Stony Brook University

Effect of Toxicity of RDP in Polymer Scaffolds on Fibroblast Cell

A Thesis Presented

by

Jae Wha Yang

to

The Graduate School
in Partial Fulfillment of the
Requirements
for the Degree of
Master of Science
in

Materials Science and Engineering

Stony Brook University

December 2015

Stony Brook University

The Graduate School

Jae Wha Yang

We, the thesis committee for the above candidate for the
Master of Science degree, hereby recommend
Acceptance of this thesis.

Miriam Rafailovich – Thesis Advisor
Distinguished Professor, Materials Science and Engineering

Jonathan Sokolov – Second Reader
Professor, Materials Science and Engineering

Tae Jin Kim – Third Reader
Assistant Professor, Chemical and Molecular Engineering

This Thesis is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Thesis

Effect of Toxicity of RDP in Polymer Scaffolds on Fibroblast Cell

by

Jae Wha Yang

Master of Science

in

Materials Science and Engineering

Stony Brook University

2015

The purpose of this thesis is to design substrates with flame retardant and clay to enhance the property of polymer and to study the effect of toxicity of a flame retardant mixed polymer, which had not been conducted in vivo. Therefore the human dermal fibroblast cells, engineered to have green fluorescent membrane (GFFB), were cultured on PS scaffolds with three different conditions: pure PS, PS-Na^{+}-Clay and RDP-Clay.

In first part of the study, the polymer samples (PS, PS-Clay and RDP-clay) that would be used as scaffolds were prepared and sterilized to use for cell culture, while testing that sterilization using autoclave was adaptive in the experiment. SEM, EDX spectroscopy and contact angle measurement were used to see if there was any change in the polymers after sterilized in autoclave.

In second part of the study, the prepared GFFB cells were cultured on the substrates used as scaffolds and the cell growth was observed by cell counting using both hemocytometer and observation under a fluorescent microscopy. The condition of cells was also observed using a confocal microscopy.

Results showed that cell proliferation rates were different for each type polymer scaffolds, and surprisingly, RDP-Clay PS polymer showed the highest cell proliferation rate and lowest doubling time, similar to the control group, which used a commercial PS tissue culture plate. It was determined that RDP was not toxic, but helpful for cell growth. Therefore further study might be needed to figure out the mechanism of RDP effects on the cell growth.

Dedicated to

My dearest family

for

Their love and support

Table of Contents

Abstract ····· **iii**

Dedication ····· **v**

List of Figures ····· **viii**

List of Tables ····· **x**

Acknowledgements ····· **xi**

1 Introduction ····· 1
- 1.1 Background ····· 1
- 1.2 Objective and Mythology ····· 7
- 1.3 Thesis Structure ····· 8

2 Preparation of Polymer Scaffolds ····· **9**
- 2.1 Experimental Materials ····· 9
- 2.2 Experimental Equipment ····· 9
- 2.3 Experimental Steps
  - 2.3.1 Preparation of 20% RDP-Clay ····· 10
  - 2.3.2 Preparation of Polymer Scaffolds ····· 11
  - 2.3.3 Sterilization of Polymer Scaffolds ····· 12
  - 2.3.4 Contact Angle Test for Polymer Scaffolds ····· 12

3 Preparation of Cell Culture ····· **14**
- 3.1 Experimental Materials ····· 14

3.2 Experimental Equipment ····· 15
3.3 Experimental Steps
3.3.1 Preparation of Solutions ····· 16
3.3.2 Cell Preparation ····· 16
3.3.3 Cell Culture on the Scaffolds ····· 17
3.3.4 Cell Counting using a Hemocytometer ····· 18
3.3.5 Cell Fixing and Staining ····· 18
3.3.6 Fluorescent Microscopy ····· 19
3.3.7 Confocal Laser Scanning Microscopy ····· 19

4 Results and Discussion ····· 21
4.1 SEM images of Polymer Scaffolds ····· 21
4.2 Contact Angles of Polymer Scaffolds ····· 22
4.2 Cell Counting using a Hemocytometer ····· 23
4.3 Cell Counting using Image J ····· 24
4.4 Condition of Cells under a Confocal Microscopy ····· 25

5 Conclusion ····· 27

6 **Figures and Tables ····· 29**

Bibliography ····· 65

List of Figures

Figure 1. The chemical formula of RDP [10]. 29
Figure 2. Chemical Interaction of RDP with PPO [11]. 30
Figure 3. Metabolites of RDP and RDP oligomers [8]. 31
Figure 4. SEM image of pure PS before autoclave at 300 X. (20kV) 33
Figure 5. SEM image of pure PS before autoclave at 1000 X. (20 kV) 34
Figure 6. SEM image of pure PS before autoclave at 300 X (5 kV) 35
Figure 7. SEM image of pure PS before autoclave at 1000 X. (5 kV) 36
Figure 8. SEM image of pure PS after autoclave at 300 X. (20 kV) 37
Figure 9. SEM image of pure PS after autoclave at 1000 X. (20 kV) 38
Figure 10. SEM image of pure PS after autoclave at 300 X. (5 kV) 39
Figure 11. SEM image of pure PS after autoclave at 1000 X. (5 kV) 40
Figure 12. EDX spectrum of the visible area of pure PS before autoclave. 41
Figure 13. EDX spectrum of the visible area of pure PS after autoclave. 42
Figure 14. SEM image of PS-Na^{+}-Clay after autoclave at 300 X. (20 kV).....43
Figure 15. EDX spectrum of PS-Na+-Clay in the area boxed in Figure 14. 44
Figure 16. SEM image of PS-RDP-Na+-Clay after autoclave at 3000 X. (20 kV) 45
Figure 17. SEM image of PS-RDP-Clay at 10 KX, enlargement of image in Figure 16.....46
Figure 18. EDX spectrum of PS-RDP-Clay in the area boxed in Figure 16. ...47
Figure 19. Diagram of polymer scaffolds placed in 24-well cell culture dish for each day from Day 1 to Day 6.....48
Figure 20. Diagram for polymer scaffolds selected for hemocytometer cell counting. 49

Figure 21. Green fluorescent fibroblast cell growth curve for first experiment with seeding density of 5000 cells/100 μL. 50
Figure 22. Green fluorescent fibroblast cell growth curve for second experiment with seeding density of 10,000 cells/100 μL. 51
Figure 23. Doubling time for proliferation rates of GFFB cell for 1st trial.....52
Figure 24. Doubling time for proliferation rates of GFFB cell for 2nd trial.....53
Figure 25. 9 division of fluorescent images taken for each polymer samples..54
Figure 26. Image at division 6 taken for DAPI dyed GFFB cells on PS (Day 6) under a fluorescent microscopy. 55
Figure 27. Image at division 6 taken for DAPI dyed GFFB cells on PS-clay (Day 6) under a fluorescent microscopy. 56
Figure 28. Image at division 6 taken for DAPI dyed GFFB cells on RDP-Clay (Day 6) under a fluorescent microscopy. 57
Figure 29. Image at division 6 taken for DAPI dyed GFFB cells on control (Day 6) under a fluorescent microscopy. 58
Figure 30. GFFB Cell growth counting using a fluorescent microscopy and ImageJ. 59
Figure 31. Doubling time of GFFB cells on polymer substrates calculated based on the data analyzed using fluorescent microscopy pictures and ImageJ.....60
Figure 32. Image of GFFB cells on PS scaffold under a confocal microscopy. 61
Figure 33. Image of GFFB cells on PS-Clay scaffold under a confocal microscopy. 62
Figure 34. Image of GFFB cells on RDP-Clay scaffold under a confocal microscopy. 63
Figure 35. Image of GFFB cells on RDP-Clay scaffold under a confocal microscopy. 64

List of Tables

Table 1. Contact Angle Measurement for the Polymer Scaffolds before and after autoclave. 32

ACKNOWLEDGEMENTS

I would like to have my greatest thanks to Professor Miriam Rafailovich, my academic advisor, for her support and guidance. I really do thank her for all the introduction and thesis ideas. It was an honor to conduct this independent research. My other reviewers, Prof. Jonathan Sokolov and Prof. Tae Jin Kim also have my thanks for the helpful comments and great attention on this study.

I also want to extend my thanks to my supervisor and lab partner, Fan Yang, who instructed me to get the ideas of this research, laboratory, other lab procedures and instruments. It was really a pleasure to work with him.

Many thanks to all the members of Miriam's group. Thanks to all! I learned a lot of useful experiences and gained lots of knowledge from your help on lab safety, polymer making procedures and other lab experiences.

Special thanks to all my good friends in Stony Brook University, who encouraged and gave me so many advices that helped me to finish my academic degree with a success. I am really going to miss you all.

All the honor of this thesis is for my parents. Without their trust, love and support me to continue my study and finish my degree with this thesis. I am really grateful to all the care and sacrifice for raise me up as their beloved daughter. I love you, my dearest Mom and Dad. Thank you!

Chapter 1

Introduction

1.1 Background

Due to advances in technology, many products have potential to fire hazard as it may explode or start fire due to any physical (ex. friction, electrical, etc.) or chemical (ex. high temperature, chemical chain reaction, volatility, etc.) activities. It is very common to see fire damages about 400,000 homes annually in the United States [1], which causes about 7 billion dollars in loss. Electric circuits and batteries, which are now installed in most of electrical products: car, laptop, smart-phones, tablets, etc., have high potential to cause fire hazard while consumers use them on daily basis. These products are not only harmful due to its potential fire sensitivity, but also toxicity by leaking of chemicals when they are on fire. Therefore, industry notices on the fire retardation because of the importance of prevention of fire and safety issue.

There are several methods of fire retardation by either physical or chemical action. For physical action, fire retardant works by cooling or forming protective layer of blocking fire or releasing water and/or carbon dioxide or other mixed substrates (ex. fire extinguisher mixture) to block air or radicals from burning. For chemical action, the substrate is either coated on the surface or mixed in the product interrupting any chemical reaction in the flame, preventing any chain reaction and/or release toxic/explosive gas or liquid in the fire, which may cause further disaster. Some retardants may break down polymers, let them melt and flow away from the flame and/or causes carbonaceous char layer forming on the surface, which makes the product blocked to the environment, quenching the flame.

In order to prevent fire rather than extinguish flame after fire starts, chemical retardants are welcomed in industry to apply on the products for safety issue, thus several chemical fire retardants are developed. The most common classes of chemical fire retardants are brominated, phosphorus, nitrogen and chlorinated and inorganic compounds [2].

Brominated compounds are welcomed at first as it helps prevent a fire from starting in the first place or slow a fire down while it does not change the property of materials when it is added. However, denomination, the reaction that removes bromine from a compound, is very poisonous [3] and may contaminate environment, therefore replace brominated flame retardants to more suitable

products [4]. Therefore, brominated compounds are now limited or inhibited in use. The non-toxic brominated compounds are showing high performance of fire retardant, used on the coating of submarine, ship or airplanes. However, the cost is very expensive, there is need of cheaper and safer substitution.

On the other hand, nitrogen compounds only limit and/or inhibit the chain reaction leading to combustion by releasing nitrogen gas and chlorinated compounds also have potential releasing toxic chlorine gas into air. Also inorganic compounds (hydrated aluminum and magnesium oxides, etc.) while the side-products of hydrated aluminum ("red mud") is damaging to the environment and highly toxic [5]. Therefore, phosphorous compounds work as the best fire retardant due to its non-toxic and environmental-friendly and high performance of fire retardant as forming char-layer on the surface. [6, 7]

Thus resorcinol di(phenyl phosphate) (RDP) is "the chosen" oligomer phosphorous flame retardant, which is used to blend to decrease flammability in consumer products (plastic, textile, paints, electronics etc.) [8]. It is also considered as a primary substitute for deca-bromodiphenyl ether (deca-BDE), a banned flame retardant [3] because of its toxicity by debromination and triphenyl phosphate (TPP), which is high volatile [9]. Furthermore, RDP is also considered as substitute of halogen (bromine and chlorine) containing flame retardants, which causes environmental concerns and end-of-life issues. RDP is halogen-free, which does not show unfavorable toxicology like other

phosphorous fire retardant, tris(dibromopropyl) phosphate [6]. Therefore it is very notable and sustainable compare to other substitutes.

Therefore RDP is used in broad application due to its good thermal stability, high efficiency and low volatility because the chemical formula of RDP contains bridged aromatic diphenyl phosphate [6], which forms in oligomer between n=1 to 7 [10]. Thus RDP is less likely to be released into environment, which can be reason to replace for tris(2-carboxyethyl)phosphine) (TCEP) and tris(1-chloro-2-propyl) phosphate (TCPP), which are fire retardants with high volatile than RDP [7].

RDP is an oligomeric phosphate ester, which has surfactant properties, since the phenol groups can be considered as nonpolar moieties and phosphoric acid groups can be considered as polar moieties, which blends into polymer by modifying surface energy [10]. This phosphorus groups, which comprise 10.7 wt % are known to react with polymer residues at high temperatures forming insoluble chars, which is the fire retardant formulation. In several studies, RDP added polymers show better performance of fire retardant when RDP-blended polymer shows lower heat release rate (HRR), mass loss rate (MLR) [10], higher char yield without change in mechanical property of the polymer (poly(styrene-co-acrylonitrile) (PC), poly-2,6-dimethylphenylene oxide (PPO)) blended [10, 11]. As the phosphorus group accumulates in the char on the surface of burning polymer, it is assumed the chemical interaction between the polymer and RDP

causes the fire retardant property through a transesterification mechanism with phenolic OH groups as shown in Figure 2 [11].

Therefore, it is assumed that the RDP leaves phenolic OH groups, which can crosslink with the polymer as well, increasing the fire retardant. However, fire retardant action is still this not well clarified to explain in the polymers as well as PPO, because PPO has natural mechanism of crosslinking, which competes with RDP because both consumes reactive OH groups [11] while it may have different chemical interaction with other polymers, which has not been clarified yet.

The physical and chemical properties of RDP is somewhat clarified. RDP has molecular weight of 574.47g/mol, vapor pressure of 6.5×10^{-8} mm Hg at 20 °C. RDP has high boiling point of 587 °C due to its structure, but it decomposes above 300 °C and flash point is 322 °C [7]. RDP is viscous, pale yellow oil-like liquid at room temperature and in case of fire, carbon oxides and phosphorus oxides are formed [7]. The phosphorus oxide, product of decomposition of RDP, transforms into phosphoric acid when they are in direct contact with humid mucous membranes, while the solubility of RDP is very poor, 1.11×10^{-4} mg/L [7]. RDP is known to dissolve in alcohol (methanol, ethanol, etc.) and chloroform [12], however, the solubility is not clarified.

The production of RDP is occurred by the reaction of phosphoric trichloride with resorcinol, 65-80% of it while 1-5% of triphenly phosphate is produced as

contaminant and the bis[3-[(diphenoxyphosphinyl)oxy]phenyl] phenyl ester of phosphoric acid comprises the remaining 15 to 30%. [13] The price of RDP is about $500~600 per gram [12] The production/usage rate in Europe is more than 1500 ton (2005), 277 ton in United States (2006) and 6 ton in Sweden (2008) [7].

However, there is no data of RDP available on occurrence or degradation products in the environment, while RDP is commonly used in consumer products as plastic, textile, paints, electronics etc. Possibility of bioaccumulation in organism is on debate because of its polar degradation products [7] and the presence of RDP in biological samples has not yet been reported [8].

Therefore, there are little toxicity and environmental data for RDP in literature and little number of researches that investigated in vivo metabolism and disposition of RDP in rats, mice, and monkeys [8]. According to these studies, RDP has a minimal effect on human health and neither mutagenicity and chromosome abnormalities nor other genotoxic effects in a mouse micronucleus assay was found. Even in Pakalin's study about 2-generation rat study with highest does of RDP (20,000mg/kg per day) is performed, no adverse effects on reproductive performance or fertility parameters are found, while there is sign of moderate accumulation in lungs and bones for a combination of RDP together with TPhP, which caused liver weight gain, liver amplification and eye irritation were find in rats after oral and inhalative tests [7]. Thus, it seems to not

problematic to use RDP in place of other toxic flame retardants according to the available data [7].

