REPUBLIC of TURKEY
HACETTEPE UNIVERSITY
INSTITUTE of HEALTH SCIENCES

STERILIZATION of GRAFTS USED in PERIODONTOLOGY by GAMMA and MICROWAVE RADIATION

Rad.Tech Saharnaz BARGH

Radiopharmacy Program

MASTER of SCIENCE THESIS

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09 Ağustos 2018

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YAYIMLAMA VE FİKRİ MÜLKİYET HAKLARI BEYANI

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Tezin kendi orijinal çalışmam olduğunu, başkalarının haklarını ihlal etmediğimi ve tezimin tek yetkili sahibi olduğumu beyan ve taahhüt ederim. Tezimde yer alan telif hakkı bulunan ve sahiplerinden yazılı izin alınarak kullanılması zorunlu metinlerin yazılı izin alınarak kullandığımı ve istenildiğinde suretlerini Üniversiteye teslim etmeyi taahhüt ederim.

Yükseköğretim Kurulu tarafından yayınlanan ***"Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"*** kapsamında tezim aşağıda belirtilen koşullar haricince YÖK Ulusal Tez Merkezi / H.Ü. Kütüphaneleri Açık Erişim Sisteminde erişime açılır.

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i "Lisansüstü Tezlerin Elektronik Ortamda Toplanması, Düzenlenmesi ve Erişime Açılmasına İlişkin Yönerge"

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor (Prof.Dr.A.Yekta Özer and Dr.Öğr.Üy. Mine Silindir Güay) and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences .

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ABSTRACT

BARGH, S, Studies on Sterilization of Grafts Used in Periodontology by Gamma and Microwave Radiation. Hacettepe University, Institute of Health Sciences, Radiopharmacy Program, Master of Science Thesis, Ankara, 2018. The aim of this work is to research the sterilization of grafts (bone and dermal grafts from human, horse and porcine sources) used in the dental periodontal operations and because of their direct contact with the mucosal tissues and blood, it is essential to assure a complete sterilization. Moreover, the quality and the efficiency of the materials after sterilization is another aspect of this study taken into consideration. In this study, the differences made by gamma radiation sterilization were investigated by doing some different analyses like (organoleptic, FTIR, TGA, SEM, SAXS and ESR) and microbiologicalal tests like (sterility, pyrogenity and determination of the Sterility Assurance Level). All tests done by gamma radiation were also performed by microwave irradiation on the grafts; then the results were compared with the gamma radiation results. Based on the results, both gamma radiation and microwave sterilization have been found effective and appropriate for dental grafts used in this study even at low irradiation doses and time as 5 kGy for gamma and 3 min for microwave, respectively. Additionally, as a new research topic, microwave radiation sterilization was evaluated as a good and practical option for grafts' sterilization.

Key Words: Gamma Radiation Sterilization, Microwave Sterilization, Periodontal Materials, Grafts, Stability

ÖZET

BARGH, S, Periodontolojide Kullanılan Graftlerin Gamma ve Microdalga Radyasyonuyla Sterilizasyonu. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Radyofarmasi Programı, Yüksek Lisans Tezi, Ankara, 2018. Bu çalışmanın amacı, dental periodontal cerrahilerde kullanılan greftlerin (kemik, dermal, insan, at ve domuz kaynaklı) sterilizasyonu ve bunların karşılaştırmalı olarak incelenmesidir. Graftlerin mukozal doku ve kan ile temas halinde olmaları nedeniyle tam bir sterilizasyon yapılması şarttır. Ayrıca, sterilizasyon sonrası malzemelerin kalitesi ve verimliliği de, dikkate alınan bu çalışmanın bir başka yönüdür. Bu çalışmada çeşitli kaynaklardan sağlanan graftlerin fizikokimyasal özellikleri (organoleptik, FTIR, TGA, SEM, SAXS ve ESR) ve mikrobiyolojik özellikleri (sterilite, pirojenite ve SAL düzeyi) gama ışınlama öncesi ve sonrası araştırılmıştır. Yapılan tüm testler, diğer bir sterilizasyon yöntemi olan mikrodalga ışınlama sonrası da tekrarlanmıştır ve iki yöntemin sonuçları birbiriyle karşılaştırılmıştır. Elde edilen sonuçlara dayanarak, hem gama radyasyonu hem de mikrodalga sterilizasyonu, bu çalışmada kullanılan diş greftleri için, gama için 5 kGy ve mikrodalga için 3 dakika, düşük ışınlama dozlarında bile, etkili ve uygun bulunmuştur.

