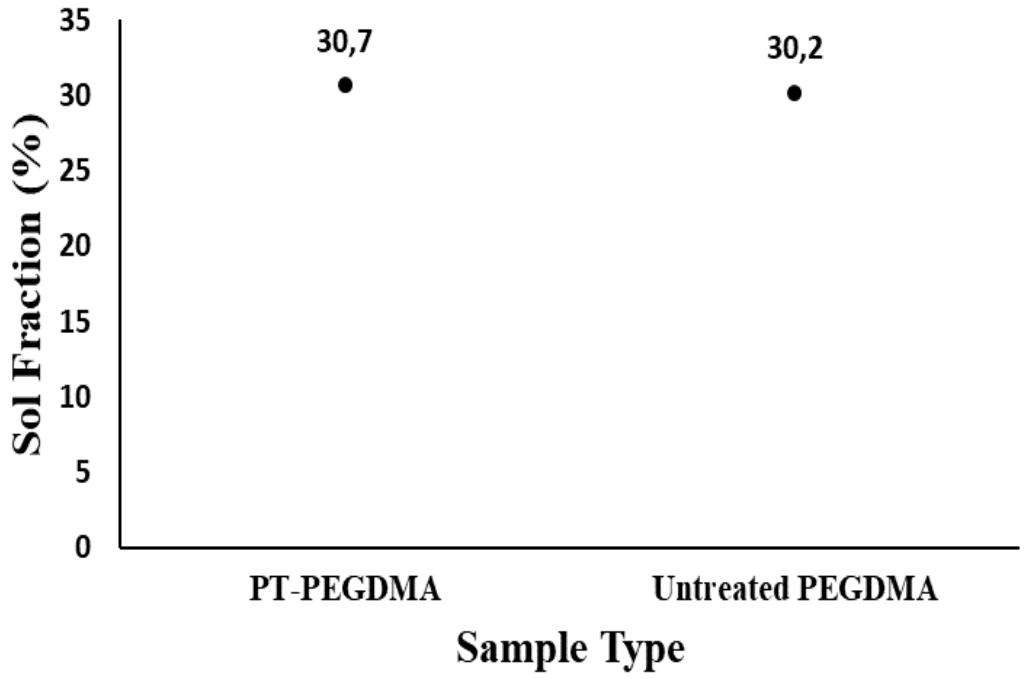


Figure 3. 8 : Sol fraction of PT-20/10% PEGDMA and Control 10% PEGDMA.

3.6 Rheological Properties

In this research, the effect of cold atmospheric plasma on PEGDMA gels was investigated. There were 2 groups of gels: control (untreated) group and PT-20/10% PEGDMA group. Figure 3.9 shows the storage (G') and loss (G'') modulus of control and PT-20/10% PEGDMA gels as function of the angular frequency. If storage modulus was compared, storage modulus of control group was higher than storage modulus of PT-20/10% PEGDMA. For loss modulus, control gels had higher loss modulus values than PT-20/10% PEGDMA gels. The storage modulus values of both control and PT-20/10% PEGDMA groups were increased with increasing angular frequency. But loss modulus was firstly decreased then increased rapidly. Similar to the loss modulus, tan (δ) values were shown a decrease and then an increase. Figure 3.10 presents tan (δ) values of control and PT-20/10% PEGDMA gels versus angular frequency.

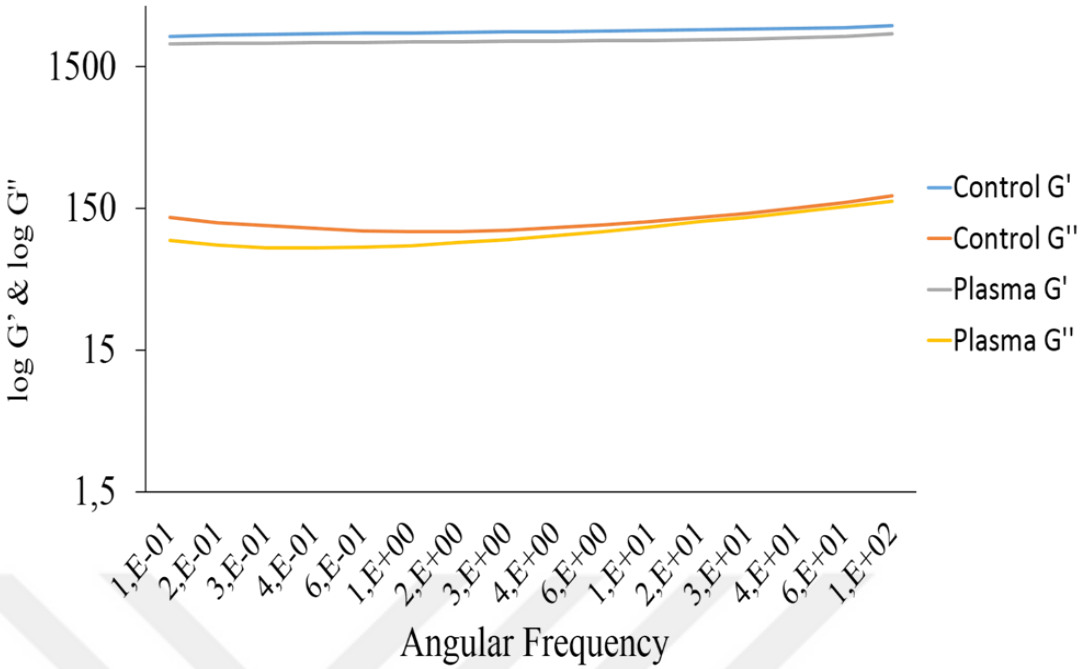


Figure 3. 9 : Storage and loss modulus of PT-20/10% PEGDMA and Control 10% PEGDMA.