However, these studies are only done in vivo, which does not quite explain the effect of RDP. As RDP contains both polar and non-polar sides, it is readily hydrolyzed (Figure 3) in 7 days into DPHD, RDP-[Phe] (loss of phenyl ring from RDP), meta-hydroxy-TPHP, meta-hydroxy-RDP and meta-hydroxy-RDPn2, which are present in RDP as impurities [8]. Even though there has been notified for almost non-toxic to the RDP in previous studies, further studies about this degraded impurities should be conducted as well to determine the toxicity of RDP in health and environment as RDP is commonly used as fire retardant in consumer products.

1.2 Objective and Methodology

This thesis is aimed to investigate the toxicity of RDP in vitro by observe the growth of cells on polymer scaffolds containing RDP, as many products of nowadays are made of plastics as well as mixture with flame retardants.

In order to obtain the data of cell growth, polymer scaffolds are made with PS (polystyrene) and 20% RDP-clay, which are physically mixed using polymer mixer (Barbender). 20% RDP-clay is chosen because it is the most effective amount of phosphorus working with the best flame retardant synergy with the clay [15].

The cells were cultured on these scaffolds, then either removed with 10x-EDTA trypsin and counted by tally counter or stained to count the cell numbers and/or see the structure of cell condition under confocal fluorescent microscopy to image the stained cells.

1.3 Thesis Structure

Chapter 2 presents how the scaffolds, either with RDP or without RDP to be compared, used for this experiment are prepared and sterilized to be used in the research. The method of sterilization is also introduced in this chapter and proves that this sterilization is safe to use for the prepared scaffolds.

Chapter 3 focuses on how the cells are cultured on these prepared scaffolds in order to qualify and quantify the cell growth. Green Fluorescent Fibroblast Cells (GFFC) are used to see the behavior of the most common skin cell easily under the microscope in order to find the best amount of trypsin to use in this research. In this chapter, the methods used to observe the cells and analyze the data is also included.

Chapter 4 explains the overall data of this research, the numerical data of living cells on the scaffolds as well as pictures of the cells are listed to show the condition of cells on each scaffolds using confocal microscopy.

Chapter 5 is the result and discussion and Chapter 6 is the conclusion section of this research.

Chapter 2

Preparation of Polymer Scaffolds

2.1 Experimental Materials

Cloisite Na^+-clay was purchased from Southern Clay Inc. Polystyrene (PS) and low-density polyethylene (LDPE) were purchased from Aldrich Sigma. A resorcinol di(phenyl phosphate), known as Fyroflex RDP is provided by ICL-Supresta Industrial.

2.2 Experimental Equipment

Polymer mixer, Barbender (C.W. Barbender) with two screw roller blades (EPL-V501) and a direct current drive (GP100) with heating chamber was used to mix the clay and RDP-clay with PS. A laboratory presses, Carver (Model#3912, Lot#11636) was used to mold pure or mixed polymers. Two 6×6-in mirror-like

finishing stainless-steel plates were purchased from McMaster-Carr to use as pressing plates. Aluminum mold of 3.5×3.5x0.2-in size and 9-0.5 in diameter holes were used as the mold to shape polymer scaffolds. Kapton film sheet (#B7VHC33297) was purchased from American Durafilm and used between the pressing sheets and mold to prevent polymer sticking on the pressing sheets. 70% ethanol and air compressor (CRC Duster) was used to get rid of any dust on Kapton film when it was used in the experiment. Research Autoclave (B4000-16, BioClave™) was used to sterilize prepared polymer scaffolds in glass petri-dishes. A powder conditioning mixer (Thinky, ARE-250) was used to mix RDP and Na^+-clay. A mortal to crush the RDP-Na+-Clay mixture and spatula (metal rod) and a digital weight (CS 200, Ohaus Corporation, USA) to measure polymer and clays were also used in this experiment. Scanning electron microscopy (SEM, Zaise LEO1550) was used to analysis the chemical and physical properties of the scaffolds. 70% ethanol and Kimwipes were used for cleaning process. Cam 2000 KSV Instrument LTd, contact angle meter and its software were used to measure the contact angle of samples.

2.3.1 Preparation of 20% RDP-Na+-Clay

To obtain the 20% RDP-Na^+-Clays (RDP-clay), the 20 wt% of RDP was first poured into a 200mL beaker and then 80 wt% Na+-Clay was poured. The mixture was then stirred manually using a spatula until the liquid was completely absorbed

into the clay powder. The mixture was then scrapped with spatula and transferred to a container for powder conditioning mixer. The mixture is mixed in the conditioning mixer in 700 RPM for 5 minutes. Then using a mortar, the unmixed sphere ball of mixture were crushed and placed back into the container. The procedure were repeated for 5-7 times until all the mixture was mixed and powdery.

2.3.2 Preparation of Polymer Scaffolds

2.3.2.1 Preparation of pure PS scaffolds

Two 5×5-inch Kapton films were cut and each placed on the mirror-like finishing side of the stainless-steel pressing plates. Then the sheets were sprayed with 70% ethanol and wiped with Kimwipe. Any dust left were blown by air compressor. Then the aluminum mold was placed on the Kapton film and filled with pure PS polymer pellets. Then the other cleaned side of Kapton film and pressing plate was placed on the mold, sandwiching. The set was then placed in the lab press, which was heated to 356 °F (180 °C). It was heated for 3 minutes to melt the polymers, then 5 tons (4000 psi) of pressure was applied to mold for 7 minutes. After the molding was done, the set of polymers was air-cooled and removed from mold.

2.3.2.2 Preparation of PS-clay scaffolds

36g of pure PS and 4g of Na+-Clay were measured. 18g of PS was added first, 4g of clay, and rest of PS were gradually added to the Barbender at rotation speed of 20 rpm at 180 °C. The mixture was further blended at 100 rpm for 15 minutes, then removed from the Barbender using a cooper spatula then cooled to room temperature. The polymer was then cut into little pieced and molded in the same procedure described above. Same procedure was done for PS-RDP-clay as well.

2.3.3 Sterilization of Polymer Scaffolds

The prepared scaffolds were marked with an X with a razor blade on a side which would not be cultured. The X sides were placed down on glass petri-dishes to sterilize the other side. Then glass petri-dishes in each separated kinds were taped with autoclave tapes. Then they are placed in autoclave, ran for an hour in temperature of 121°C. After autoclave, the petri-dishes were sprayed with 70% ethanol and then placed in the lab hood.

2.3.4 Contact Angle Test for Polymer Scaffolds

The contact angles of polymer samples were measured using a contact angle meter, Cam 2000 KSV Instrument LTd. Two 5 µL droplets of DI water was placed on the

surface of each polymer scaffolds and the angles and covered to in dark to obtain the best result. Then angles were measured using the contact angle measurement software for the instrument. Both left and right angles were measured separately.

Chapter 3

Preparation of GFFB Cell Culture

3.1 Experimental Materials

Green fluorescent fibroblast cells (passage 9) were donated by Dr. Simon from Stony Brook Dental School. The cells were frozen in liquid nitrogen (-191°C). Standard Dulbecco's modified eagle medium (DMEM, 11965-092 from GIBCO) was used as cell growth medium. Fetal bovine serum (FBS, HyClone) was used to provide essential proteins for the cell growth. Penicillin/streptomycin (P/S, GIBCO) to provide protein and prevention of bacterial contamination for the medium. Dulbecco's Phosphate-Buffered Saline (PBS, 14190-144, GIBCO) was used for cleaning. 0.05% Trypsin-EDTA (1X) solution (GIBCO) was used to trypsinize cells from the polymer scaffolds, cell culture flask and dishes. EDTA, ((Ethylenedinitrilo)tetra-acetic acid, disodium salt, dehydrate) from J.T. Baker was

used to make 10X Trypsin-EDTA solution. 37% formaldehyde in H_2O (Sigma) was used to freeze the cells. Triton X-100 (28314, Thermo Fisher Scientific) was used to permeabilize the membranes of cells. Alexa Fluor® 488 Phallloidin (Thermo Fisher Scientific) was used to dye actin-F. Propidium iodide (PI, R37108 from Thermo Fisher Scientific) was used to dye the nuclei of cells. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (D1306, Thermo Fisher Scientific) was used to dye the nuclei of cells in blue for count under fluorescent microscopy. 70% ethanol was used for cleaning and sterilization.

3.2 Experimental Equipment

$75cm^2$ tissue culture flask (T-75, Falcon) was used to culture the cells for the experiment. 24-multiwell tissue culture plate (Falcon) was used as scaffold container as well as a control scaffold. Easy grip tissue culture dish (35mm, Falcon) was used to culture control cells for confocal microscopy. Pipets of 1-10μL, 10-200μL and 100-1000μL from Pipet-Lite XLS were used to transfer medium. Lab hood used in this experiment was from Labconco, purifier class II biosafety cabinet. Cell culture incubator was from Napco 5400 and the carbon dioxide level was 5% while the temperature was 37 °C. Waterbath was from Isotemp 210 and temperature was 37.4 °C. Hemocytometer (Hausser Scientific) was used in cell counting. Centrifuge was from Damon/IEC Division (IEC HN-SII) and used to separate the trypsinized cells from medium. TCS SP2 confocal microscopy from Leica was used

to observe stained cells. 15mL and 50mL Falcon tubes were also used in this experiment. Disposable round-bottom PS tubes were used for cell counting. Optical microscopy used to cell counting was Olympus CKX41. Olympus Introduces the IX51 Inverted Microscope with fluorescent light was used for cell counting using ImageJ. TCS SP2 sectral confocal & multiphoton system from Leica was used to observe stained cells.

3.3 Experimental Steps

3.3.1 Preparation of Solutions

Full medium with 10% of FBS and 1% P/S was prepared by addition of 50mL FBS and 5mL P/S to 500mL of DMEM. 3.7% formaldehyde solution was prepared by dilution of 1mL of 37% formaldehyde with 10mL of PBS. 0.4% Triton solution was prepared by dilution Triton X-100 in PBS. PI was diluted by PBS for concentration of 5×10^{-3} mg/mL. Alexa Fluor® 488 Phallloidin was diluted by PBS for concentration of 5×10^{-3} mg/mL. DAPI stock solution was prepared by dilution by deionized water for concentration of 5 mg/mL. 10X EDTA-Trypsin was prepared by adding 18 mg of EDTA with 10 mL of trypsin.

3.3.2 Cell Preparation

All medium/trypsin were warmed to 37°C before in use. GFFC was cultured in T75 tissue culture flask with Full DMEM in humidified incubator with 5% CO_2 at 37 °C

for 3 days or until the cells grew enough to use in experiment. Cell culture medium was changed every 2 days. Then the cells were washed twice with PBS after the flask was taken out from incubator and placed in the hood. Then 2 mL of 1X EDTA-trypsin was added and the flask was placed in incubator for 4 minutes for trypsinization. After 4 minutes, the flask was gently tapped to make sure all cells were floating. 4 mL of Full DMEM was added to the flask to stop trypsinization. Then the medium was transferred to a 15 mL falcon tube and centrifuged at speed of 800 RPM for 10 minutes. After aspirating out the medium, not touching the supernatant, the cells were diluted with Full DMEM for the concentration of 10,000 cells/100 μL per a scaffold.

3.3.3 Cell Culture on the Scaffolds

Each autoclaved polymer scaffold was placed on a well of 24-cell culture dish. 5 of each PS, PS-clay and PS-RDP-Clay was placed in each dish, shown in Figure 18 and 19. The experiment was repeated twice: for first trial, 100 μL of 5,000 cells (recommended seeding density for 24-well dish) were seeded for each scaffolds and wells for control up to Day 4. For 2nd trial, 100 μL of 10,000 cells were seeded on each scaffold and wells for control up to Day 6. Then the dish was placed in the incubator for 30 minutes to make cells settled down and attached on the surface of scaffolds. Then 1 mL of Full DMEM was added on each well and incubated. 300 μL of 30,000 cells was seeded on 35 mm petri dish for confocal control samples

and also incubated. The samples were prepared for Day 1, 2, 3, 4, 5 and 6. Cell culture medium was changed every 2 days.

3.3.4 Cell Counting using a Hemocytometer

The 24-well dish with seeded polymer scaffolds was taken out to the lab hood and the medium was aspirated out and washed 3 times with PBS. Then in another new 24-well dish, 100 μL of 10x-EDTA trypsin was added to each well. Facing the cell-seeded face down, each polymer scaffolds picked from 1st and 3rd row (Figure 19) were transferred to the new 24-well dish with 10x-EDTA trypsin. Then the set was placed in the incubator for 4 minutes for trypsinization. 200 μL of medium was then added to each well to deactivate the trypsin, pipetted and transferred to disposable PS round-bottom tubes. Then each tubes were vortexed before 10 μL of each samples were pipetted to each side of hemocytometer and then observed under Olympus optical microscope and counted cells on only both sides of 4 corners and the center squares with a tally counter every day up to Day 6.

3.3.4 Cell Fixing and Staining

After the cell counting, the cell culture dish with rest of samples was taken out from the incubator and washed with PBS twice. Then 500 μL of diluted 3.7% formaldehyde was added on each well to soak the samples and waited for 15 minutes to fix cells. Then samples were washed with PBS twice again. 200 μL of

Triton was added to each for cell membrane permeation for 7.5 minutes, then washed with PBS twice again. Then the cell membranes were stained to green with 200 μL of Alexa Fluor for 20 minutes and covered with aluminum foil to block light. Then it is washed with PBS twice. For confocal microscopy, 200 μL of PI was added to the sample for 3.5 minutes to dye the nuclei to red. For cell counting using Image J, DAPI was added to the sample to dye the nuclei to blue for 3.5 minutes. Then the samples are washed twice and soaked in PBS, then stored in a refrigerator.

3.3.5 Fluorescent Microscopy

The DAPI-dyed cells on the polymer scaffolds and cell culture dishes for control were observed using a fluorescent microscopy with a 5X lens. The images for each samples were taken for 9 sections, and then the pictures were analyzed using a software called ImageJ to automatically analyze the dark-spot, which was the dyed cell, and obtain the number of cells plated on the surface for each image. Then the numbers were added for total.

3.3.6 Confocal Laser Scanning Microscopy

The PI-dyed cells on the polymer scaffolds and cell culture dishes for control were observed using a confocal microscopy with 5X and 10X water lenses. Two samples of each polymers were picked from the 24-well culture dishes. Then each sample

was placed on a 35 mm petri-dish with PBS filled, then observed for results and imaged.

Chapter 4
Results and Discussion

4.1 SEM images of Polymer Scaffolds

The colors of polymer scaffolds changed after autoclave. The transparency of PS changed to unclear and the colors of PS-clay and PS-RDP-clay polymers were darkened. SEM microscopy was used to observe any physical changes in 20 kV (inside structure) and 5 kV (surface) for PS before and after autoclave, concerned about the low melting point of PS, unlike high boiling point of RDP at 587 °C and of decomposition which occurs at 300 °C. Some holes were observed on the surface of PS after autoclave, which was formed by trapped air escaped in the process due to high temperature and pressure. However, the holes were about size of 3 μm, which was smaller than the green fluorescent fibroblast cells (10~15 μm) [16]. Therefore such holes would not affect any effects on the cell growth and could be ignored. On the other hand, EDX (Energy-dispersive x-ray spectroscopy) spectra were measured for chemical change before and after autoclave, however, there was no difference. The SEM images for 20 kV and 5 kV for before and after autoclaves

are shown in Figure 4 through Figure 11. The EDX spectra for before and after autoclave are shown in Figure 12 and Figure 13. The SEM images of PS-clay and PS-RDP-clay were also captured as well as the EDX spectra after the samples were autoclaved, which are shown in Figure 14 through Figure 18.