Anahtar Kelimeler: Gama Radyasyon Sterilizasyonu, Mikrodalga Sterilizasyon, Periodontal Malzemeler, Greftler, Stabilite

CONTENT

SYMBOLS and ABBREVIATIONS

DSC	Differential Scanning Calorimetery
TGA	Thermogravimetric Analysis
SEM	Scanning Electron Microscopy
SAXS	Small Angel X Ray Scattering
ESR	Electron Spin Resonance Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
FTM	Fluid Thioglycolate Medium
G	Gause
GHz	Giga Hertz
kHz	Kilo Hertz
MW	Microwave
SCDM	Soybean Casein Digest Medium
TAEK	Turkish Atomic Energy Agency
TCP	TriCalcium Phosphate
GTR	Guided Tissue Regeneration
GBR	Guided Bone Regeneration
RTM	Regenerative Tissue Matrix
FDA	Food and Drug Administration
SAL	Sterility Assurance Level
USP	United States Pharmacopoeia
EP	European Pharmacopoeia
GVHD	Graft Versus Host Disease
UV	UltraViolet
ADM	Acellular Dermal Matrix
RW	Reagent Water
ATR	Attenuated Total Reflectance
RG	Radius of Gyration
STV	Single Test Vial
RS	Endotoxin Reference Standard
SANS	Small-angle neutron scattering
PPC	Positive Product Control

FIGURES

TABLES

1. INTRODUCTION

In last decade, sterilization with gamma radiation has been accepted as a safe, efficient, easy, economic and reliable method of sterilization. In this way, ANSI/AAMI/ISO 111371994 "Sterilization of health care products–Requirements for Validation and Routine Control–Radiation Sterilization" guideline, prepared by Association for the Advancement of Medical Instrumentation (AAMI) has an important role. In 1954 Johnson and Johnson company initiated the way of sterilization of medical devices for sterilizing of their sutures by gamma radiation. The rate of using gamma radiation for sterilization raises from 5% to 50% lately. 47 of 160 countries use ^{60}Co as sterilization radiation source among worldwide. Injectors, surgical gloves, masks, plasters, surgical cloths, packaged foods, pharmaceuticals raw materials and cosmetics can be sterilized by gamma radiation (1).

Nowadays, Periodontological regenerative treatment known as Guided Tissue Regeneration (GTR) treatment is used for many operations for regenerationç The aim of GTR is to make migration of only the cells that have the potential of regeneration. In recent years, grafting as a tissue regeneration process has an important progress under the title of xenografting and allografting. The transplantation of an organ or tissue from one individual to another of the same species with a different genotype is called allografting. Allografts come from donors who died in accidents or from a sudden illness. Generally, just one donor's gift can help and change many people's life. The other kind is xenograft wich is a graft of tissue taken from a donor of one species and grafted into a recipient of another species. For both of the grafting method, because of the direct contact of the materials with the recipient body tissue and the process of regenerating, the sterility of the implanted materials before operation has a huge importance in the medical science. Obviously, this issue is more important for allografting because allografts are procured from humans and there is always a risk of the transmission of the diseases from the same genotype (2). Therefore, the safety of allograft materials became a very important and sensitive subject in tissue banking and grafting.

So by studying and considering the transmition cases, we can have an idea about the transmission process and provide some strategies for inhibiting this situation. First of all, the tissue banks have to follow the standards of the American Association of Tissue Banks (3). These standards were developed in 1984 and it has the basis of

an examination and certification program offered by the American Association of Tissue Banks. Following the standards, safety of allografts can be assured. The regulations for tissue banks recently published by the Food and Drug Administration (FDA) which is another way of controlling the safety of the grafts. These regulations, which all tissue banks in the United States and any foreign tissue banks exporting tissues into the United States must follow, are similar to the standards of the American Association of Tissue Banks. Another way to reduce the risk of transmission of viral disease is to use processed allografts whenever possible. A processed graft is the one that has been thoroughly cleaned from blood, bone marrow, and soft tissues, including periosteum and freeze-dried bone chips by washing with high-pressure sterile water, alcohol rinses, and traditional treatments in order to destroy proteins (2). The best and final way of blocking the transmission is sterilization of graft.So, before placing them in the body, the Sterility Assurance Level (SAL) for them should be 10^{-6} for all kinds of bacteria and viruses. This can be achieved by different sterilization methods. Irradiation with gamma ray, microwave, ethylene oxide, autoclave and stove (4,5) are some of the sterilization methods. However; in recent years sterilization by gamma radiation is accepted as the most reliable sterilization method. The best sterilization method can be chosen depending on the material type, as well as the properties not causing adverse or undesired effects (6). For example a patellar ligament graft cannot be processed with high-pressure washes with water, because such cleaning cause shreding of the tendon or, soft tissues cannot be treated with alcohol without fixing the tissue (7) . So, generally irradiation can be the best method of sterilization for materials like these grafts because of its low effects on the materials and its reliability, although there is some hesitations that the irradiation can have some effects on the materials (8).