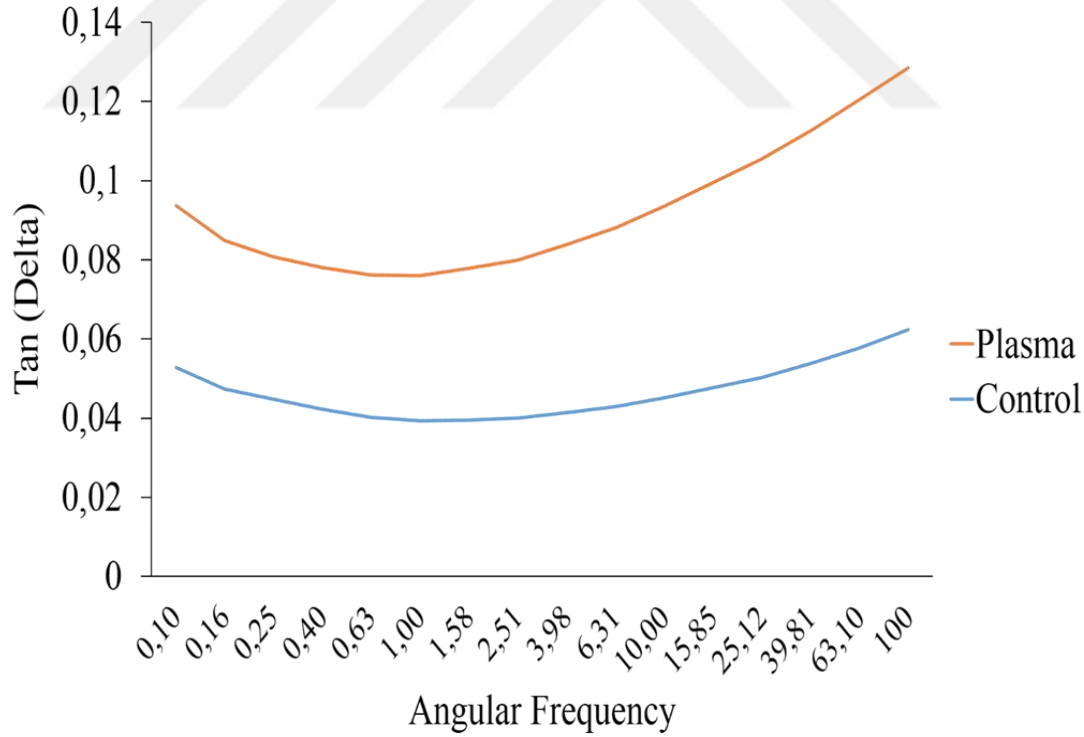
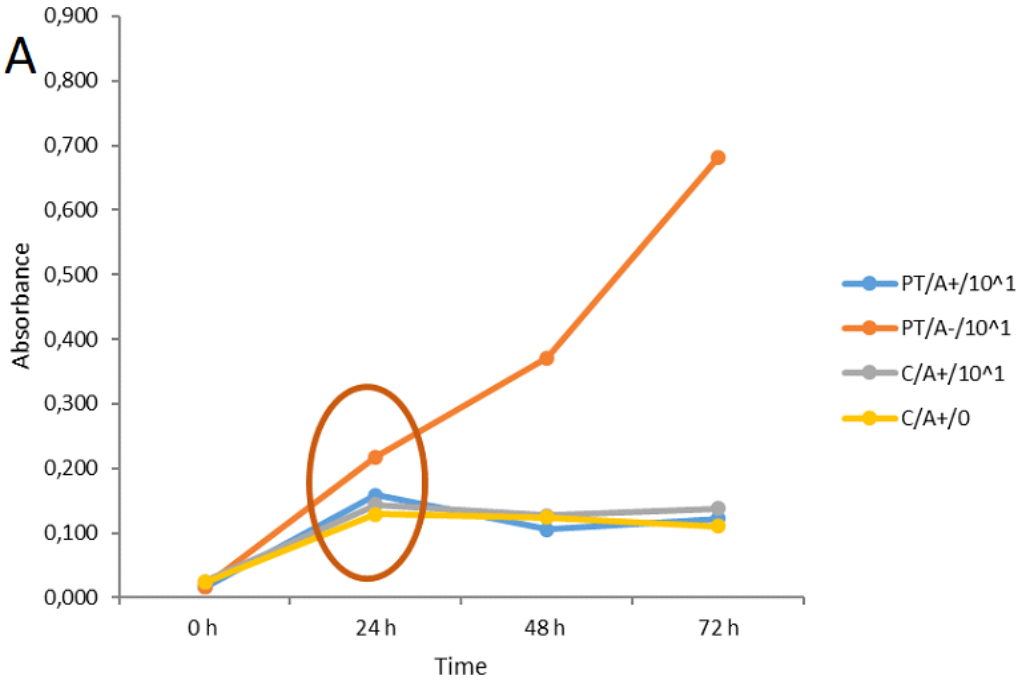