The PS-clay sample did not show peak for phosphorus, however, PS-RDP-clay sample showed the sign of phosphorus, which was in the chemical composition of RDP. The spectra were the proofs that the clay and RDP-clay did stay in the polymer after autoclave, no leaking or decomposing. Therefore autoclave at 121 °C was determined as a safe method to sterilization in this experiment.

4.2 Contact Angles of Polymer Scaffolds

The contact angle between DI water and polymer scaffolds were listed in Table 1 to see if there was difference before and after the sterilization using autoclave. According to the measurement, there was very slight increment in angle for 1~2 ° for PS and RDP-clay, while PS-Clay had the largest difference about 3° for left angle and 7 ° for right angle after autoclave. However, such differences could be ignored as the polymers were still hydrophobic, less than 90 ° [17].

4.2 Cell Counting using a Hemocytometer

The first two samples were picked from 1st and 3rd row of the 24-well dish (shown in Figure 19 and Figure 20) for the cell counting using a hemocytometer and a tally counter. The cell numbers were counted three times using a hemocytometer and a tally counter. Then the counted numbers were averaged and then analyzed to compare the conditions among control, PS, PS-clay and PS-RDP-Clay groups. The proliferation rates of cells on each scaffolds were analyzed as well as the doubling time by the data obtained from cell counting. On the other hand, the cell condition were observed under a confocal microscopy and also analyzed to see the images of the cell growth on substrates. For first trial of this experiment with recommended seeding density of 5000 cells/100 μL, growth curves of the cells were plotted with standard deviation in Figure 21. The second trial growth curve of seeding density with 10,000 cells/100 μL was plotted with standard deviation in Figure 22 as well. For the 2nd trial, data after Day 4 was discarded because the number of cell exceeded the maximum confluency (0.2×10^6) for 24-well culture dish. The cell culture doubling time was calculated using a formula ($T_d = Duration\times\frac{\ln(2)}{\ln(C_{final})-\ln(C_{iniial})}$) as growth rate is $\mathrm{N}(t) = \mathrm{N}(t_{initial})e^{growth\ rate\times time}$ [18] for first and second trial and shown in Figure 23 and 24.

The counted cell number was averaged and calculated from Day 1 to Day 4 for each substrates. Cells on control and on RDP-clay scaffolds showed dramatic increment

in Figure 21, while PS and PS-clay did not perform as well as control and RDP-clay group. RDP-clay showed about 2 folds better performance than PS, 3 folds better than PS-clay in 1st trial and 4 folds better than PS and PS-clay in 2nd trial on Day 4. RDP-clay even had more cell numbers than control on Day 3 and Day 4 in 1st trial while 33% less than the control in 2nd trial according to the plotted graphs. The doubling time of RDP-clay showed 1.01 and 1.26 days (shortest), control for 1 and 1.38 days, PS for 2.41 and 3.69 days, PS-clay for 2.33 and 13.90 days (longest) for 1st trial and 2nd trial, consequently. This showed that RDP-clay provided the best environment compare to other substrates according to the high number of cell growth and lowest doubling time while PS- clay showed the lowest number of cell growth and highest doubling time, indicating the worst condition as a scaffold.

4.3 Cell Counting using Image J

After 2 rows of samples were used for hemocytometer cell counting, rest two rows of samples were fixed and dyed to observe the cells under either a fluorescent microscopy or a confocal microscopy. The second row was selected for the image under fluorescent microscopy and pictures were taken for 9 division as shown in Figure 25 for each day. Then using a software called ImageJ, the total cells numbers from 9 pictures were counted and doubling time was calculated. The fluorescent microscopy pictures used for the counting for PS, PS-clay, RDP-clay and control were shown in Figure 26 through Figure 29 on Day 6. The growth curve based on

the number of cells analyzed and calculated using ImageJ was shown in Figure 30. The doubling time was shown in Figure 31. The growth curve had similar tendency as the growth curve plotted by cell numbers counted by hemocytometer; control had the greatest increment from Day 1 to 6, while RDP-clay has the second highest rate. The doubling time was also similar results as both RDP-clay and control showed the lowest and similar data close to 1.8 days. However, PS in fluorescent image counting had worse performance than PS-clay unlike previous cell counting. Also, PS had the doubling time was highest, around 21 days. This value was even 7 days longer than of PS-clay, which was close to 14 days. Such long double time indicates that the cells barely grow on the scaffolds. Therefore it was indicated that RDP-clay PS showed the best result for the cell growth.

4.4 Condition of Cells under a Confocal Microscopy

The images were taken using a confocal microscopy to observe the condition of cell. The cells were fixed and dyed on Day 3 to image under a confocal microscope. The images were Figures 32 through Figure 35 for PS, PS-clay, RDP-clay and control. The nuclei of cells shown in Figure 32, PS sample, were barely visible and the cell were not stretched out or connected to each cells. In Figure 33, the nuclei of cells were visible in color red on PS-clay scaffold, however, there was no green colored cell membrane around the nuclei. The cell did not look normal or healthy.

However, cells on RDP-clay substrate in Figure 34 looked very normal, well distributed and both nuclei and membranes were clearly visible. Furthermore, Figure 34 and Figure 35 (control) looked very similar, both images showed cells were well stretched, connected and clearly visible. This indicates that RDP-clay scaffold provided very similar environment for the cell growth as much as commercial tissue culture plates.

5 Conclusion

In this study, RDP contained polymer scaffolds were created and used as substrates for cell culture in order to test its toxicity. RDP was currently known as flame retardant and commonly used, however, its toxicity data had not been studied thoroughly in vivo. According to the cell counting and cell condition images using a confocal microscopy, 2% RDP in PS was determined as it did not only harm the cell growth, but also enhanced the cell growth even in hydrophobic condition. While PS and PS-Clay both showed worst condition as polymer scaffolds due to high doubling time and low cell numbers, RDP-clay scaffolds showed similar performance to current commercial tissue culture plate (control). This result was against the hypothesis that RDP might have negative effects on the cell growth. Based on this result, RDP may be used addition to commercial PS tissue culture plates as it provoked the cell growth very similar to current commercial PS tissue culture plates or flask. The disadvantage might be the transparency, however, RDP could be used for dishes that does not need to be transparent or requires block of

light. Therefore it would be believed to have potential to provide optimized environment for cell growth. On the other hand, further studies would be need to see how the chemical and/or physical mechanism of how RDP evokes the cell growth and might have the same effect on bacteria culture that may endanger the health of human body. RDP could be an effective and non-toxic flame retardant commonly used in plastics, and the source for the bacteria culture and virus infection.

6 Figures and Tables

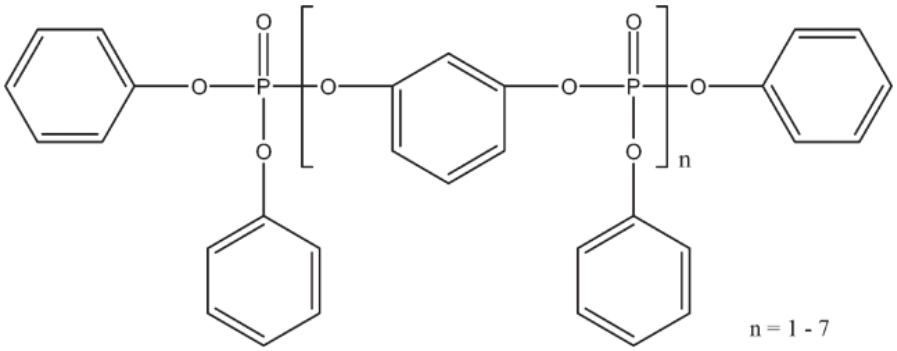

Figure 1. The chemical formula of RDP [10].

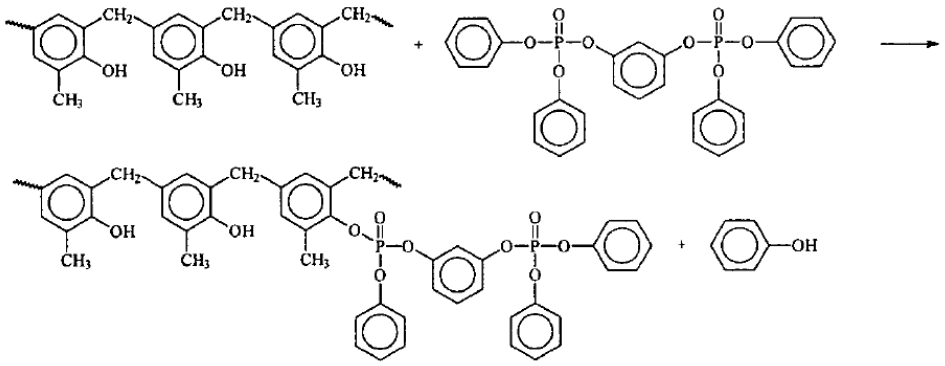

Figure 2. Chemical Interaction of RDP with PPO [11].

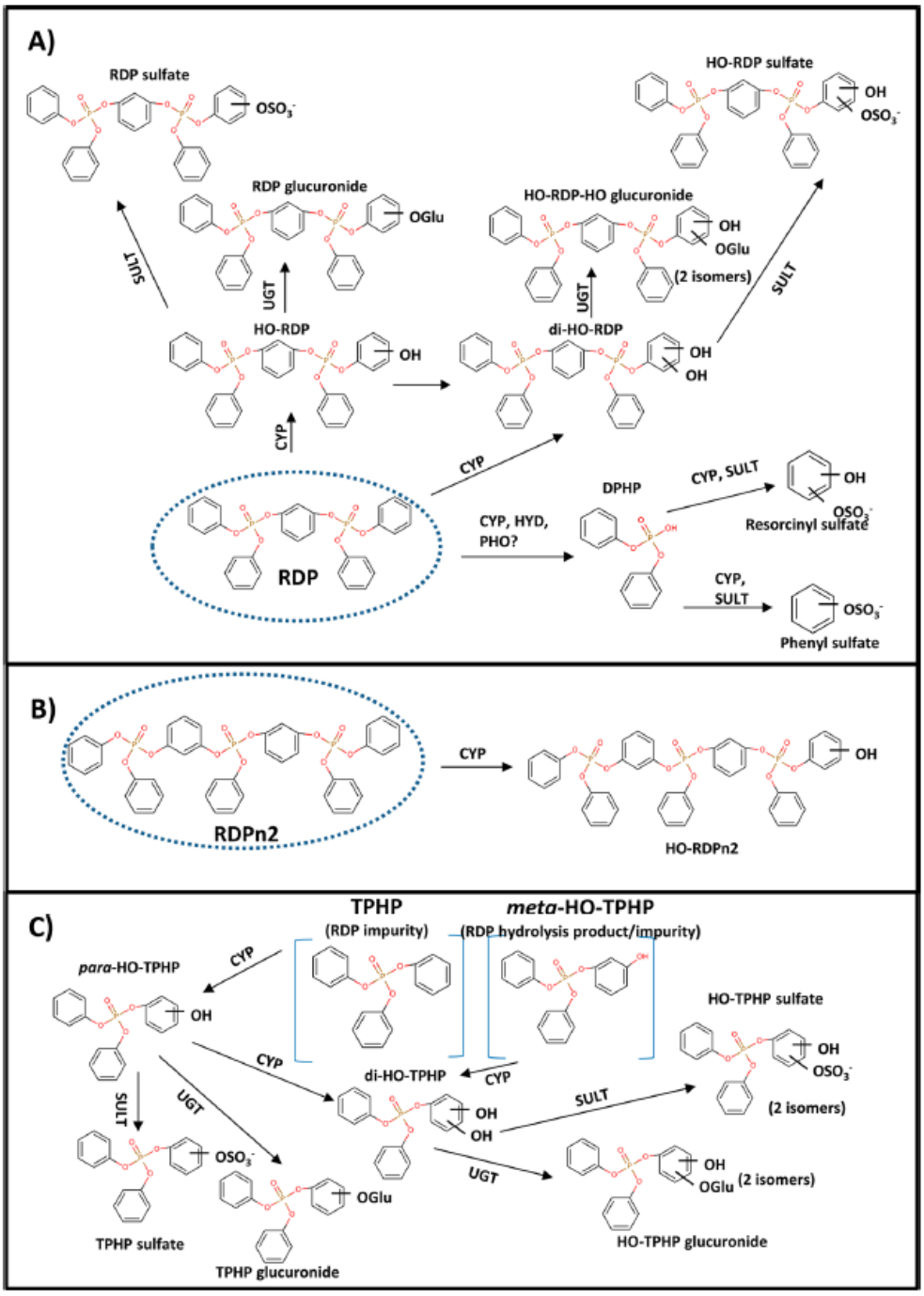

Figure 3. Metabolites of RDP and RDP oligomers [8].

Samples	Left Angle	Right Angle
PS	92.15 °	92.80 °
PS (autoclaved)	93.49 °	93.38 °
PS-Clay	88.83 °	86.90 °
PS-Clay (autoclaved)	91.06 °	93.60 °
RDP-Clay	88.78 °	90.38 °
RDP-Clay (autoclaved)	91.86 °	91.40°

Table 1. Contact Angle Measurement for the Polymer Scaffolds before and after autoclave.

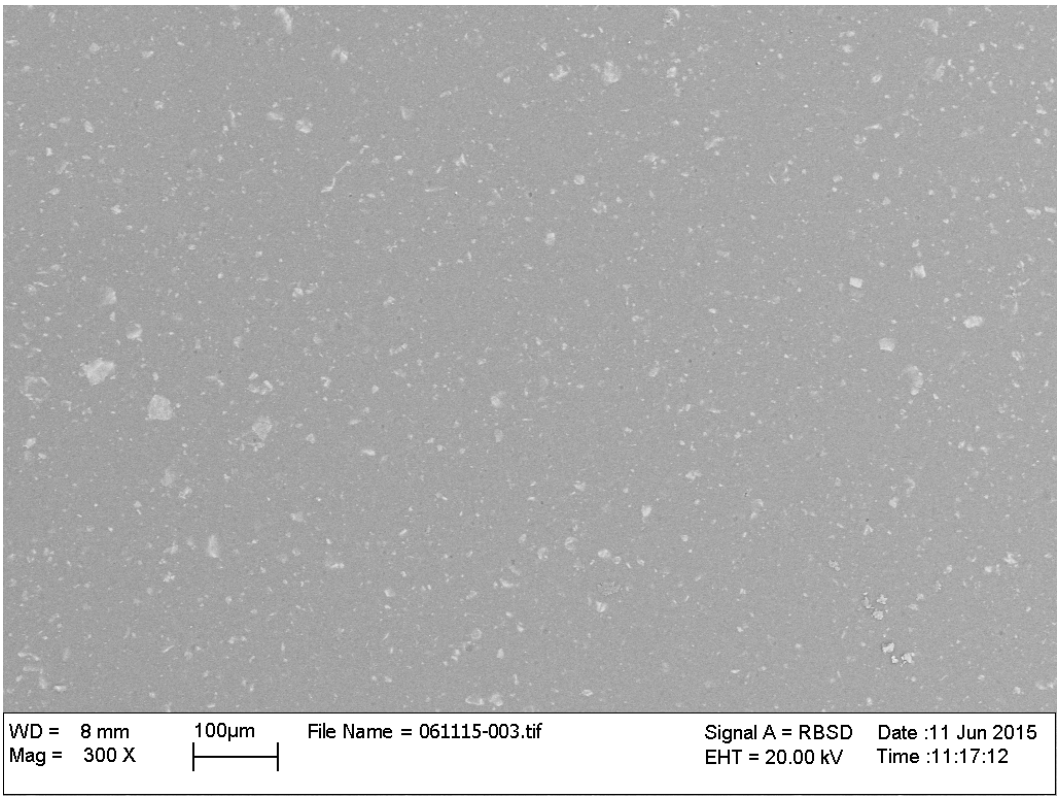

Figure 4. SEM image of pure PS before autoclave at 300 X. (20kV)