The aim of this study is to investigate the effects of gamma ray and microwave sterilization on the materials and compare the structural characteristics of grafts pre- and post sterilization by both methods and evaluating the feasibility of these sterilization methods for the availibility of grafts (coming from different sources) used in a periodontology clinic.

2. GENERAL INFORMATION

2.1. Radioactivity and Gamma Radiation

Radioactivity is a natural phenomenon in atoms with unstable energy state. These atoms try to reach to a stable energy state by emitting invisible but energetic radiations, spontaneously. Radioactivity can happen in some materials like potassium-40 availabile naturally, also radioactivity as an artificial process was discovered in 1896 by the French physicist, Henri Becquerel.

Atoms with high nucleus weight are unstable and broke down into smaller atoms and during this process nucleus particles and wave type radiation are produced as a result. This process is called radioactive decay and these elements are called radioactive atoms. Radioactive atoms try to be stable by giving their extra energy in this way. Radioactive elements that are produced during radioactive decays are usually three types as: alfa, beta and gamma rays. In alpha decay, a group of two protons and two neutrons are ejected from the nucleus. The beta particle can pass through matter easier than alfa, because beta particles (electrons) are lighter and only singly charged, they produce less dense ionization than alpha particles and are much more easily deviated from a straight line as they ionize atoms in the material through which they pass. Gamma rays and X-rays are not particles like alpha and beta, but are examples of electromagnetic radiation (like high energy light) and consequently interact with matter in a rather different way. Radioactivity can be categorized into two types as a particle and wave radiation. The particle radiation type is known as masses with energy that move with a special speed and on the other hand, no mass with a certain energy radiation is the wave type radiation. These type of radiation transfer the energy by vibration like electric and magnetic energy waves (9).

2.2. Sterilization

The beginnings of preservation and sterilization techniques go back to ancient years. It took a long time to find out about the presence of different kinds of microorganisms and their relationship with diseases. In 1665, Robert Hooke evaluated the microorganisms in the tissue cells for the first time. In 1666, Isaac Newton

discovered light microscope. Afterwards, in 1673 Antony van Leeuwenhoek discovered microorganisms named “animalcules”. In the beginning of 19th century, Louis Pasteur´s studies about the higher efficiency of the humid heat than dry heat on microorganism death and sterilization, were performed. In 1864, Joseph Lister approved that in the surgical process the microorganisms may cause infections so they started the antiseptic preservation. In the same year, Robert Koch has approved that humid heat sterilization was more effective by showing anthrax fungi rupture in the 100 ^{0}C water. In 1984, Charles Chamberland has built the foundation of the Chamberland-Pasteur filter systems and later on the autoclave system (9,10,11).

Later on by using sterilized surgical cloths, gloves, masks and materials in the aseptic condition in surgeries, the rate of death for a patient was decreased. During this time different scientists were working on different kind of sterilization methods; so, gradually the methods for sterilization have been progressed (9).

Sterilization is a physical and/or chemical process which make a material or an object free of all kind of living microorganisms that are with them. In practice, sterility assurance level (SAL) is essential for sterility. A SAL of 10 $^{-6}$ represents a one in a million chance of an organism surviving after the sterilization process and at this level, it is considered ‘sterile’, or it can be said that in a million preparations that have been sterilized, one of them could have the microorganisms (the possibility is 1 in 10^{6}). So, if the sterility can provide the sterility assurance level, it is safe to use (10).

Medical devices and grafts as a part of medical devices, are required to be sterile because of their placement in the body and their direct contact to the body tissue and blood. Naturally, different types of microorganisms, generally bacteria and in lower frequency fungi are found in the medical materials. Moreover, different kinds of viruses like hepatitis C and HIV as a more dangerous situation for grafts with human resources or animal resources, can be transferred from the donor to the recipient (1,12,13) .

2.3. Sterilization Methods

Sterilization can be done by one of the methods listed below (Table 2.1.). Modifications or combinations of these processes may also be used, provided that the effectiveness and integrity of the selected process, including the container or packaging. In all sterilization methods, the critical conditions of the process are followed to confirm that the entire set of sterilization processes has been carried out in accordance with the pre-determined requirements (14). Table 2.1. gives a general view to all types of sterilization methods advantageous and disadvantageous.

Table 2.1. Advantages and Disadvantages of Sterilization Methods (15)

Sterilization Method	Advantages	Disadvantages
Dry heat sterilization	It has no toxic effects and safe for the environment. Proper for Powders, glycerine soft parafin sterilization.	It needs a high heat and long time for penetration specially for larg devices . Not proper for plastics and heat sensetives.
Pressured vapor sterilization	It has no toxic effects . A short processecing time is need. And it is economic.	Not proper for heat and moisture sensetive materials, also oily materials like soft parafin, liquid materials and electrical devices can not be sterilized by this method.
EtO sterilization	A good option for heat sensetive materilas. Good penetration by use of the permeable gas. It is important to define the SAL with the use of biological indicators.	It is toxic, cancerogenic, flammable, explosive. It needs an Quarantina period after sterilization because of the formation of ethylene chlorohydrin.
Formaldehyde sterilization	Proper for heat sensetive materials and no need for vetelation after sterilization.	It is toxic and carcinogenic so it can not be used for the sterilization of liquids.