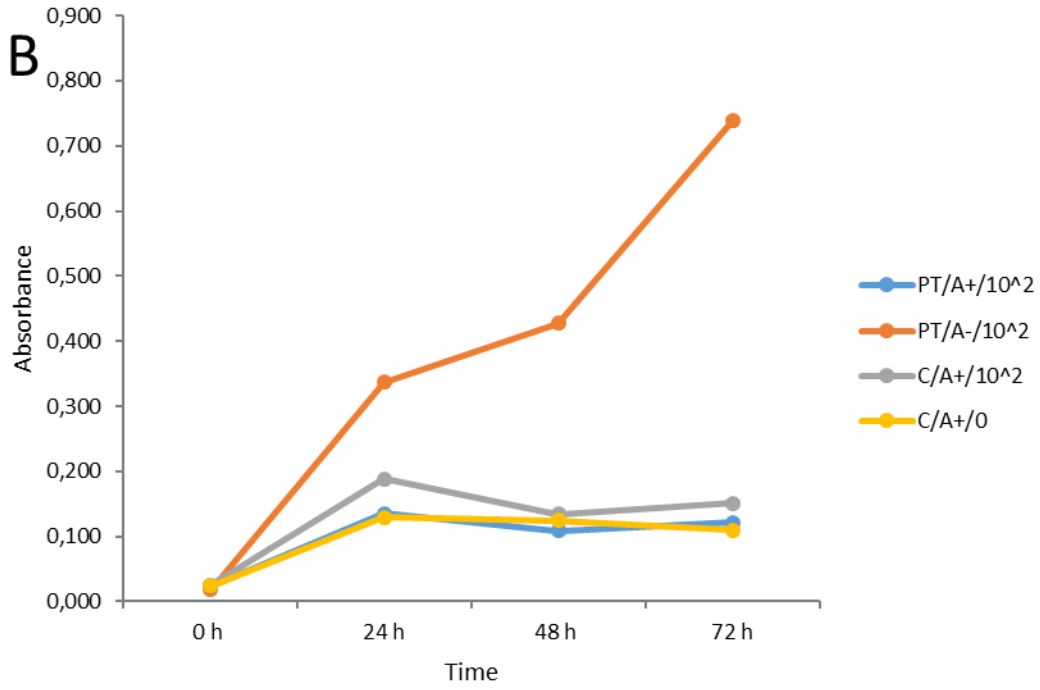
Figure 3. 10 : Tan (Delta) values of PT-20/10% PEGDMA and Control 10% PEGDMA.

3.7 Antimicrobial Effect of Plasma Treated and Gelated PEGDMA

20% PEGDMA solutions were treated with DBD plasma for 1 minute. After treatment, PEGDMA solution was diluted with PBS at 1:1 ratio and diluted solution was added to with and without antibiotic. After incubation for 24, 48, and 72 hours, absorbance of solutions was obtained with multiplate reader at 600 nm.

Control group without bacteria with antibiotic (C/A+/0), control group with bacteria with antibiotic (C/A+/10^1), and plasma treated group with bacteria without antibiotic (PT/A+/10^1) showed similar absorbance for 72 h period. As shown in Figure 3.11, the absorbance values of C/A+/0, C/A+/10^1, and PT/A+/10^1 decreased with time. On the other hand, plasma treated group with bacteria without antibiotic (PT/A-/10^1) shows increasing absorbance values with time but for first 24 h, absorbance value of PT/A-/10^1 is lower than other bacteria concentrations. For *E. coli*, all bacteria concentrations (10^1, 10^2, 10^3, and 10^4 CFU/mL) show increase absorbance regime for time.