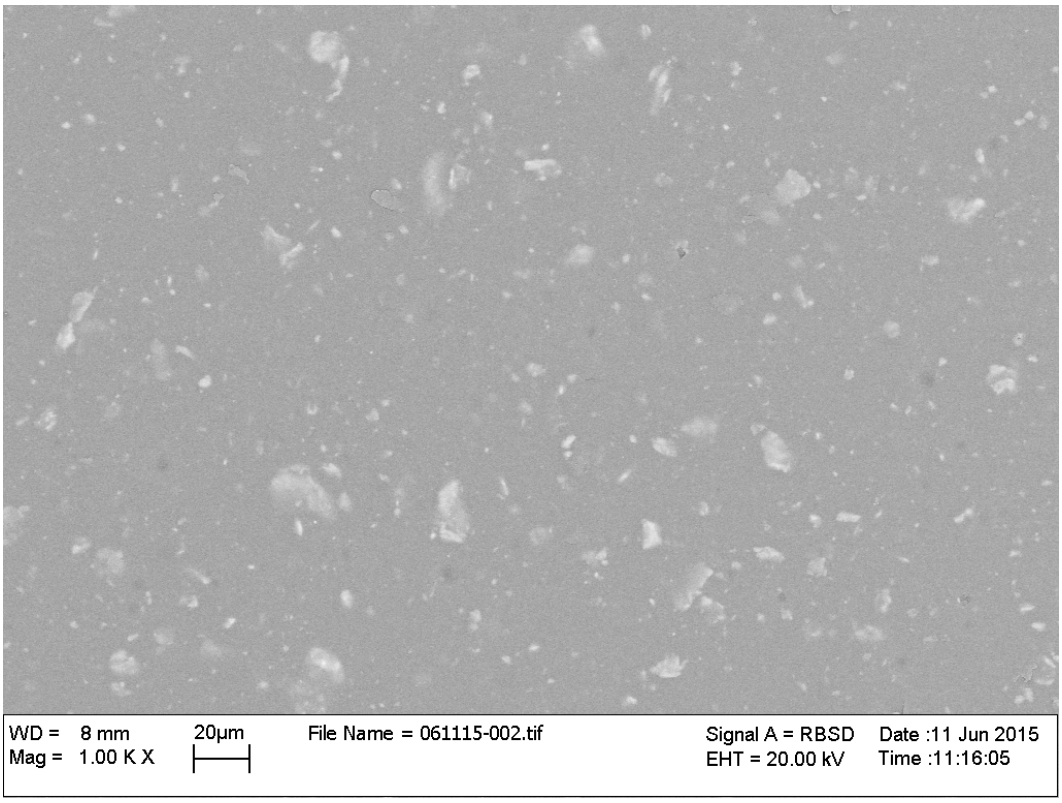

Figure 5. SEM image of pure PS before autoclave at 1000 X. (20 kV)

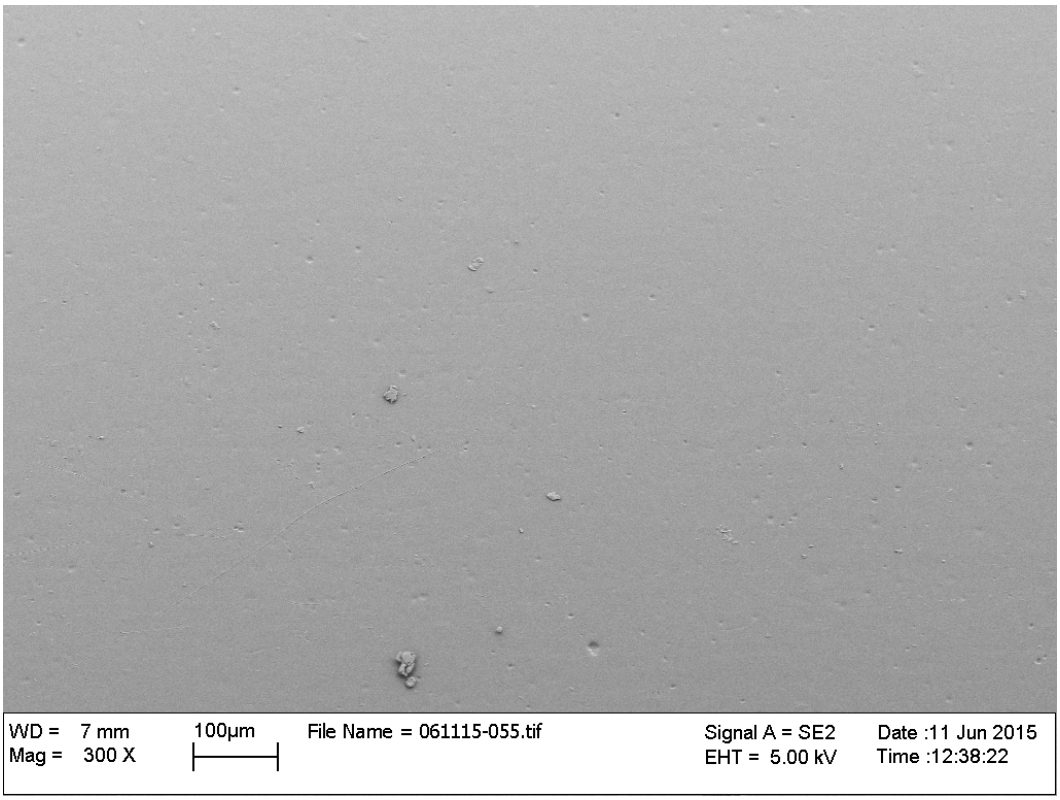

Figure 6. SEM image of pure PS before autoclave at 300 X (5 kV)

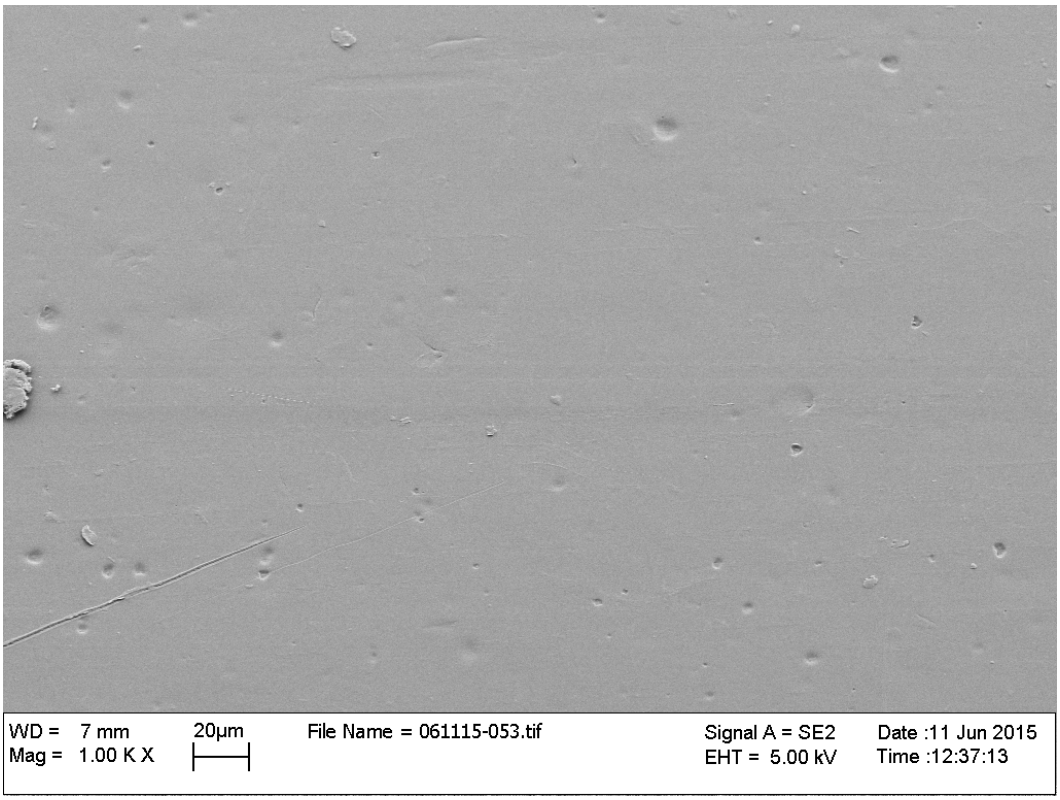

Figure 7. SEM image of pure PS before autoclave at 1000 X. (5 kV)

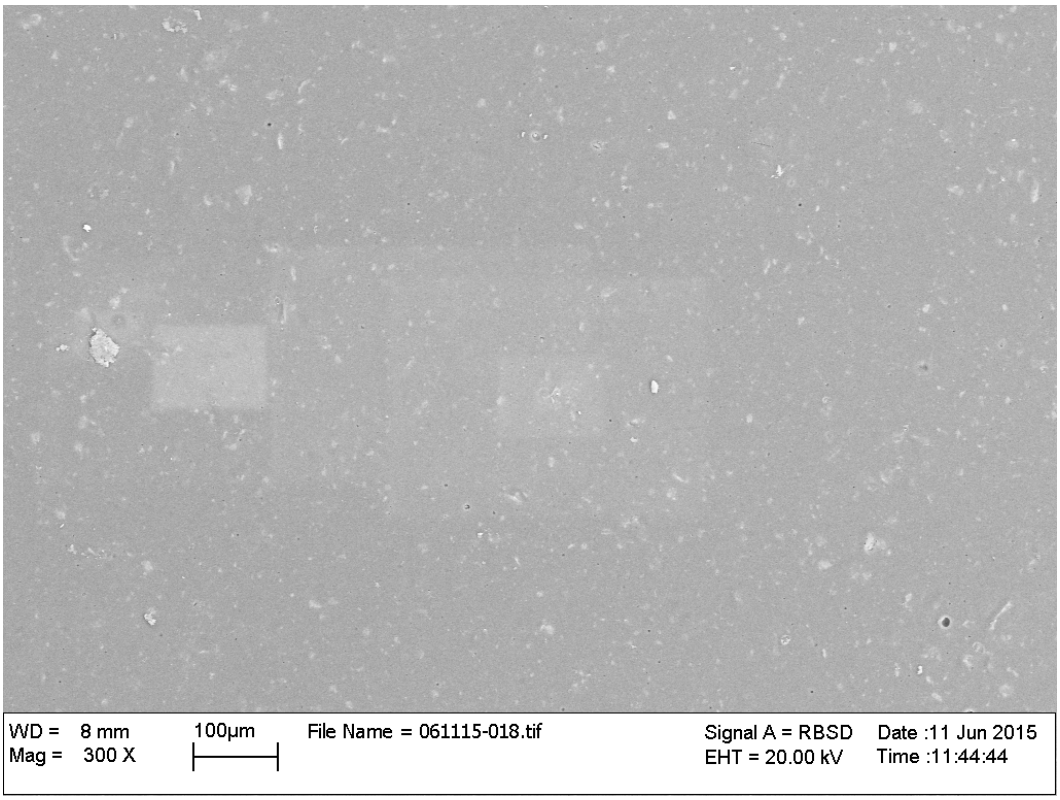

Figure 8. SEM image of pure PS after autoclave at 300 X. (20 kV)

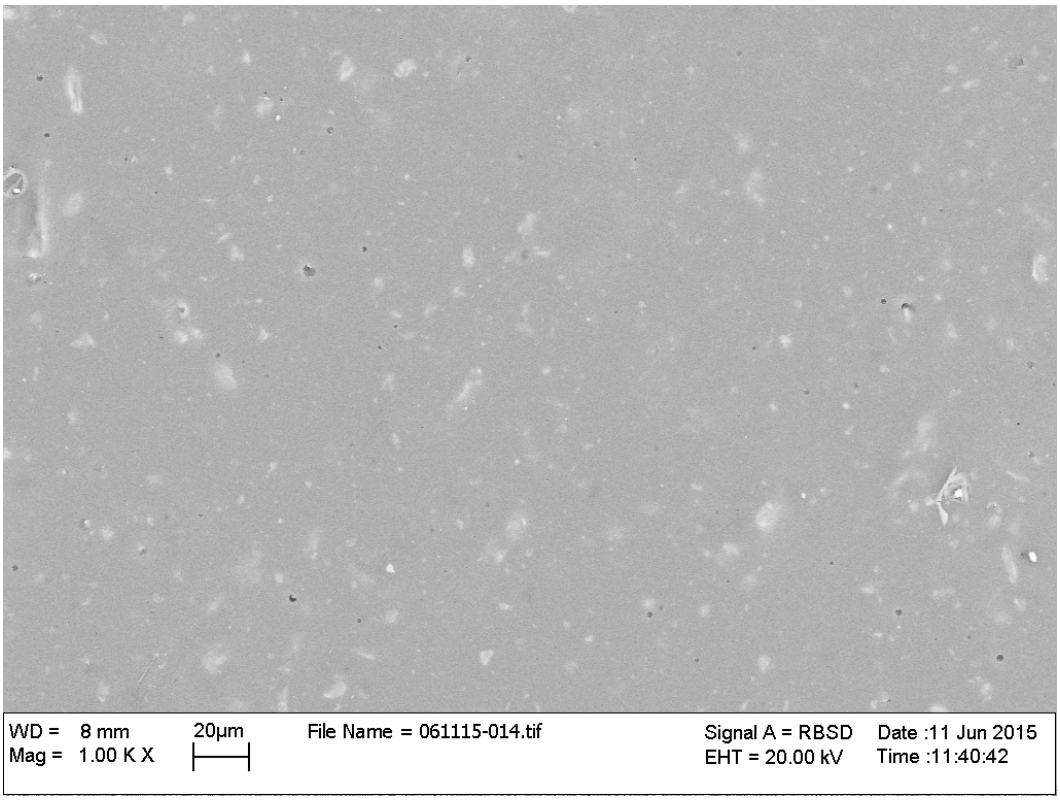

Figure 9. SEM image of pure PS after autoclave at 1000 X. (20 kV)

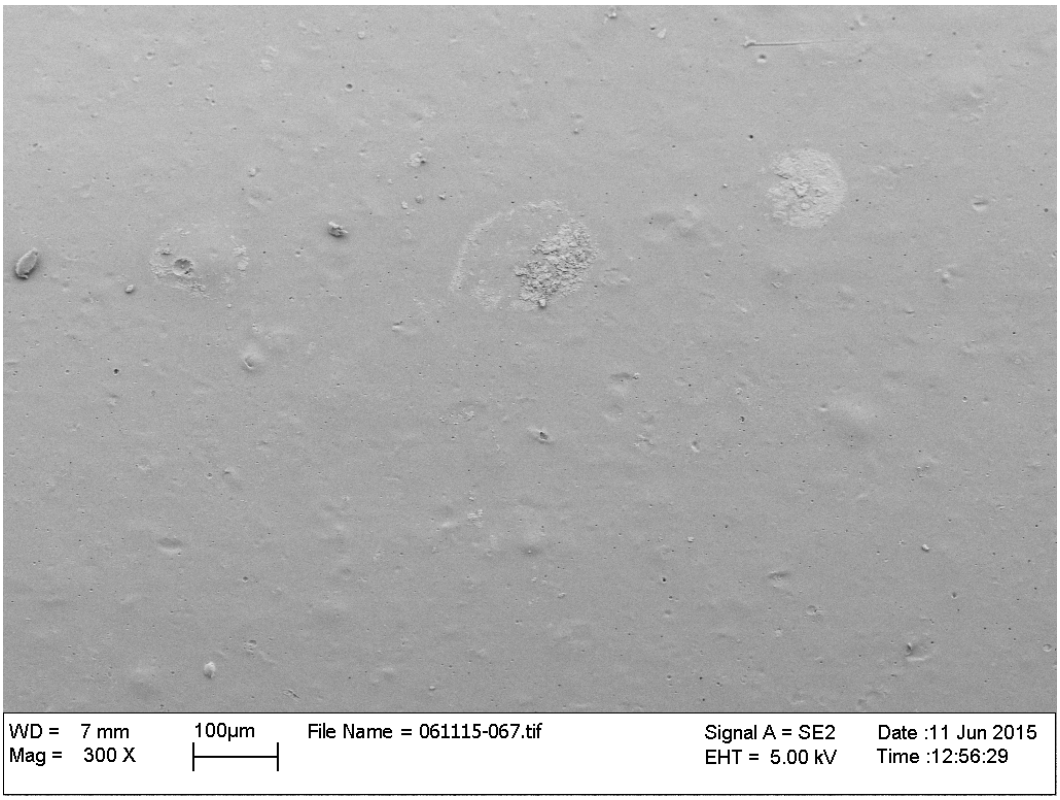

Figure 10. SEM image of pure PS after autoclave at 300 X. (5 kV)