Table 2.1. Advantages and Disadvantages of Sterilization Methods (15) (continued)

Gas plasma (H_2 O_2) sterilization	Hydrogen peroxide has less hazardous to work with for workers. Sterilization process need a short time between 28 min to 74 min. There is no need for the ventilation concentration within the isolator during sterilization. Appropriate for heat sensetive materials.	It is not proper for liquid materials. Measuring the hydrogen peroxide concentration within the isolator during sterilization cycles in real time may also be a problem.
Peracetic acid sterilization	It is safe to work with and safe for enviroment. It is more proper for delicate materilas than steam sterilization and it can be a good sterilization option for a wide variety of materials-plastics, rubber, and heat-sensitive items. It is a single-use process,there is no possibility of contamination.	Only one or a small number of instruments can be processed in a cycle. Using of the materials after sterilization process is not possible.
Gamma radiation sterilization	It is a reliable sterilization method. It is a cold method, there is not a significant change in temperature, so it is proper for all heat sensitive materials. It has a high SAL. Control of the method is very easy that can be made only by the parameter of applied dose.It leaves no radioactive residue.	Dose rate is lower than electron beams. It has no dose flexibility.
E-beam sterilization	Very safe method. It is an advanced technology method. It is a cold method, increase in temperature is so slight. It has a high SAL. Control of the method is very easy that can be made only by the parameter of applied dose.	It needs an electron accelerator that is very rare.

Terminal sterilization methods based on United States Pharmacopoeia (USP) 30 (14) and European Pharmacopoeia (EP) (16);

a.<u>Pressured vapor sterilization</u>: (otoclave heating, minimum 121 ^{0}C for 15 min): is a simple yet very effective sterilization method. Sterilization is achieved by exposing products to saturated steam at high temperatures (121°C to 134°C).

Product(s) are placed in a device called the autoclave and heated through pressurized steam to kill all microorganisms including spores. The device's exposure time to steam would be anywhere between 3 to 15 min, depending on the generated heat. This method is using for aqueous solution more often than others. All measures and procedures should provide SAL 10^{-6} or better. High-temperature heat and moist can cause any structural and metabolic effects of microorganisms replication and destroy them (6).

b. Dry heat sterilization: (minimum 160 ^{0}C for 2 hours): Dry heat sterilization uses high temperatures to kill microorganisms and bacterial spores. Dry heat sterilization requires higher temperatures and longer exposure times than moist heat sterilization.

c.Ionizing radiation sterilization: The materials irradiated with a radiation by radioactive sources like ^{60}Co or by an electron accelerator. For this aim, the suggested and standard references dose is 25 kGy.

d.Gas sterilization (Ethylene oxide): Gas sterilization sterilization by means of a bactericidal gas, frequently used for items that are heat and moisture sensitive. Ethylene oxide is the most used gas; it is highly explosive and flammable in the presence of air, but these hazards are reduced by diluting it with carbon dioxide or fluorinated hydrocarbons. Gas sterilization is a chemical process resulting from reaction of chemical groups in the bacterial cell with the gas. Factors influencing gas sterilization include time of exposure, gas concentration, penetration of the gas, and temperature and humidity in the sterilizing chamber. Automatically controlled ethylene oxide sterilizers are usually heated to a temperature of 54°C (130°F). A humidity level of 35 to 70 % is recommended. This method is usually used as an alternative method and it is not often used because of its toxicity effect after the sterilization process.

e.Filtration sterilization (0.22 µm or smaller pores) under aseptic conditions: This sterilization method is used to obtain sterile products, such as those containing the microbial impurity in the amounts specified in the European Pharmacopoeia (16) category 1 and the European Pharmacopoeia (2002) categories 2-4 (14,16,17).

Other methods that are used recently;

- Gas Plasma (Hydrogen peroxide): The oxidation effect of the H_2O_2 helps the sterilization process.
- Signaling light system: High energy electric is used to produce strong light and use for sterilization. This method is limited to superficial sterilization.
- Ozone gas sterilization: Although fast cycling time of this method is an advantage its low penetration ability and the reactive characteristic is a limitation and disadvantageous for it (18).