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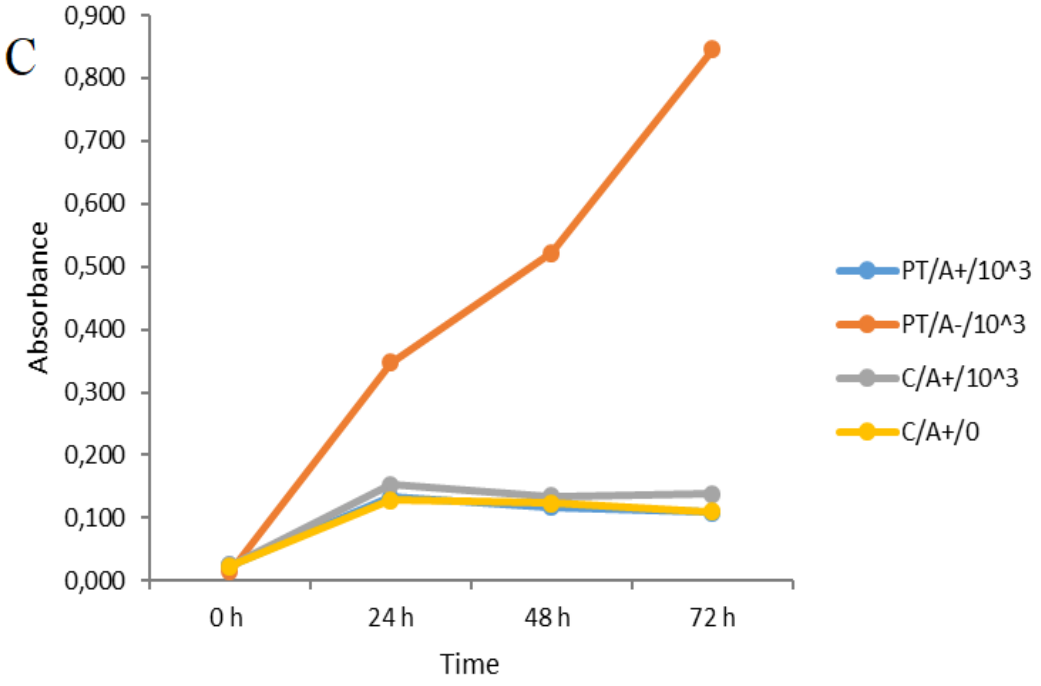
72 h
Time
PT/A+/10^2
PT/A-/10^2
C/A+/10^2
C/A+/0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C/A+/10^3
C/A+/0

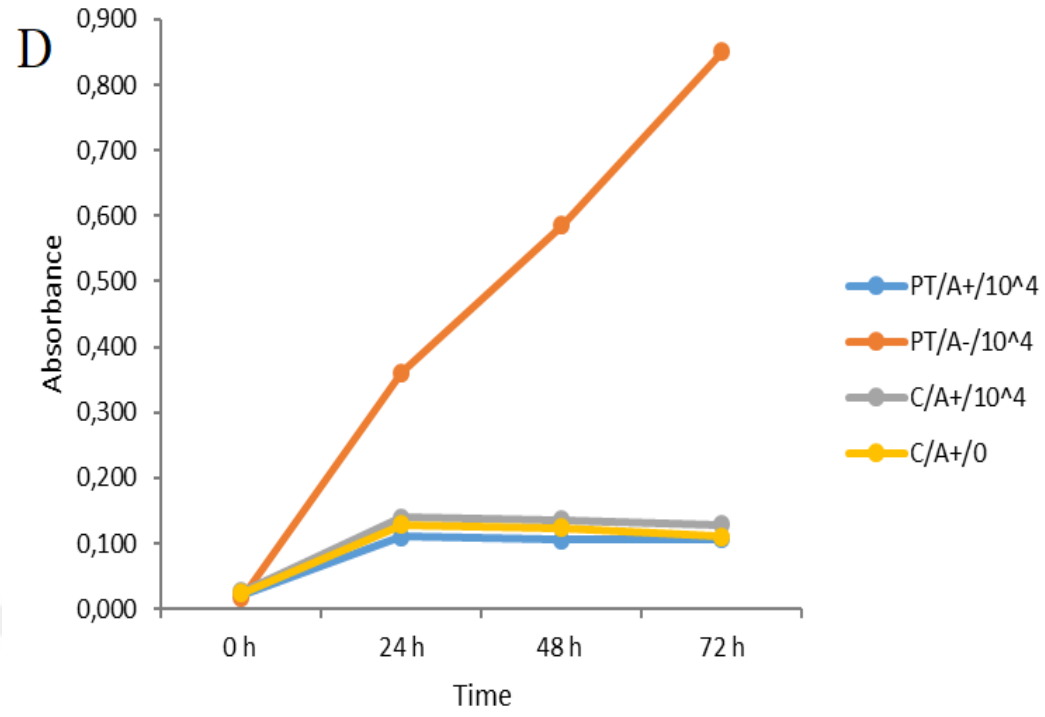
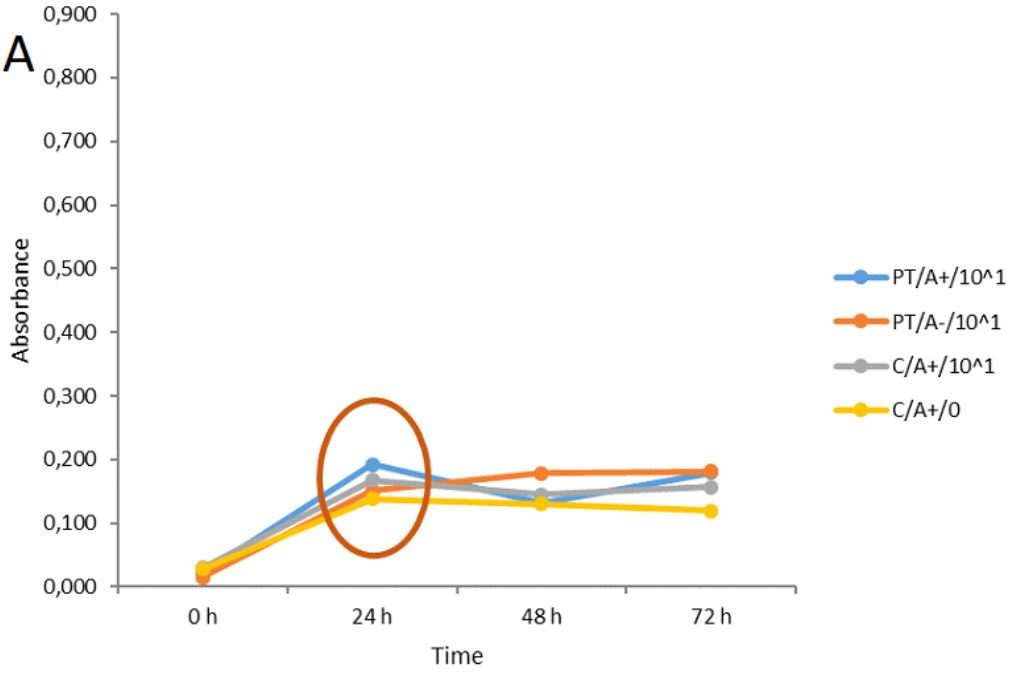