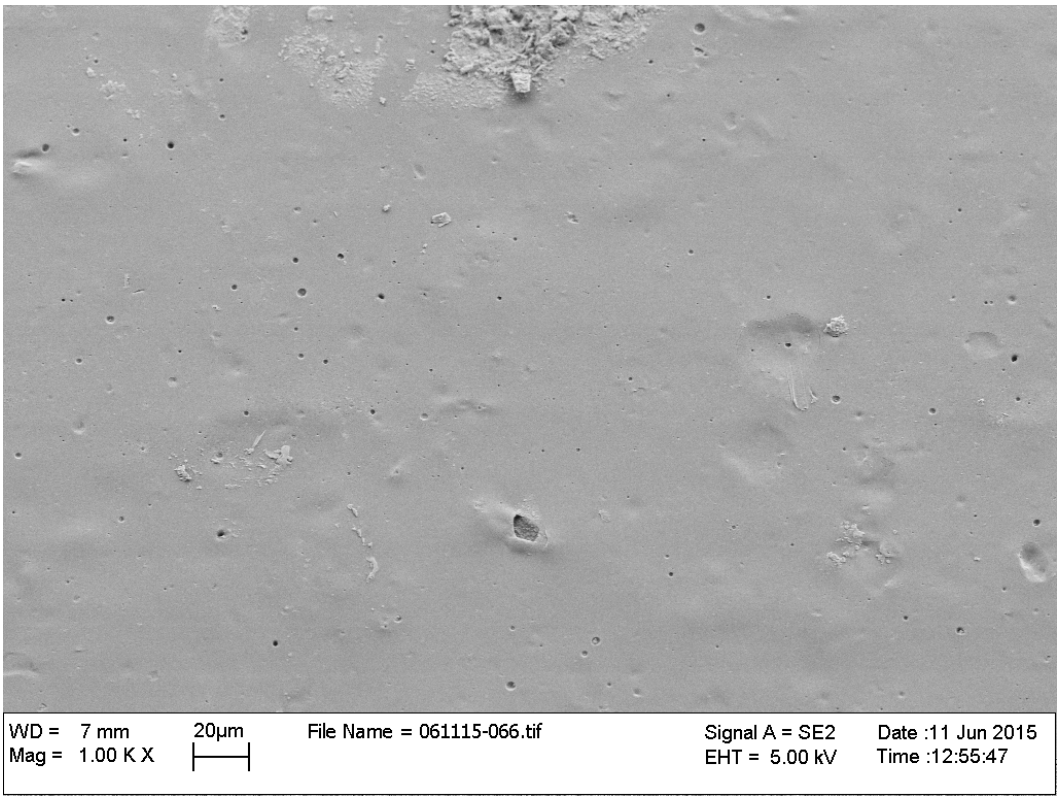

Figure 11. SEM image of pure PS after autoclave at 1000 X. (5 kV)

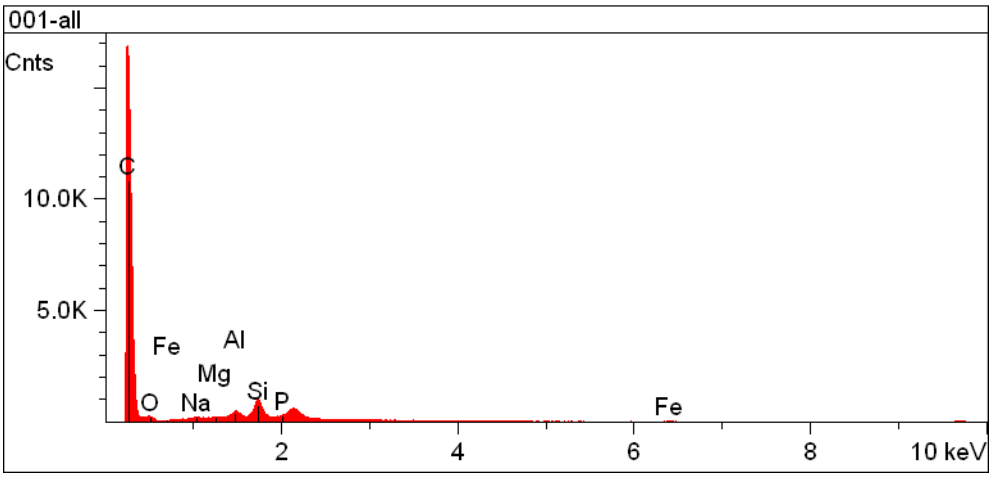

Figure 12. EDX spectrum of the visible area of pure PS before autoclave.

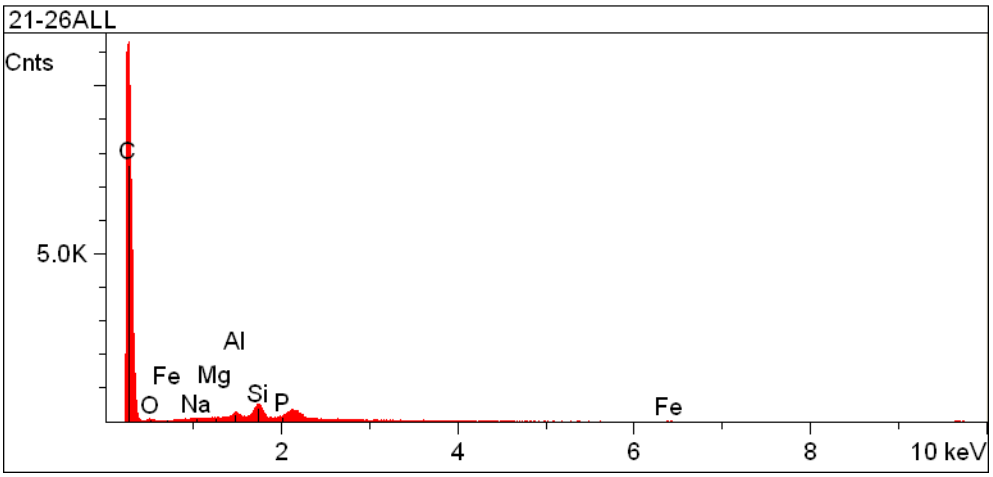

Figure 13. EDX spectrum of the visible area of pure PS after autoclave.

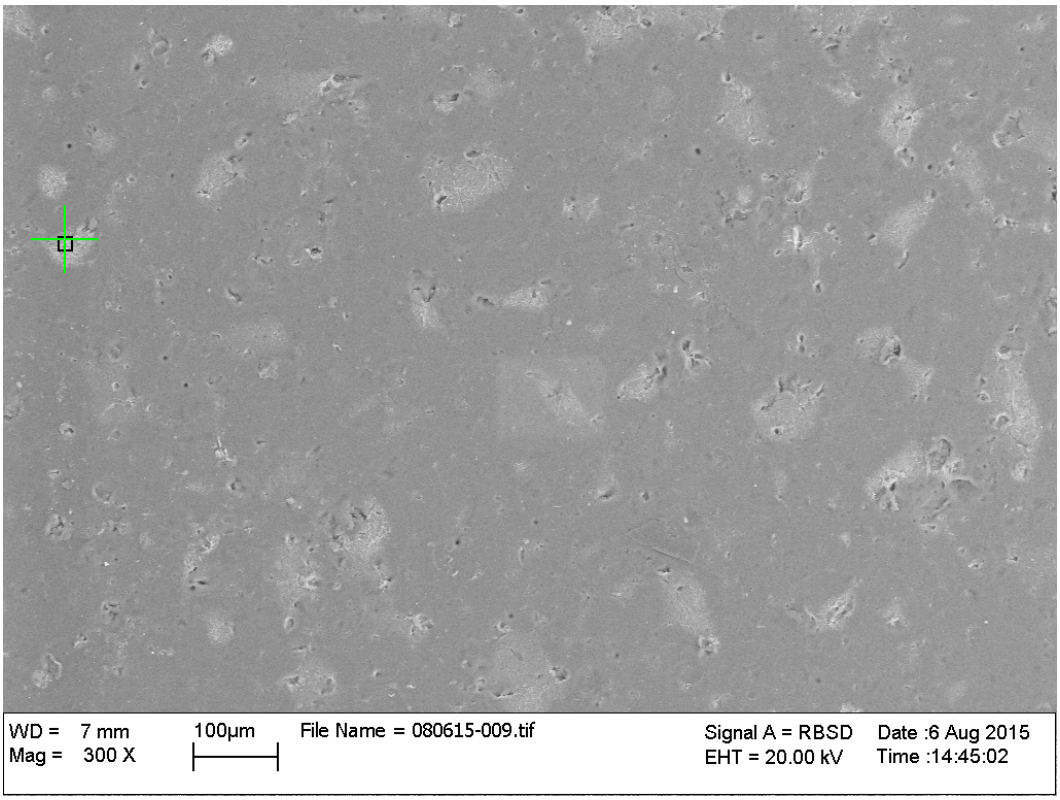

Figure 14. SEM image of PS-Na^{+}-Clay after autoclave at 300 X. (20 kV)

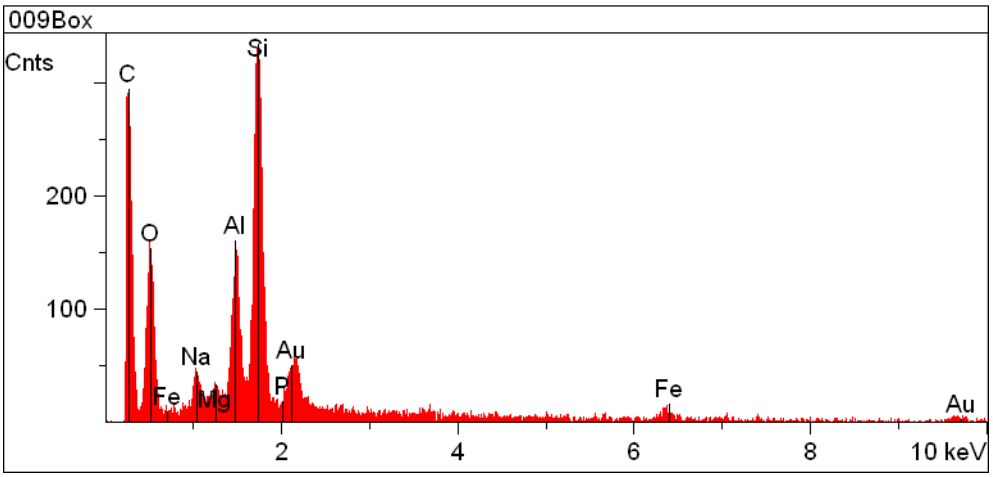

Figure 15. EDX spectrum of PS-Na+-Clay in the area boxed in Figure 14.

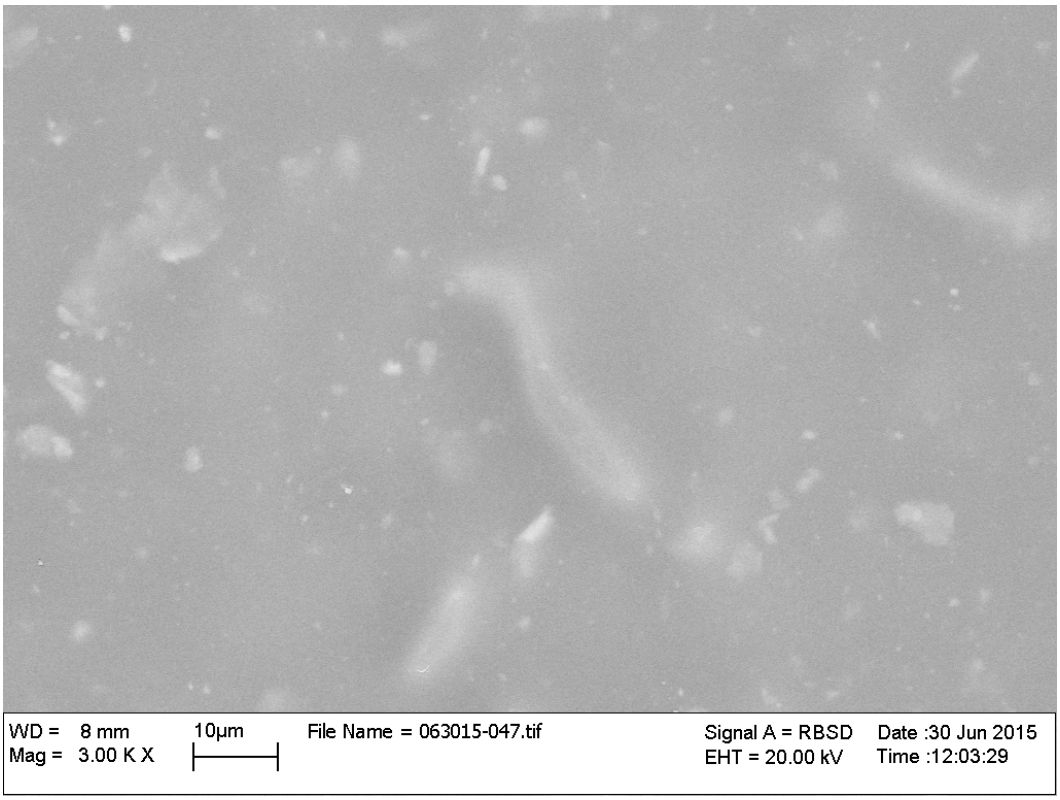

Figure 16. SEM image of PS-RDP-Na+-Clay after autoclave at 3000 X. (20 kV)

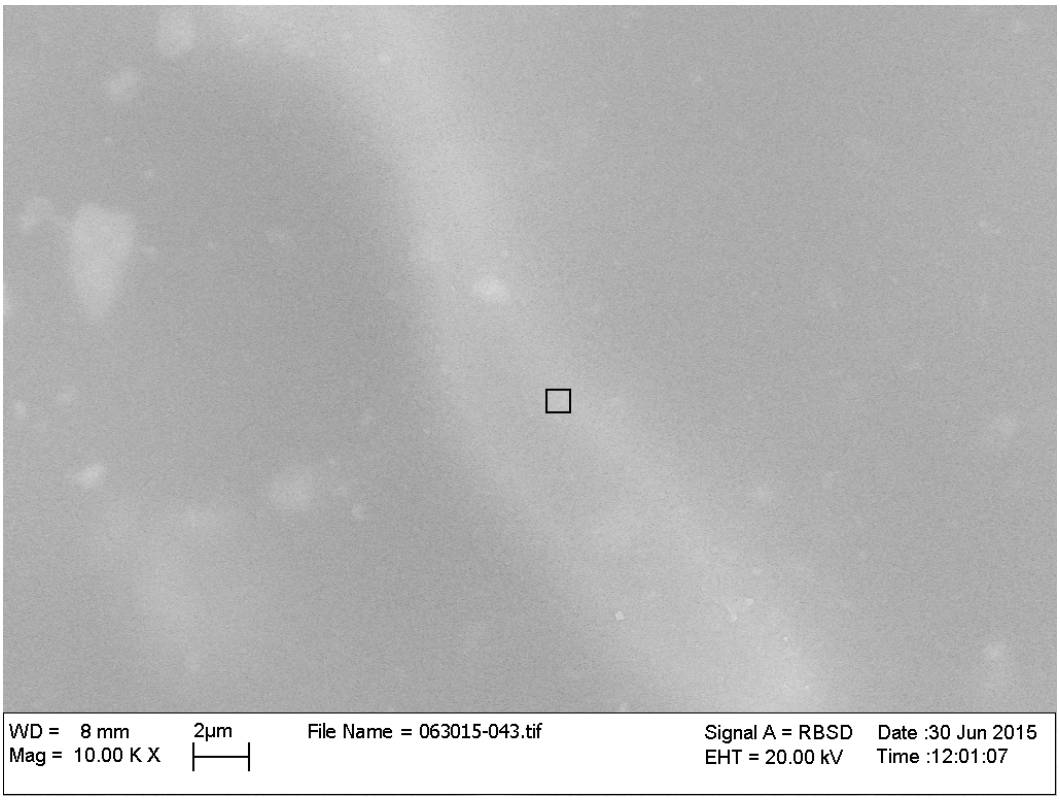

Figure 17. SEM image of PS-RDP-Clay at 10 KX, enlargement of image in Figure 16.