2.3.1. Sterilization with Gamma Radiation

Gamma radiation had been founded by Pierre Curie and Marie Sklodowska-Curie in the 19th century, but the bactericidal characteristics of the gamma radiation and its effects was founded 30 years later by H. Lacassagne and Marie Sklodowska-Curie. In 1953 for the first time, gamma ray had been used for sterilization goal. Sterilization for the food industry as a protective method and antimicrobial effects in spices were the first use of gamma-ray for sterilization purposes. Years later, it was used for the sterilization and decontamination of medical devices, disposable materials, implants and cosmetics (17,18,19).

Gamma radiation is generally known for its high penetration and low dose rate. It is a cold method, it makes a very minute heating change, so it can apply to the materials in their packages. Gamma radiation sterilization can be applied to a variety of materials including foods, raw materials, nutritions, grains, cosmetics, pharmaceutical ingredients, pharmaceutical preparations, formulations, medical devices, etc. Therefore, in many countries, it is used as a sterilization method for the

packaging materials (16,17). For this purpose, ^{60} Co as a reference source or accelerated electrons have been used for the sterilization of medical devices and others.

This method is the best option for the sterilization of the materials that are sensitive to heat. The general principle of sterilization process with gamma ray is that the material is turning around the gamma-radiation reference such as ^{60}Co to irradiate all the points of the materials. 25 kGy of irradiated dose is generally used for the sterilization of pharmaceutical ingredients, pharmaceutical preparations, formulations and as well as medical devices (6).

Gamma radiation sterilization is accepted as a reliable and economical method for the sterilization of the materials that are related to human being health and day by day the importance of this radiation in the sterilization of healthcare products increases. There are various studies and projects about this method among the world.

In our radiopharmacy department, there are different theses and projects about this subject too: Gamma radiation sterilization of Sulpha group ophthalmics and parenteral preparations (20), medical devices (surgical suturs, surgical covers, aprons, caps, and gloves) (21), cephalosporin parenteral preparations (22), cosmetic products and raw materials that are used in cosmetics (23) and also sterilization of medical devices used in periodontology (membrane, graft, syringe) with gamma radiation (24) were previously performed. The use of this method in the market has increased from 5% to 50% during last decades. The reliability, low cost and easy application are the reasons for its increased popularity. A chosen committee in Association for the Advancement of Medical Instrumentation (AAMI) has an important role in controlling the process of gamma radiation sterilization. ANSI/AAMI/ISO 11137-1994 an American National Committee Sterilization of health care products (25) "Requirements for development, validation, and routine control of a sterilization process for medical devices" was accepted as a source for both industry and legal regulations. Dose determination methodology tests are required after the sterilization process and the permission for using of the products after sterilization without quarantine (26).

Gamma Irradiation Center and Gamma Cell

Gamma irradiation center is made from three parts as gamma source unit, a conveyor for carrying the products and storage unit. The crucial part of this facility is ^{60}Co as the gamma-ray source. The ^{60}Co source which is used in this center is produced from bombardment of the ^{39}Co with a neutron in the reactor. ^{39}Co is a stable isotope which is available in nature. In this way, the unstable ^{60}Co nucleus releases two gamma rays with 1.17 ve 1.33 MeV energy and one beta particle during the decaying process and transfer to a stable ^{60}Ni element. The beta particles are captivated in the source unit, so the radiation comes out from the source unit is pure gamma ray that is exposed to the materials which are turning around the gamma source on the conveyers (20). This facility is for the industrial sterilization in a large amount and big products in their packages. Beside this there is a gamma cell which is just specialized for only research aim having a capacity to sterilize limited amount of materials. So, it is possible to give a certain dose in a certain time to the material in gamma cell.

^{60}Co source in the center of the facility is usually in a form of a stick that is covered by a two-layer stainless steel capsule to protect the source from external factors and prevent it from leaking to out of the facility Figure 2.1. The half life of ^{60}Co is 5.27 years, after 15-20 years of radioactivity ^{60}Co source is returned to the company where it has taken from.

Radiation unit is usually covered by 1.5-2.2 m thickness concrete and when the machine is not active it is preserved in a pool full of water or in a shielded storage. The preservation method is divided in two methods comprising dry and wet method (20,27). Water as a shielding material is very advantageous because of that it is an economic and available material moreover it is capable of transferring the heat that is produced and it is transparent. In this facility, there are units for the storage of the irradiated and unirradiated products. Moreover, there are units for dosimetry studies, microbiological tests and material tests (27). In Figure 2.1 There s is a schematic representation of gamma radiation center.