Figure 3. 11 : Antimicrobial effect of PT-20/10% PEGDMA on *E. coli* 10^1 CFU/mL (A), 10^2 CFU/mL (B), 10^3 CFU/mL (C), and 10^4 CFU/mL (D). Orange circles indicate same absorbance values in the presence of 10^1 CFU/ml *E.coli* which suggest PT-PEGDMA is capable of inactivating bacteria that could be responsible for contamination in cell culture systems.

As shown in the Figure 3.12-A and B, for *S. aureus*, all groups showed similar absorbance values and bacterial growth of *S. aureus* is lower than other concentrations. In Figure 3.12-C, C/A+/0, C/A+/10^3, and PT/A+/10^3 showed similar absorbance profile that increased at 24 h then decreased with time. However, PT/A-/10^3 group showed continuous increase with time. As shown in Figure 3.12-D, all groups for *S. aureus* showed similar absorbance profile, increasing for 24 h, then decreasing for 48 h. But only C/A+/0 group showed increasing absorbance values.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72 h
Time
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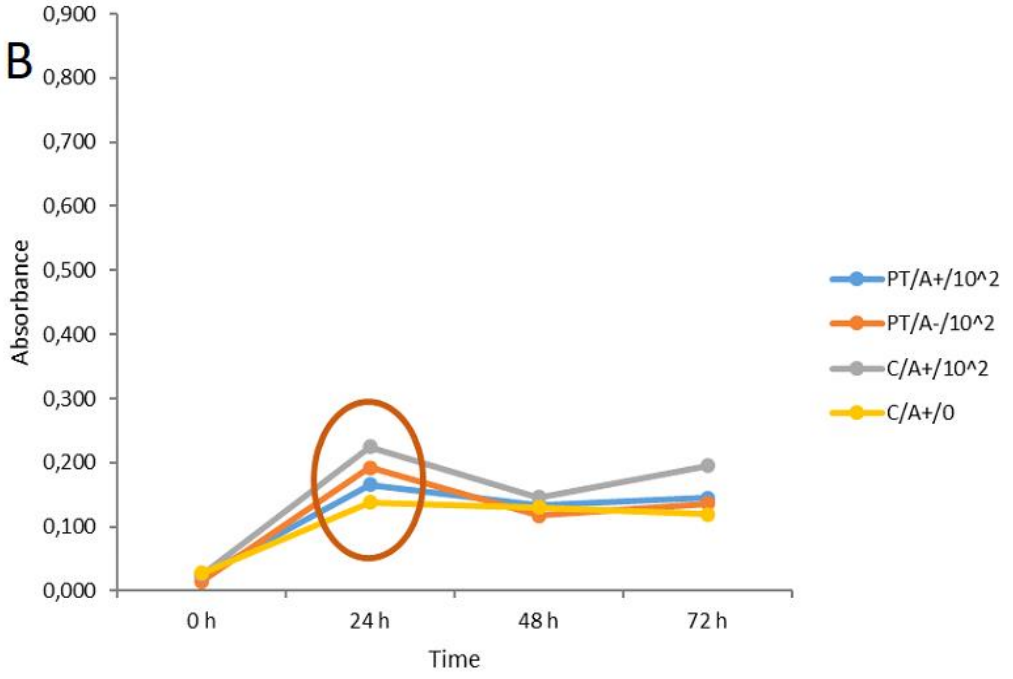
PT/A-/10^1
C/A+/10^1
C/A+/0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Time
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PT/A-/10^2
C/A+/10^2
C/A+/0