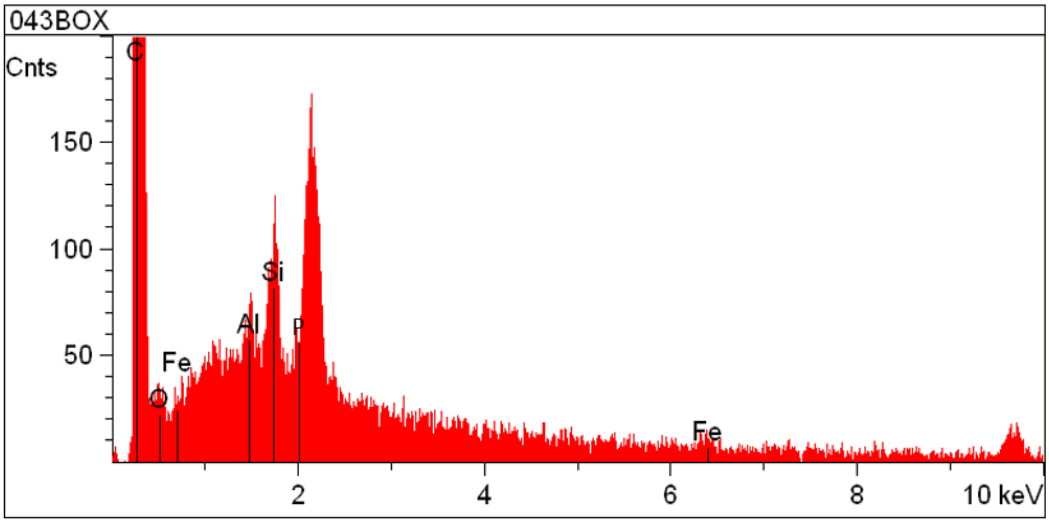

Figure 18. EDX spectrum of PS-RDP-Clay in the area boxed in Figure 17.

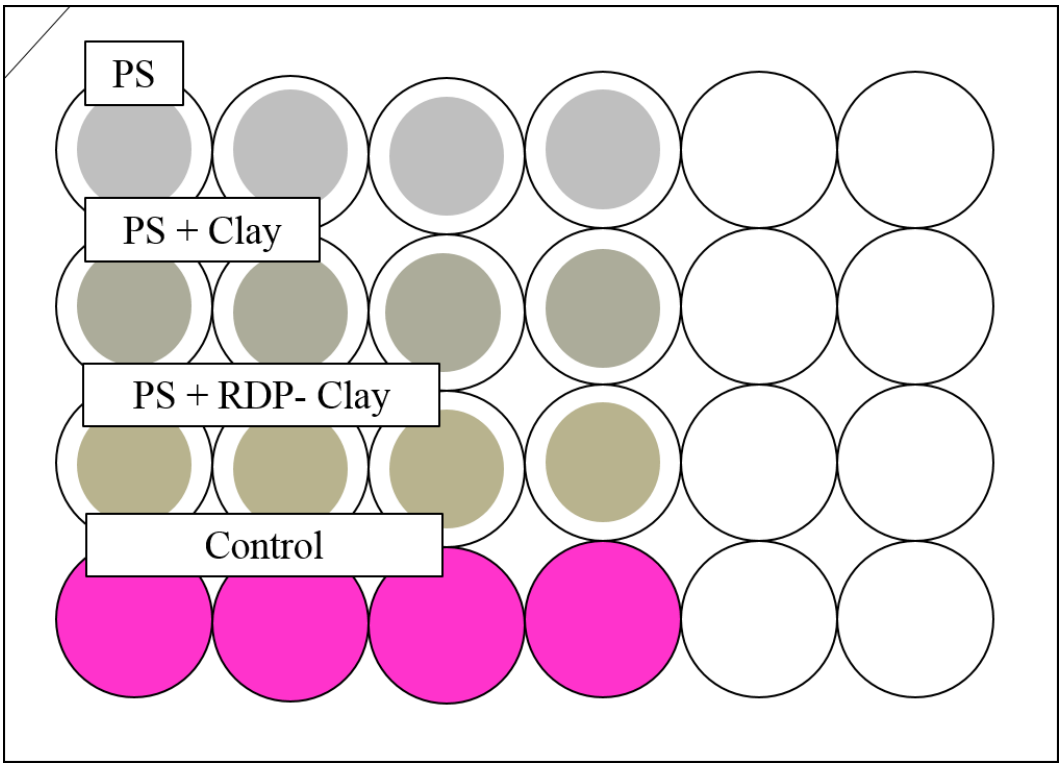

Figure 19. Diagram of polymer scaffolds placed in 24-well cell culture dish for each day from Day 1 to Day 6.

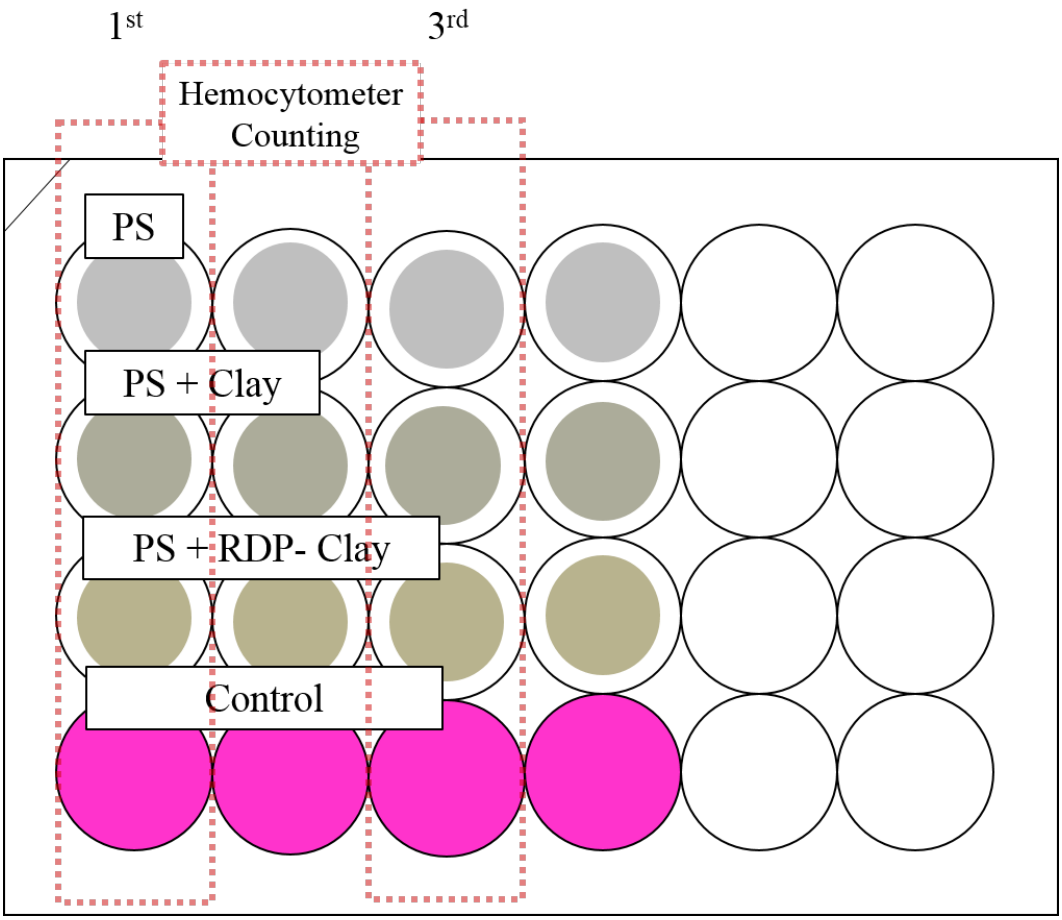

Figure 20. Diagram for polymer scaffolds selected for hemocytometer cell counting.

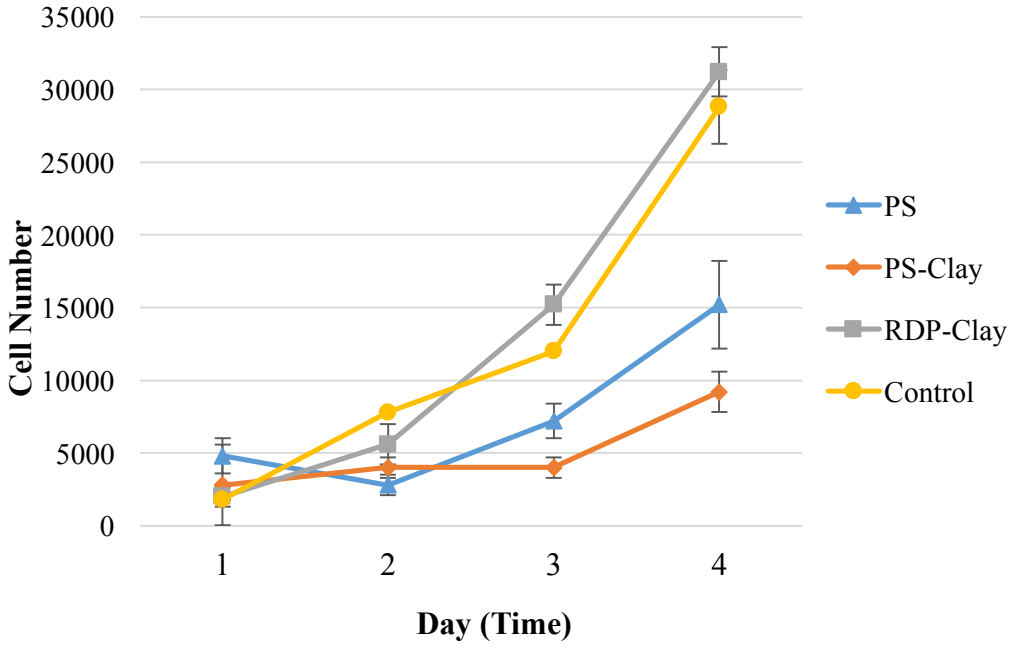

Figure 21. Green fluorescent fibroblast cell growth curve for first experiment with seeding density of 5000 cells/100 µL.

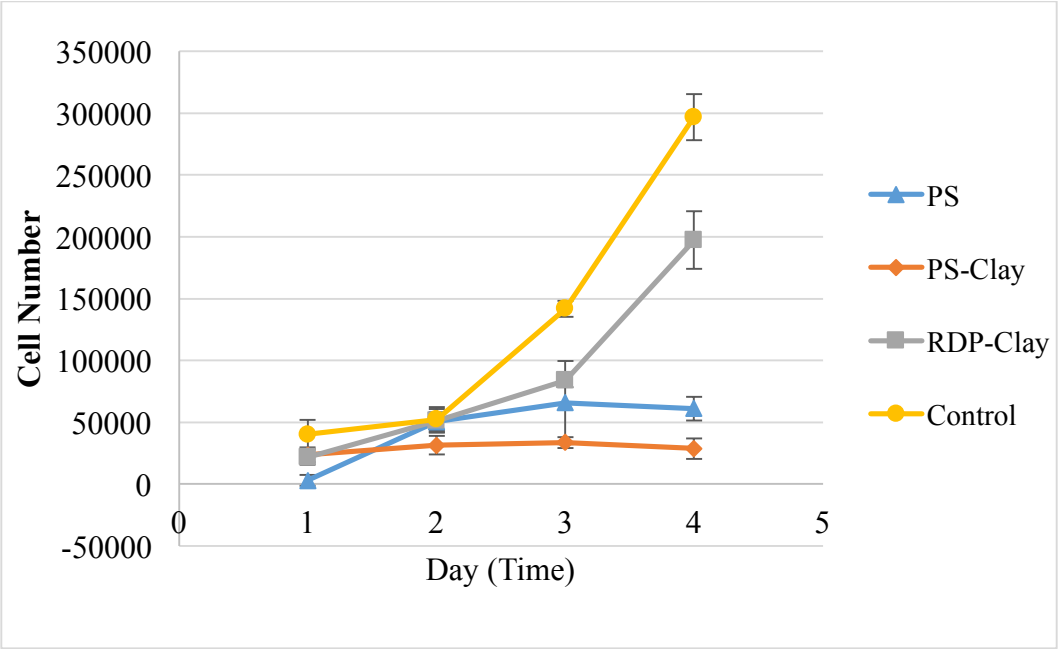

Figure 22. Green fluorescent fibroblast cell growth curve for second experiment with seeding density of 10,000 cells/100 µL.