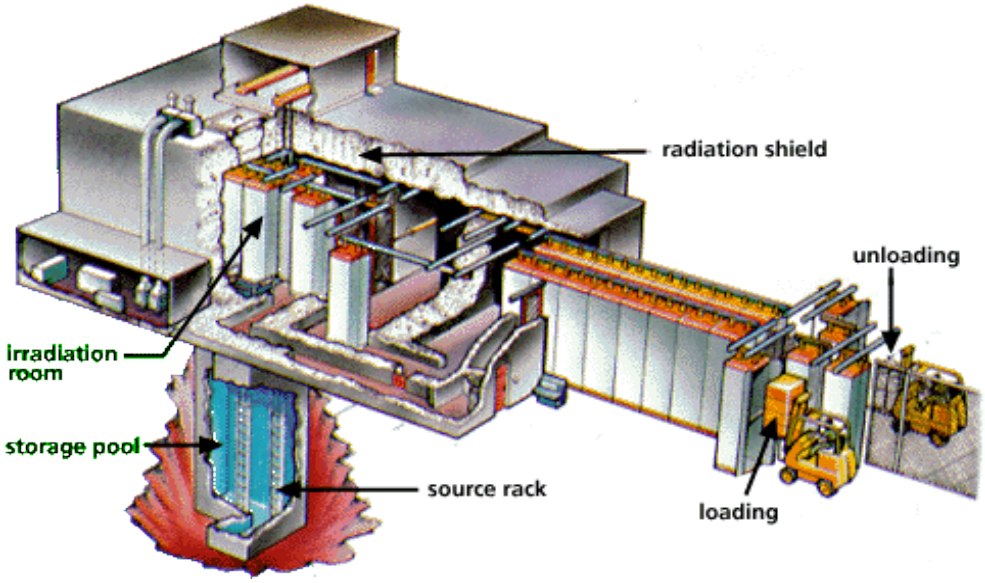

Figure 2.1. A schematic representation of gamma radiation center(22).

There are some other type of irradiators like, Self-contained irradiators are specially designed for research and for applications that need small doses like sterilizing tissue grafts, blood irradiation for preventing transfusion induced graft versus host disease (GVHD), and reproductive sterilization of insects for pest management programmes and relatively small throughputs. A large majority of these are dry storage irradiators and the source activity is limited to several kCi's (e.g. about 25 kCi for ^{60}Co). Figure 2.2 is a photograph of self-contained dry storage gamma irradiator used for research and small dimention materials. This irradiator surrounds the source with a lead shield and it has a mechanism to move the sample from the loading position to the irradiation position. The advantage of self-contained irradiator is that, it can be placed and used in an existing laboratory or a room with no need for extra shielding. Moreover, they are easy to install and operate, and that they provide high dose rate and good dose uniformity, which is essential for radiation research. The disadvantage of this irradiators can be their limitation for size of samples and the maximum capacity of it is generally around 1-5 L. These self-contained irradiators are classified by the IAEA as Category I (dry storage) and Category II (wet storage) (27,28,29) .

Figure 2.2. Self-contained dry storage gamma irradiator used for research and small dimention materials. (In preparation for irradiation, a sample holder is being placed in the irradiation chamber when it is in the loading position. Depending on the dose rate of the day, the timer on the control panel (bottom right) is set to give the desired dose) (27).

Advantages of Sterilization with Gamma Radiation

The advantages of gamma radiation sterilization are given below (1,17,18):

- It is a cold method. It provides sterilization for all temperatures, including below 0°C, so that heat-sensitive materials can be safely sterilized.

- It has deep penetration power which allows sterilization of all kinds of medical equipments and pharmaceutical products with dense packing material.

- Easy to operate and simple to control, only one process variable (exposure dose i.e duration) needs to be controlled.

- No toxic residue and quarantine time are required.

- Dosimetric release may occur immediately after the procedure.

- Materilas can be sterilized in batches because it is a continuous process.

- It eliminates the microorganisms on the compound without causing heat increase, so that the properties of the compound are preserved.

- Radiation sterilization is safe and reliable. It does not leave residues on the product.

- The continuous nature of the process ensures that the sterilization of products are mechanically and completely automatic, so that the human factor is eliminated during the process.

- No harmful effects are exposed to the environment.

- This procedure can be used for all packaging forms.

Some of the advantageous that are mentioned above can be given in detailed as follows (1,17,18):

a. It is applicable for high concentration materials in their package forms

Gamma-ray can penetrate to any concentration of a material and any place of the package without any residual. Even injectors can be sterilized safely that were filled before.

b. Package effectiveness and integrity

There is no change in the package material after sterilization. Because there is no need for vacuum or pressure or stress on the package throughout the sterilization process. Also, there is no need for the penetrable package in this method. Packaging and producer company of materials have some new projects to produce radioresistant materials and packages after realizing the importance and effectiveness of radiation sterilization.

c. High reduction in bioburden level

Gamma radiation can kill microorganisms by affecting them in two methods including direct and indirect method and inhibits the reproduction of the living microorganisms. In direct method, DNA of microorganisms is targeted. As an

alternative, the water in the cell can be targeted and radiolysis of the water and production of free radical can be formed afetrwards. Due to this ability of gamma radiation, it is known as not only a safe method but also easy and no need for control after the process. Also, the only factor to control the sterilization process is time.