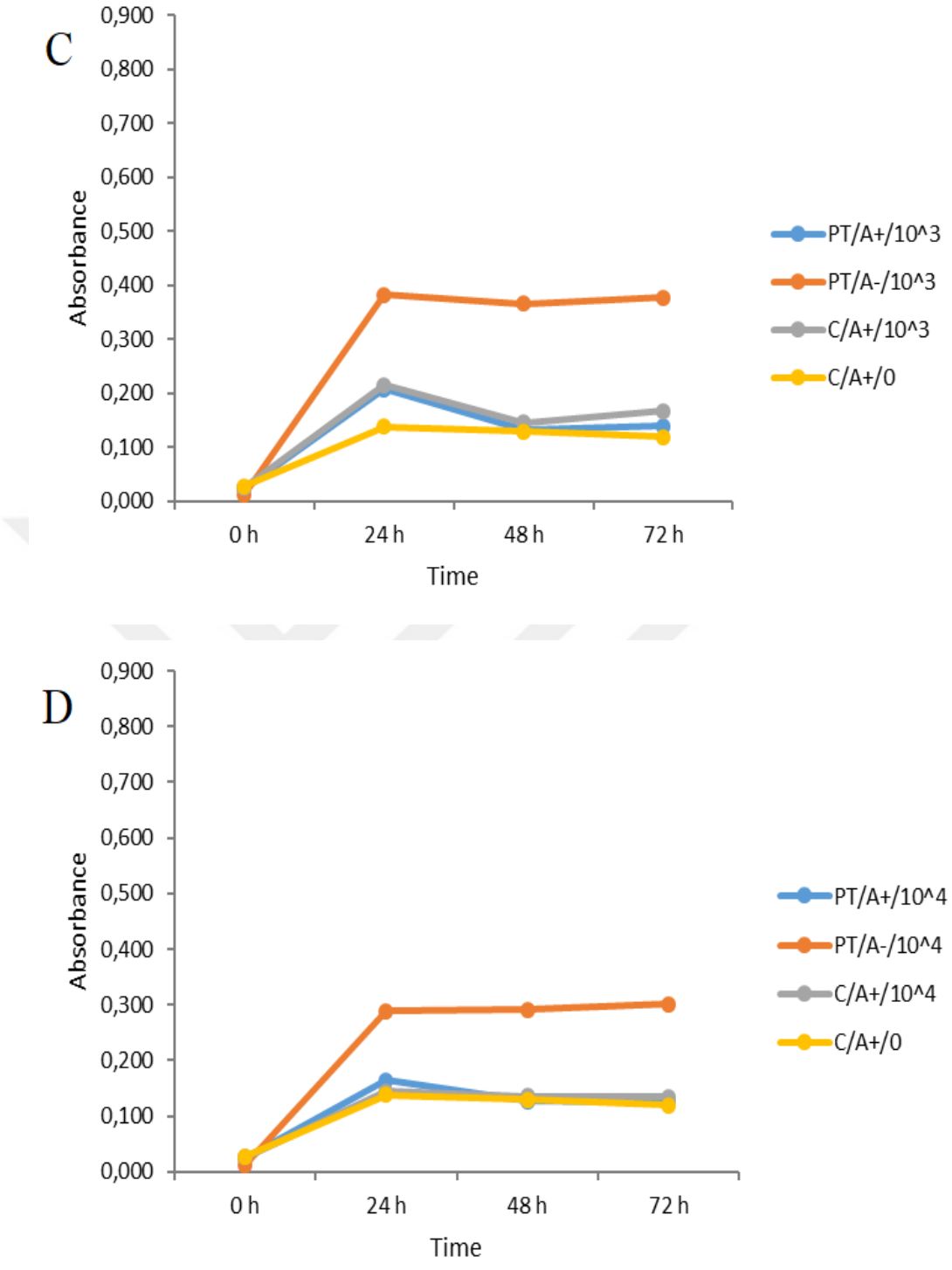

Figure 3. 12 : Antimicrobial effect of PT-20/10% PEGDMA on *S. aureus* 10^1 CFU/mL (A), 10^2 CFU/mL (B), 10^3 CFU/mL (C), and 10^4 CFU/mL (D). Orange circles indicate same absorbance values in the presence of 10^1 and 10^2 CFU/ml *S. aureus* which suggest PT-PEGDMA is capable of inactivating bacteria that could be responsible for contamination in cell culture systems.

4. DISCUSSION

There are several polymer sterilization techniques that are used in tissue engineering applications. But these techniques that are filtration, dry heat, steam, gamma and UV irradiation, and EtO, can be ineffective and mostly hazardous for chemical and mechanical properties of polymers [52]. High heat, irradiation and EtO might change the polymer structure and chemical properties, filtration does not change chemical properties or structure but it can alter polymer concentration [59]. Cold atmospheric plasma is an alternative technique for sterilization of polymers [52]. In the present study, we have examined the cold atmospheric plasma effect on PEGDMA based hydrogels. Firstly, sterilization effect of CAP on PEGDMA was determined with treatment of both contaminated and uncontaminated solutions. Secondly, PT-PEGDMA solution were mixed to determine the sterilization effect on medium with and without antibiotic. To investigate proliferation effect of CAP on L929 fibroblast cells, PT-PEGDMA solution and cells were mixed and gelated. MTT test was used to determine cell proliferation on incubated gels. Swelling ratio, sol fraction and rheological measurements were done to determine the mechanical effect of CAP on PEGDMA hydrogels.