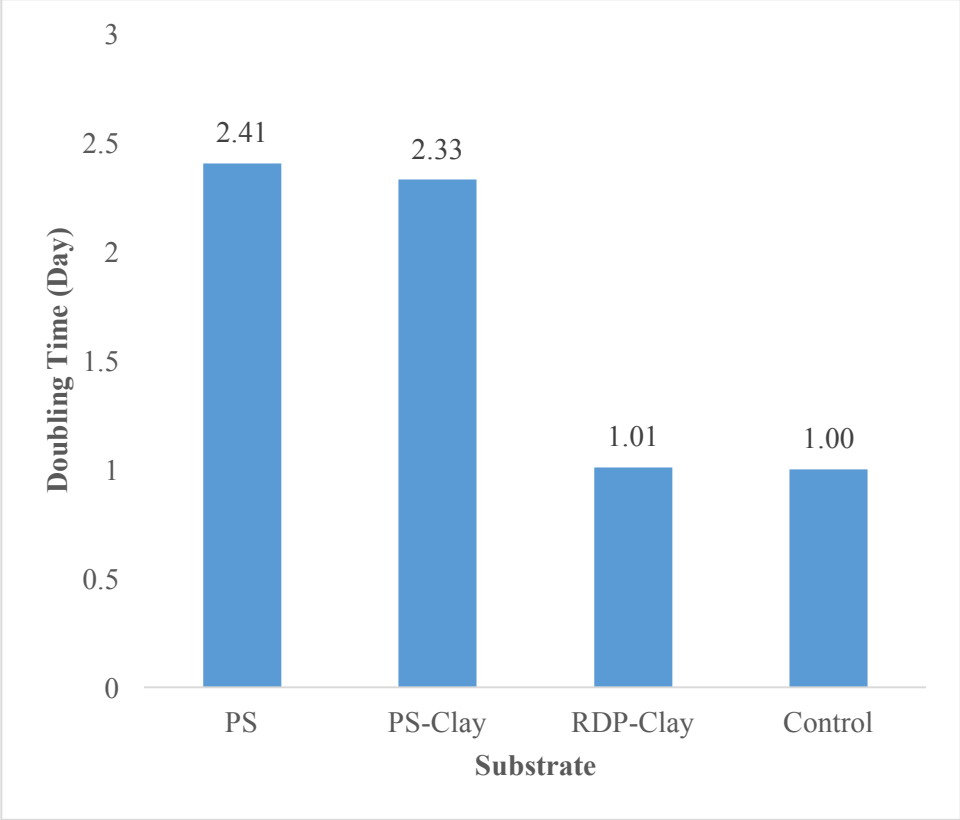

Figure 23. Doubling time for proliferation rates of GFFB cell for 1st trial

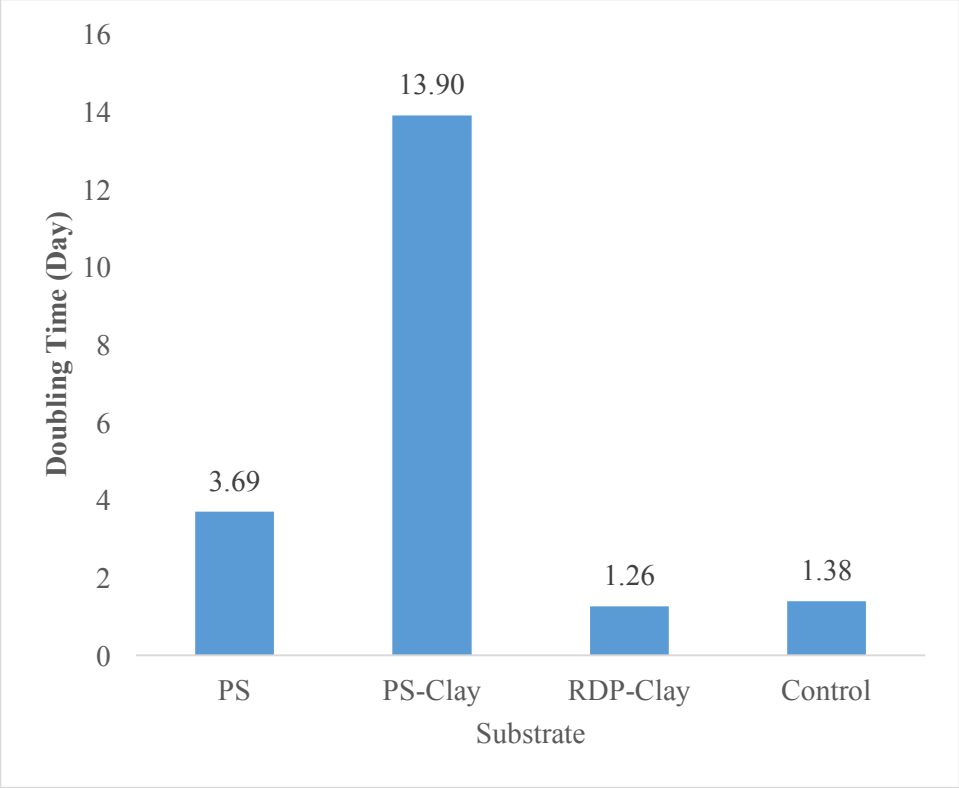

Figure 24. Doubling time for proliferation rates of GFFB cell for 2nd trial

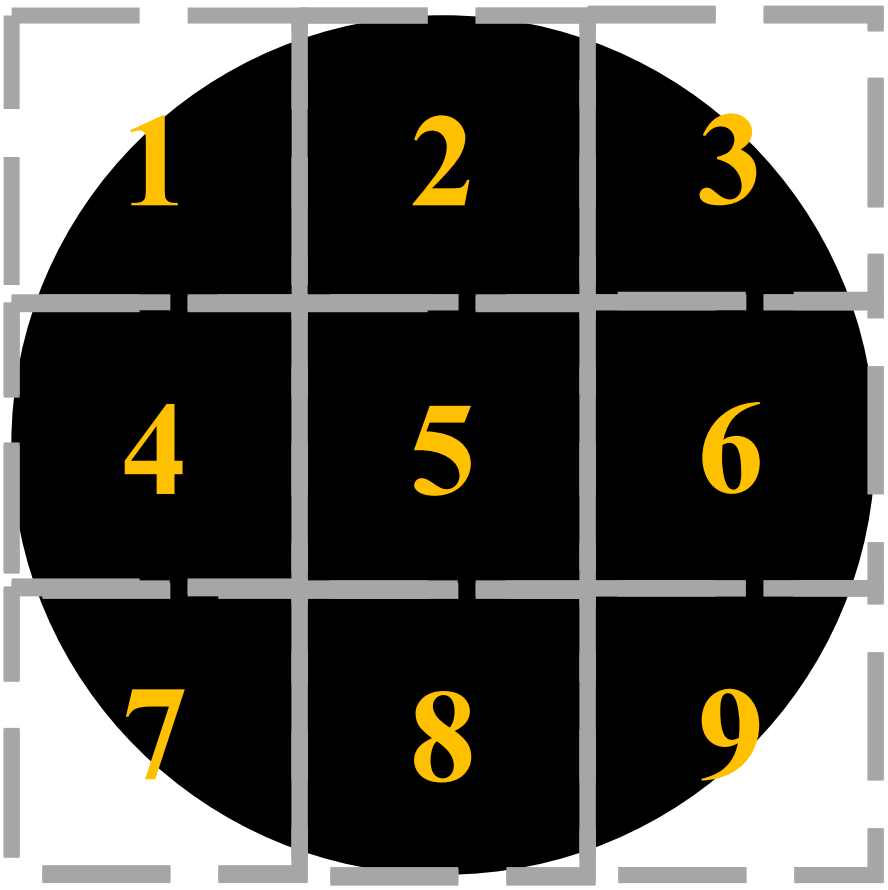

Figure 25. 9 division of fluorescent images taken for each polymer samples

Figure 26. Image at division 6 taken for DAPI dyed GFFB cells on PS (Day 6) under a fluorescent microscopy.

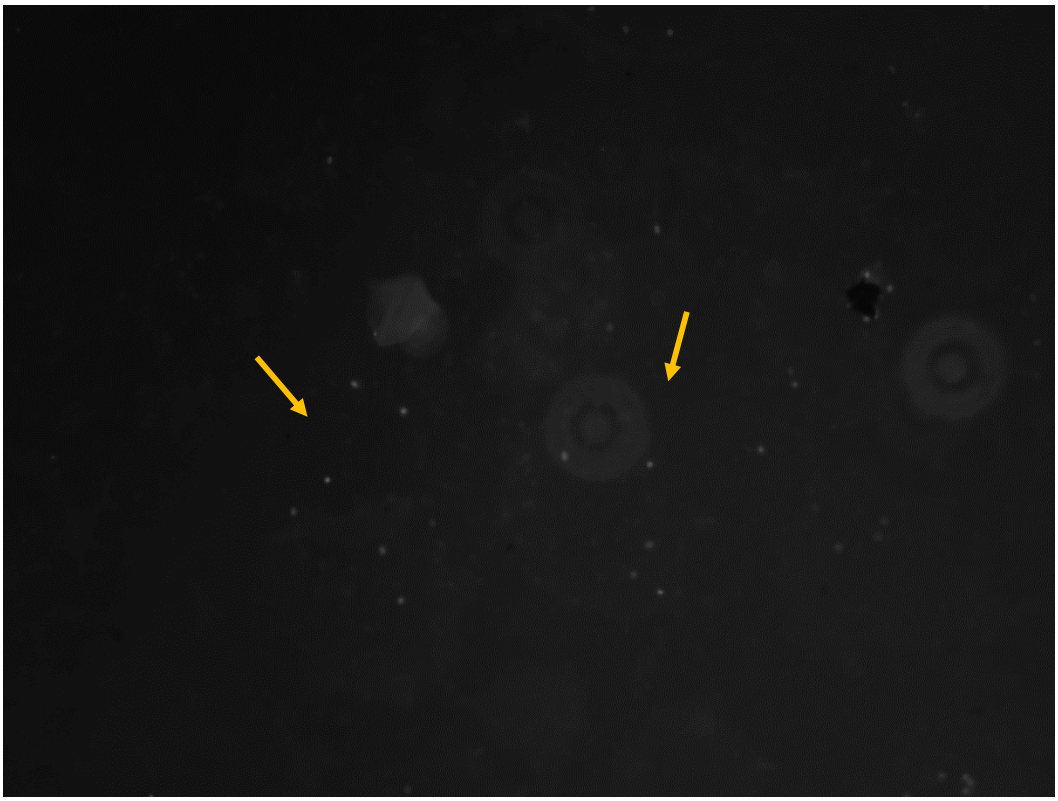

Figure 27. Image at division 6 taken for DAPI dyed GFFB cells on PS-clay (Day 6) under a fluorescent microscopy.

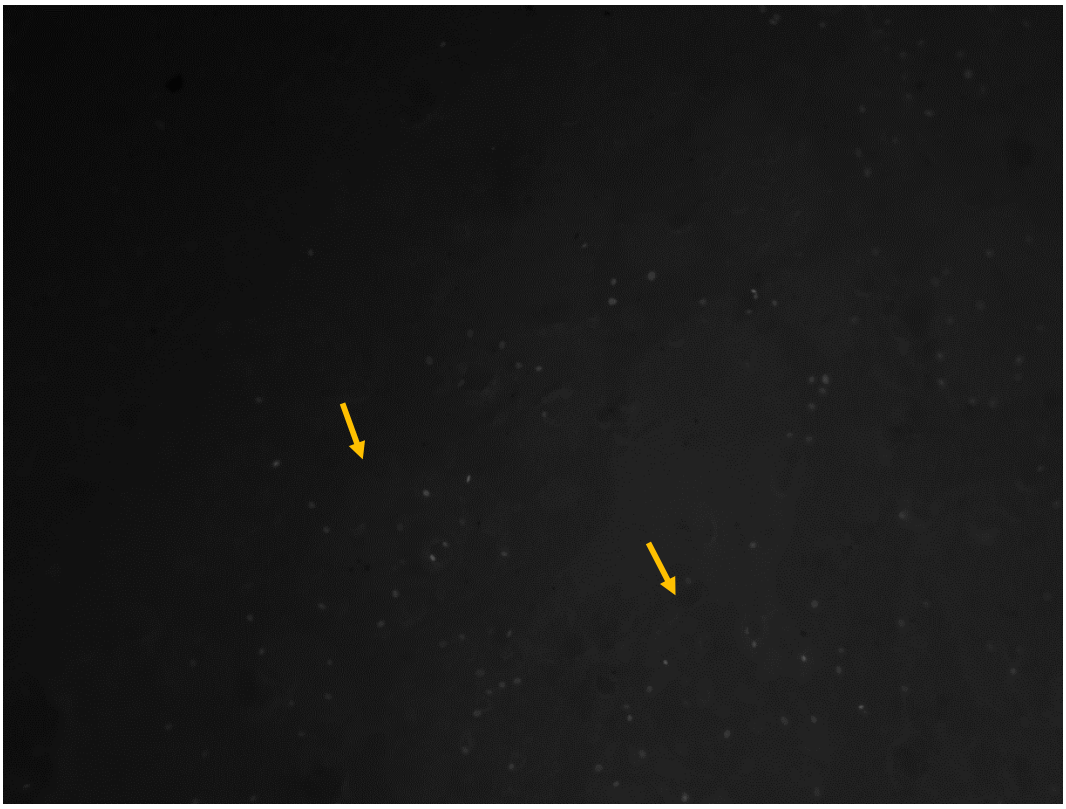

Figure 28. Image at division 6 taken for DAPI dyed GFFB cells on RDP-Clay (Day 6) under a fluorescent microscopy.

Figure 29. Image at division 6 taken for DAPI dyed GFFB cells on control (Day 6) under a fluorescent microscopy.

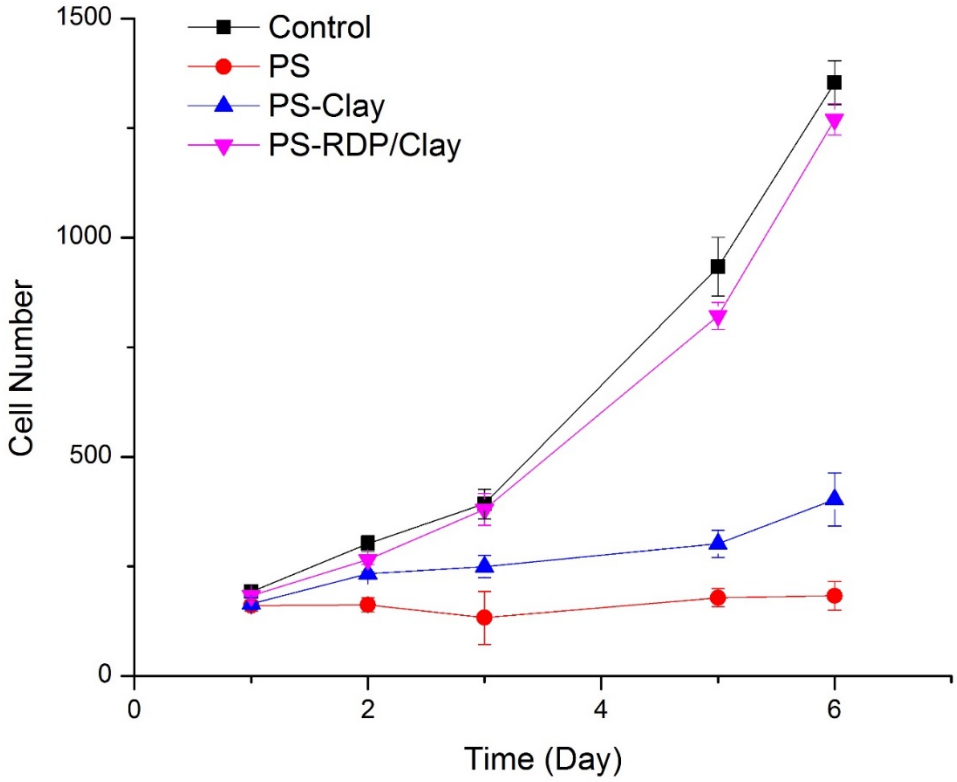

Figure 30. GFFB Cell growth counting using a fluorescent microscopy and ImageJ.

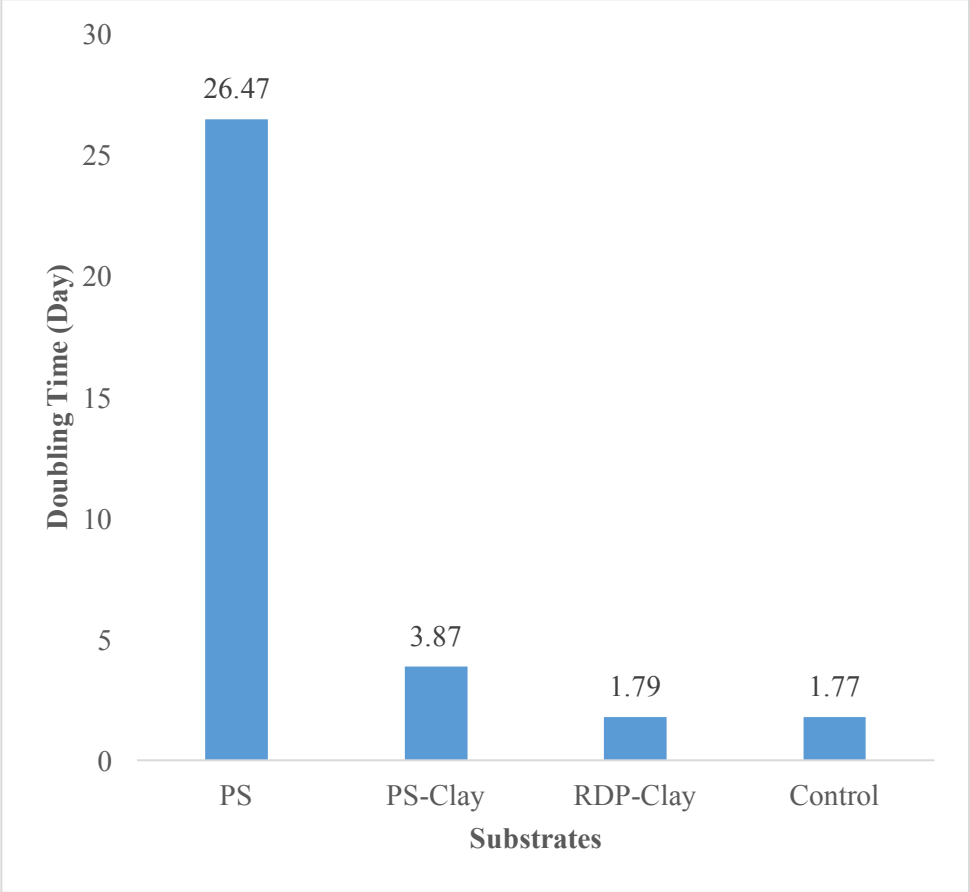

Figure 31. Doubling time of GFFB cells on polymer substrates calculated based on the data analyzed using fluorescent microscopy pictures and ImageJ.