d. Dosimetric release immediately after sterilization

Materials that are sterilized by gamma radiation are allowed to use by customers immediately after sterilization process this is called dosimetric release. This measurement is based on the gamma radiation dose that is given to the material during the sterilization and, kGy is used as measurement unit and it is measured by the dosimeters that are placed on the material during the sterilization process. After sterilization process, the dosimeters are taken out of the cells and the minimum and maximum amount of given radiation is measured by special devices like spectrophotometer and colorimeter. After measurement of the given dose, the materials are released for use. In the scope of dosimetric release, gamma radiation sterilization safety is documented by ANSI/AAMI/ISO 11137-1994 in detail (21) so it is accepted by FDA (1,17,18).

e. No residual and no radioactivity

The gamma rays emitted from the ^{60}Co source are pure energies similar to microwaves and X-rays from many directions. Gamma rays, during radiation sterilization, interact with electrons and atomic structures to break chemical bonds. Although, gamma rays are very effective in breaking down microorganisms, they do not leave residues (1, 14, 15).

f. Cost -effective

Gamma radiation provides a fast, flexible and highly cost-effective sterilization. The reduction in cost is largely due to the elimination of sterility tests used by biological indicators (because FDA has accepted dosimetric release) and also because there is no need for quarantine and pre-sterilization procedures (1, 14, 15).

The Influence of Gamma Radiation on Microorganisms

A sterile product is free from all kinds of viable microorganisms or the existing possibility of viable microorganisms is one in a million. This process is happened by killing microorganisms. Many hypotheses have been proposed for the mechanism of cell damage by radiation. Some scientists thought that toxic substances produced in the irradiated cells are responsible for killing the cells. On the other hand, others proposed that radiation was directly damages the cellular membranes. Radiation effects on enzymes or on energy metabolism were postulated. It is now universally accepted that the deoxyribonucleic acid (DNA) in the chromosomes represents the most critical 'target' of ionizing radiation. The effect on the cytoplasmic membrane appears to play an additional role in some circumstances. The direct effect on DNA can be given by gamma radiation energy accumulation on the target and inducing of breaking down the chain or affecting it by interaction with molecules and atoms in the cell. The indirect effect of radiation on DNA is that after interacting with water, radiation interacts with water which can cause free radical formation (hydrogen atoms H•, hydroxyl radical OH• and solvated electron e−). These free radicals have the capability of damaging DNA. 90 % of DNA damage is made by the OH• radical in which these radicals formed in the hydration layer around the DNA molecule so it is approved that in a living cell, the indirect effect is especially significant (1). The direct effect and indirect effect of radiation is given in Figure 2.3.

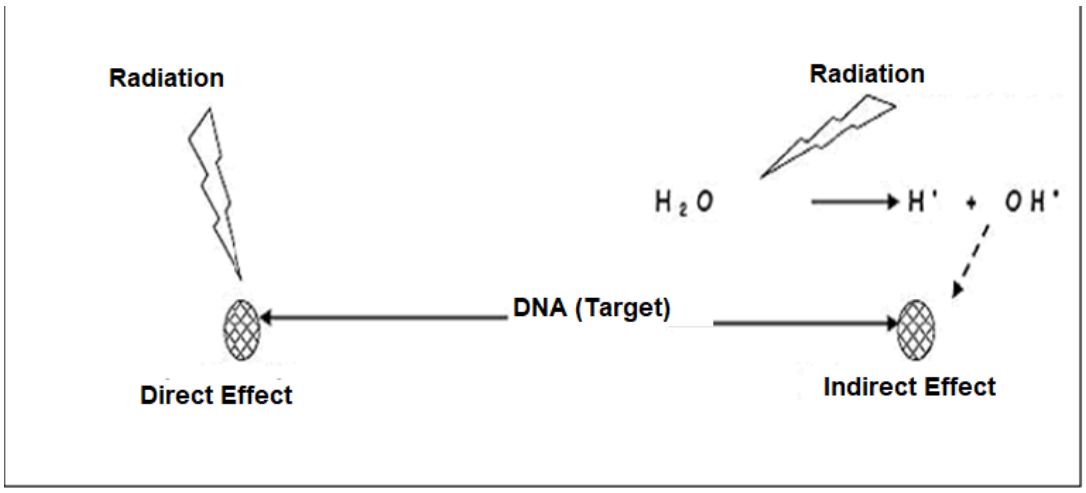

Figure 2.3. The direct effect and indirect effect of radiation (1).