Ikawa et al has showed that plasma jet was applied to distillated water that contains *E. coli*, after 120 s of plasma treatment, decrease of bacteria concentration (more than 6-log) was shown and it is noted that the presence of superoxide anion is critical for antibacterial activity, which reacts with H^+ to produce hydroperoxy radicals that can penetrate and damage cell membrane and intracellular components [25]. Oehmigen et al has shown that 7 min plasma treatment of NaCl solution completely inactivated 7-log *E. coli* and authors concluded that the antimicrobial activity of plasma treatment is because of superoxide anion radical that is converted to hydroperoxyl radical in acidic conditions and penetrate cell membrane then converted back to superoxide anion in neutral cytoplasm and react with intracellular components [60]. Ercan et al has shown that 3-min plasma treatment of N-acetylcysteine (NAC) solution completely inactivated 9-log *E. coli* and as a result of

plasma exposure to NAC, that contains strong antimicrobial agents like RNS and ROS, intracellular oxidative and nitrosative stress were observed with damaging lipids, nucleic acids and proteins. Also it is concluded that plasma-generated RNS was more dominant for antimicrobial effect than ROS [61]. Ziuzina et al has shown that direct plasma treatment on both maximum recovery diluent (MRD) and PBS. For PBS, all treatment times (10-300 s), complete inactivation was shown on *E. coli*, on the other hand, for MRD, all times except 10 s, complete inactivation was shown. In MRD, which is consists of low concentration of peptone and sodium chloride (NaCl), acidification and plasma-generated reactive species were effective for antimicrobial activity. In PBS, which is consists of NaCl and phosphates, plasma-generated ozone and saline reacted and hydrochlorous acid that is known as an excellent bactericidal compound was generated. Hydrochlorous acid can show toxic reactivity with protein –SH groups, amino groups, DNA, RNA and lipids [22]. It has been reported that organic molecules in liquid enhance effect of cold atmospheric plasma and allow longer plasma effect [62]. In the present study, we have treated PEGDMA solutions and contaminated PEGDMA solutions with different concentration in order to investigate antimicrobial effect on *E. coli* and *S. aureus*. For PEGDMA solution treatment, PT-PEGDMA was mixed with bacteria solutions. Results showed that all plasma treatment times were effective on both bacteria. On the other antimicrobial test, contaminated PEGDMA solutions were treated with cold atmospheric plasma. Plasma treated groups showed no bacterial growth at all treatment times for both bacteria.

As a result of plasma treatment, pH of plasma treated liquid decreases. When plasma exposure times increases, pH of solution decreases. But unlikely non-buffered solutions, plasma treatment of buffered solutions, pH of solutions does not drop and thus, antimicrobial activity is decreased [14; 26]. It was reported by other authors, pH decrease, as a result of plasma treatment, antimicrobial effect of plasma treatment is accelerated, however, only acidity is not effective for inactivation of bacteria [26; 63; 64]. It is noted that plasma generated NO_2 and NO can be the cause of acidification of treated liquid [63; 65]. In the present study, we have observed that plasma treatment decreased the pH of PEGDMA solutions. In addition, pH of PEGDMA solutions decreased while increasing plasma treatment time.

Poor et al treated alginate gels with non-thermal plasma and gels showed better decontamination rate and cell proliferation [66]. Kalghatgi et al. has shown that 30-s

non-thermal plasma treatment increased the cell viability when it compared to untreated control group, but treatment longer exposure time induces apoptosis [67]. Arjunan et al has shown that plasma treated endothelial cells had more proliferation rate than untreated control group [68]. It is noted that cell proliferation effect of cold atmospheric plasma is related to membrane damage by ROS and this damage leads to secretion of fibroblast growth factor 2 (FGF2) that induces endothelial cell proliferation [67; 68]. Liu et al has shown that argon atmospheric pressure plasma jet treatment on L929 fibroblast cells was effective to increase cell proliferation for less than 20 s. Cell proliferation rate was related with level of intracellular ROS which induced the nuclear transcription factor κB (NF-κB) that regulates gene expression in host defense, immune response, cell proliferation and cell survival [69]. In the present study, cell proliferation was investigated with using 10% PT-PEGDMA and PT-20/10% PEGDMA solutions. Firstly, 10% PT-PEGDMA was used, only, cell proliferation and encapsulation tests. There are 3 different plasma exposure times such as 1, 2, and 3 minutes. Results showed that increasing plasma exposure time decreases cell viability and proliferation rate. 10% PT-PEGDMA-1 min is the optimum solutions to test. But plasma treatment decreased the pH of solution and fibroblast cells prefer higher pH range between 7.4-7.7 [70]. To improve the cell viability and proliferation rate, we used PBS as a buffer solution to dilute 20% PT-PEGDMA to PT-20/10% PEGDMA. After dilution with PBS, gels were prepared and cell viability was determined. Results showed that PT-20/10% PEGDMA gels had higher cell viability and proliferation rate than 10% PT-PEGDMA gels. PBS buffered the solution and pH increase was observed.