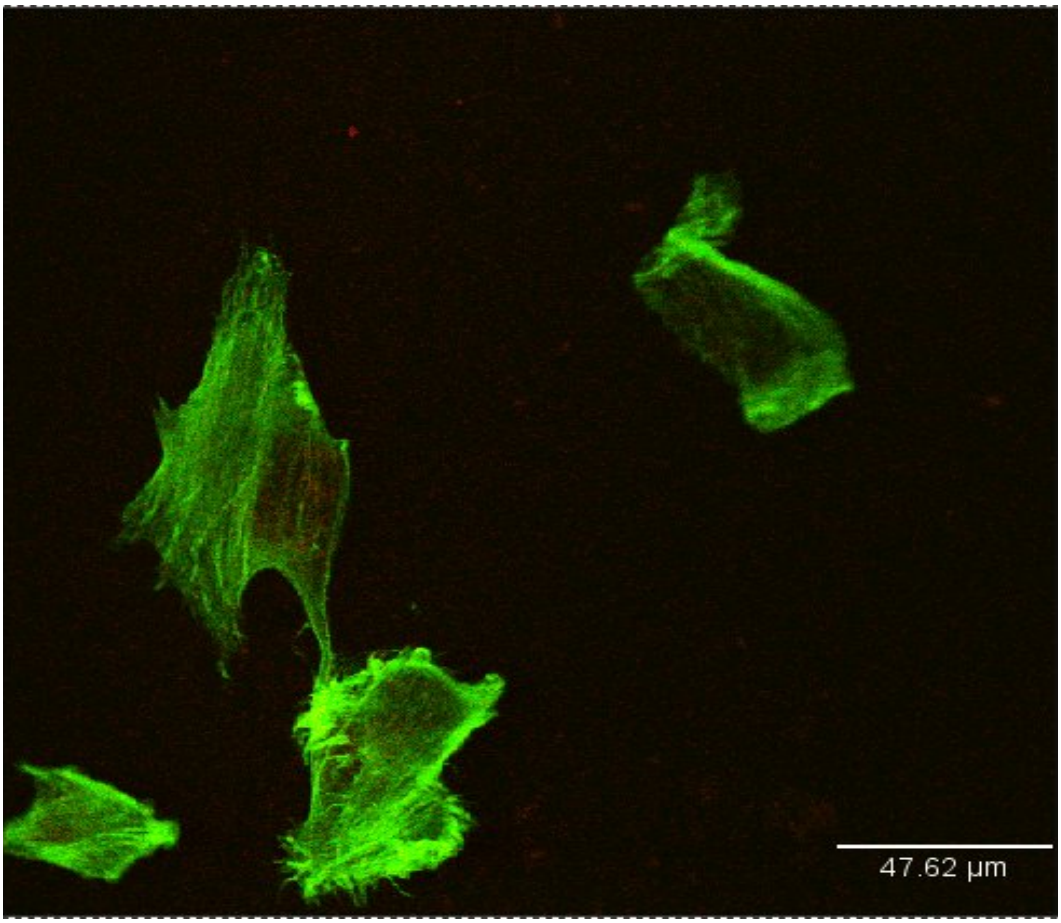

Figure 32. Image of GFFB cells on PS scaffold under a confocal microscopy.

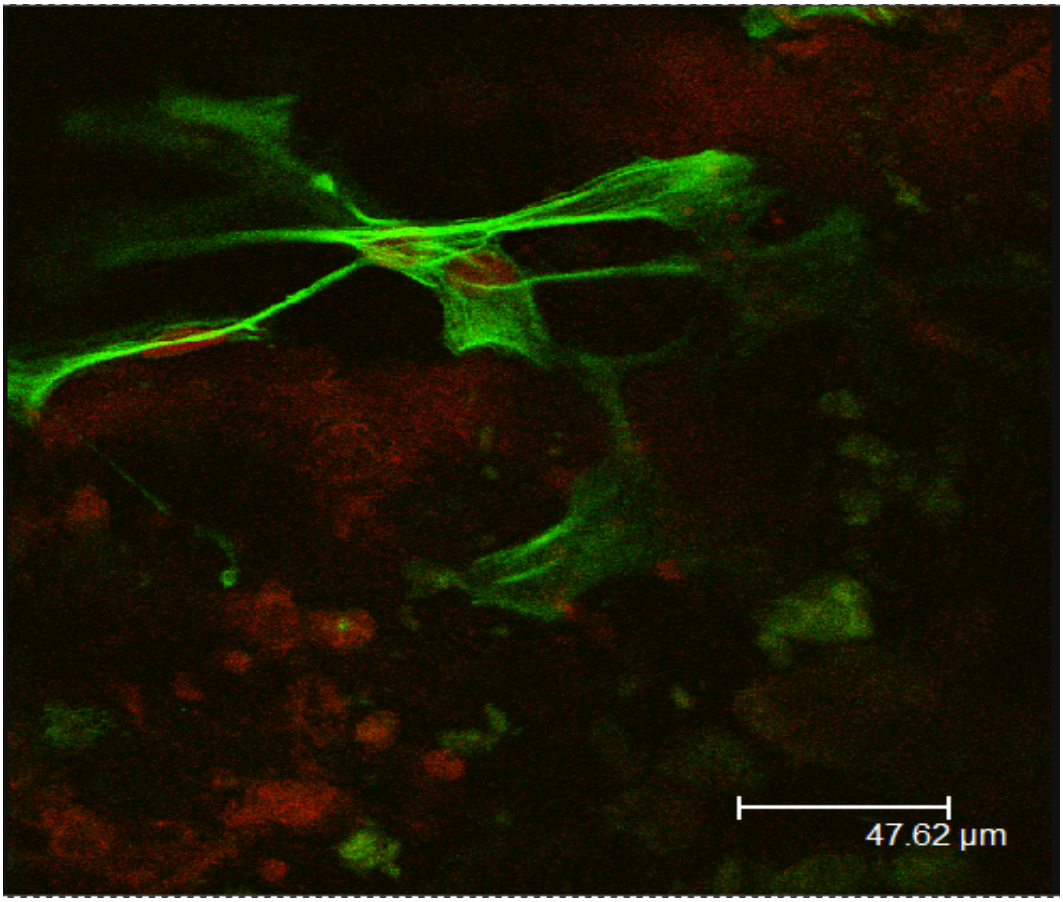

Figure 33. Image of GFFB cells on PS-Clay scaffold under a confocal microscopy.

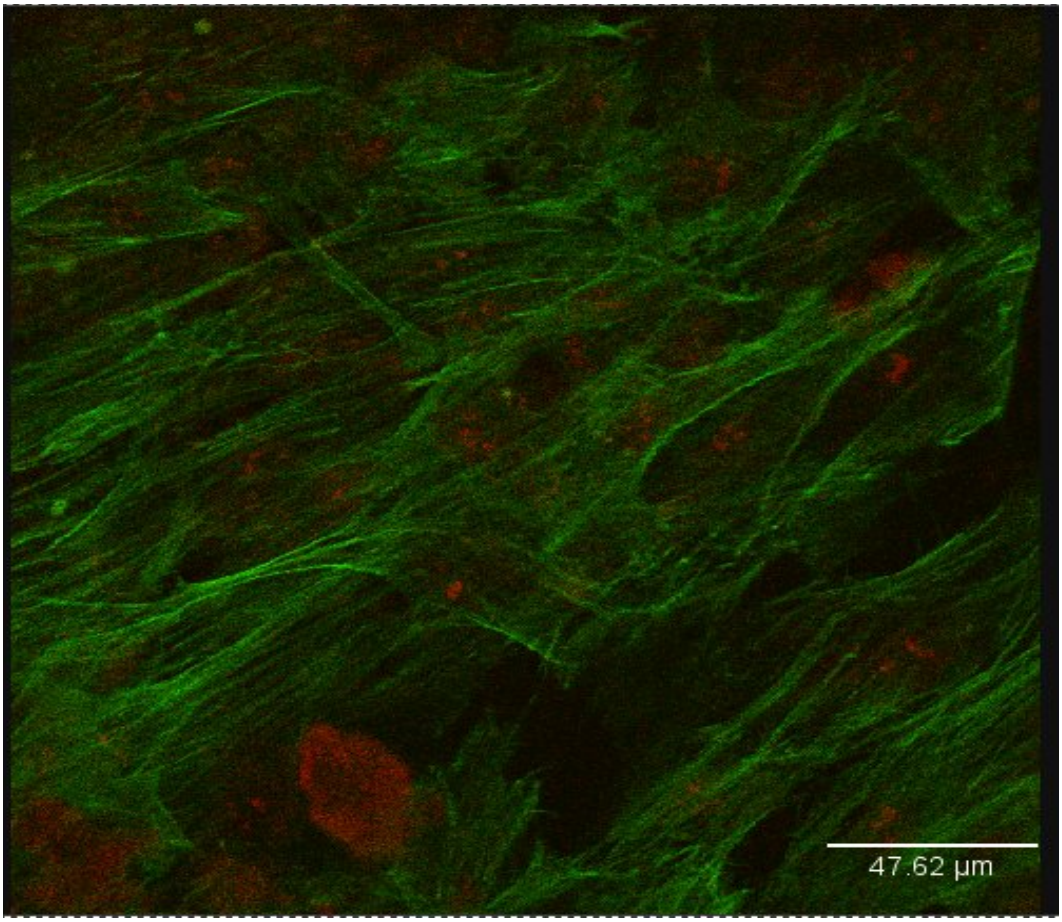

Figure 34. Image of GFFB cells on RDP-Clay scaffold under a confocal microscopy.

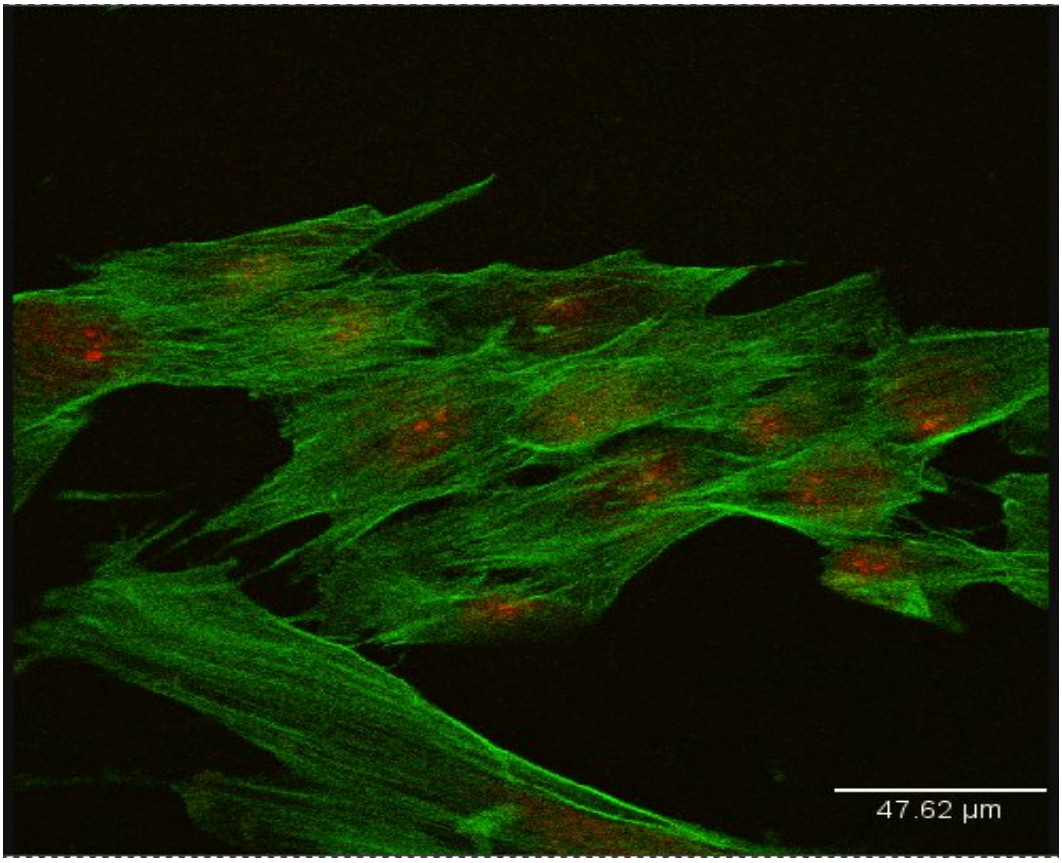

Figure 35. Image of GFFB cells on RDP-Clay scaffold under a confocal microscopy.

Bibliography

[1] "U.S. home structure fires". National Fire Protection Association. Retrieved 2007-08-22.

[2] “Classes of Flame Retardants”, North American Flame Retardant Alliance, American Chemistry Council. Web. Available on May 5. 2015.

[3] T. Reemtsma, et al. Organophosphorus flame retardants and plasticizers in water and air I. Occurrence and fate, Trends in Analytical Chemistry (2008); 27, 9

[4] D. Santillo and P. Johnston, Playing with Fire: the global threat presented by brominated flame retardatns justifies urgent substitution, Environmental International 29 (2003) 725-734.

[5] "Hungary Battles to Stem Torrent of Toxic Sludge". BBC News Website. 5 October 2010.

[6] S. Levchik, et al. A Review of Recent Progress in Phosphorus-based Flame Retardants, Journal of Fire Science (2006); 24

[7], I. Veen, et al. Phosphorus Flame Retardants: Properties, Production, Environmental occurrence, toxicity and Analysis, Chemosphere (2012)

[8] A. Ballesteros-Gomez, et al. In Vitro Human Metabolism of the Flame Retardant Resorcinol Bis(diphenylphosphate) (RDP), Environmental Science and Technology (2015); 49, 3897-3904

[9] K. Pawlowski, et al. Flame retardancy mechanisms of triphenyl phosphate, resorcinol bis(diphenyl phosphate) and bisphenol A bis(diphenylphosphate) in polycarbonate/acrylonitrile-butadiene-styrene blends, Polymer International (2007); 56, 1404-1414

[10] S. Pack, et al. Role of Surface Interactions in the Synergizing Polymer/Clay Flame Retardant Properties, Macromolecules (2010); 43, 5338-5351

[11] E. Murashko, et al. Fire Retardant Action of Resorcinol Bis(Diphenyl Phosphate) in PPO/HIPS Blend, Journal of Fire Science (1998), 16

[12] Toronto Research Chemical Inc. Web. Available on May 5, 2015.

[13] "Non-halogenated Aromatic Phosphate", Scientific Guidance Panel (SGP) and Bimonitoring California, 2012.

[14] N. Eede, et al. Age as a determinant of phosphate flame retardant exposure of Australian population and identification of novel urinary PFR metabolites, Environment International (2015), 74, 1-8

[15] C. Chigwada, et al. Synergy between conventional phosphorus fire retardants and organically-modified clays can lead to fire retardancy of styrenics, Polymer Degradation and Stability (2003), 80, 551-557.

[16] R. Freitas. Nanomedicine, Volume I: Basic Capabilities, Landes Bioscience, 1999.

[17] N. Patankar, On the Moldeling of Hydrophobic Contact Angles on Rough Surfaces, Langmuir (2003), 19, 1249-1253.

[18] Roth V. Doubling Time, http://www.doubling-time.com//compute.php, 2006. [Available on December 1, 2015].