Tran et al concluded that mechanical properties of scaffolds affect the tissue regeneration, host inflammatory response, angiogenesis and wound healing properties. Also, mechanical properties induce metabolic activities and extracellular matrix components. Scaffolds should be similar mechanical properties with native tissues [71]. Kanjickal et al has shown that EtO and gamma sterilization decreased the swelling ratio of PEG-based hydrogels but H_2O_2 sterilization increased. Also gamma sterilization and H_2O_2 sterilization decreased the surface roughness [72]. Karajanagi et al has shown that sterilization of PEG hydrogels with steam and gamma irradiation was unsuitable because they affected the hydrogel structure [73]. In the present study, for all hydrogels, storage modulus values were ranged between 10^3-10^4 Pa, it indicates that both control and PT-PEGDMA gels behaved like an

elastic solid. Storage and loss modulus increased with increasing angular frequency, it indicates that gels have a relaxation behavior of the network at increasing frequencies. For tan (δ) values, tan (δ) decreased first then increased rapidly for both control and PT-PEGDMA gels. However, tan (δ) of PT-PEGDMA gels was higher than that of control gels. This difference between values of tan (δ) indicates that PT-PEGDMA gels become tighter and bonds within PT-PEGDMA gels increased the viscosity. Also, energy consumption of PT-PEGDMA gels was greater than control gels at high frequency.

In the present study, swelling ratio and sol fraction of gels was measured. Although swelling ratio of control gel is higher than that of PT-20/10 % PEGDMA gel and sol fraction of control is lower than that of PT-20/10% PEGDMA gel, the difference between them is non-significant. Thus, we can note that plasma treatment does not affect the swelling ratio and sol fraction. It can be concluded that CAP treatment does not change the properties of PEGDMA hydrogels and it can be used as a sterilization technique for polymers.

In the antimicrobial effect of plasma treated and gelated PEGDMA test, we have shown that plasma treatment is effective for low bacterial concentrations. For *E. coli*, at 10^1 CFU/mL concentration, low absorbance value was obtained. It can be concluded that CAP treatment shows bactericidal effect in low bacteria concentrations for *E. coli*. Also, for *S. aureus*, 10^1 and 10^2 CFU/mL concentrations had lower absorbance values when compared with 10^3 and 10^4 CFU/mL concentrations. It can be said that CAP treatment is effective on *S. aureus* at low bacteria concentrations. When both bacteria compared, CAP is more effective on *S. aureus*. Han et al has shown that inactivation of *E. coli* was related with cell envelope damage-induced leakage, and inactivation of *S. aureus* was related with intracellular damage [74]. *S. aureus* is a Gram-positive bacterium that has a thick wall made of peptidoglycan on out of cell membrane and *E. coli* is a Gram-negative bacterium that has both outer membrane and peptidoglycan membrane on out of cell membrane. Thus, it can be concluded that reacting cell membrane of *S. aureus* for ROS is easier than of *E. coli* and CAP can be used on Gram-positive bacteria sterilization more effectively. In addition, Nygaard et al has shown that antibiotics in cell culture media reduce the proliferation rate of normal human keratinocytes and block 3D skin model [75]. Ryu et al has shown that penicillin-streptomycin was effective on drug response, cell cycle regulation, differentiation, and proliferation by

affecting gene transcription [76]. Thus, CAP treatment can be used instead of antibiotics to reduce side effects.

5. CONCLUSION

Sterilization techniques of polymeric scaffolds are uneffective and harmful for its structure and chemistry. Cold atmospheric plasma is a candidate with antimicrobial effect for scaffold sterilization. In summary, we investigated effect of cold atmospheric plasma on PEGDMA, bacterial growth and cell proliferation. Results showed that cold atmospheric plasma is effective on both *E. coli* and *S. aureus* inactivation for all plasma exposure times. For cell viability, PT-20/10% PEGDMA gels has the highest cell proliferation rate. In 10% PT-PEGDMA gels, increasing time decreased the cell viability with decreasing pH. Plasma treatment does not affect the swelling ratio and sol fraction. But rheology results showed that viscosity and energy consumption of PT- 20/10% PEGDMA gels was higher than control gels. In addition, PT-PEGDMA solution was added to contaminated medium with *E. coli* and *S. aureus*. Results showed that bacterial growth is inhibited at low concentrations of both bacteria. Cold atmospheric plasma application may be an adequate technique for polymeric scaffolds.

In future studies, the effect of plasma on differentiation may be observed by using stem cell cultivation in plasma-treated gels for use in bone tissue engineering. In addition, cell proliferation in contaminated medium can be observed by long-term cell viability in antibiotic and antibiotic free mediums.

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Yıldırım Ç, Onak G, Karaman O, Ercan UK. Decontamination of PEGDMA with Cold Atmospheric Plasma for Cell Encapsulation Purpose, ESB - 29th European Conference on Biomaterials September 9-13, 2018, Maastricht, Netherlands.

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