Normally, bacteria, moulds and yeasts are capable of repairing many of different DNA breaks (fractures) but the microorganisms that are sensitive to radiation can not repair double-strand breaks, whereas radiation resistant species have some capability to do so (1). The absorbed radiation energy which is needed for inactivation of different microorganisms depends on the radioresistancy of the microorganisms. The radioresistance is even variated between groups of similar microorganisms based on their physical and chemical structures (1,30). To sum up, the death of microorganism is because of the irreversible damage of microorganism DNA (1).

In a study, growth patterns of *Saccharomyces cerevisiae*, *Escherichia* coli, *Bacillus pumilus* and *Bacillus stearothermophilus* spores were evaluated after gamma irradiation. Delays in the growth of Bacillus pumilus and Bacillus stearothermophilus spores due to the bactericidal action of gamma rays and due to the toxicity have been recorded. In Saccharomyces cerevisiae, the growth rate decline and delay due to bactericidal and bacteriostatic effects were determined (31).

The chemical methods used in the inactivation of viruses depends on keeping the virus solution at room temperature or higher temperature and in these conditions the physical and chemical agents can cause the breakdown of the antigenic proteins of the viruses. In contrast, ionizing radiation at low temperatures does not cause such a breakdown and allows for the study of different viral functions (32).

A study about the effect of gamma radiation on enzymes and microorganisms in fruit juice has shown that gamma radiation sterilization of lipoxygenase enzyme is easier than polyphenoloxidase and peroxidase enzymes and that these three enzymes maintain their activity even at 5 kGy irradiation dose. It was noted that the investigation of the presence of microorganisms indicated that *Escherichia coli* was sensitive to gamma radiation and could be reduced by seven log cycles of 1 kGy irradiation dose (33).

Radiation Resistance of Microorganisms and Factors that Affect the Radioresistancy

The absorbed energy that is required for inactivation of microorganisms on a material is based on its radioresistancy and it varies among different microorganisms. It could show variation among microorganisms from the same groups too because of their differences in their physical and chemical feature and the rebuilding process and mechanisms after radiation damage (1,18).

Radioresistance of microorganisms depends on:

- The nature, number and lifespan of the resulting reactive chemical changes,
- The ability of the cell to genetically repairing damage or repairing it properly,
- Internal and external environmental effects (1).

According to the different studies about the radiation effects on the microorganisms, it can be said :

- Generally, bacterial spores are known more radiation resistant than vegetative bacteria,
- Vegetative bacteria are more resistant than gram-positive bacteria,
- Vegetative cocci are more resistant than vegetative bacilli,
- Radiation sensitivity of moulds is of the same order as that of vegetative bacteria,
- Yeasts are more resistant to radiation than moulds and vegetative bacteria,
- Anaerobic and toxigenic *Clostridium* spores are more radiation resistant than the aerobic non-pathogenic *Bacillus* spores,
- Radioresistancy of viruses is much higher than that of bacteria or even bacterial spores (1,34).

Most important factors affecting radiation resistance of microorganisms includes:

- *Size and structural arrangement of DNA in the microbial cell*;
- *The compounds that are associated with DNA:* in the cell also has an important role in the radiation resistance of the microorganisms because these compounds such as basic peptides, nucleoproteins, RNA, lipids, lipoproteins and metal ions can have influence in the indirect effect of radiation.
- *Oxygen*: The studies have shown that the peresence of oxygen can increase the lethal effect of radiation on the microorganisms.
- *Water content*: Water molecules are radiolized by radiation and free radicals are produced in the results of this interaction and these free radical can cause problems for the cell and it can consider as an indirect effect on the DNA for radiation. So presence of water can decrease the radiation resistancy of the cell.
- *Temperature*: The radiation resistancy of microbial cell is higher in the subfreezing tempreture than an ambient one, because in lower temperetures there is an decrease in water activity and diffusion of radicals is very much restricted in frozen state.
- *Medium*: The composition of the medium surrounding the microorganism can have an important role in the radiation resistancy of the cell.
- *Post-irradiation conditions*: The enviroment of the microorganisms that were survived after irradiation can also has influence on the cell viability due to their sensitivity to the enviromental conditions like temperature, pH, nutrients, inhibitors, etc. (1).

Radiation Effects on Polimer

All kinds of radiation as natural or artificial, as particle or electromagnetic radiations can have different interactions with materials. The mechanism of radiation interaction with materials may vary in different materials. The interaction of the high

energy radiation with materials, is generally divided into three categories as photoelectric effect, compton scattering and pair production. As a result of all the mentioned interaction mechanisms, atom excitement or ionisation can be happened and some fundamental chemical phenomena can be happened in the material and these happening could cause some reversible or irriversible effects (1,13).

The polymers were first developed as bioprocessing surgical thread (suture) materials. Biodegradable polymers are very useful in this area because they are degredaded into biologically inert and compatible compounds within the body. Once the dosage regimen is complete, the polymer does not need to be removed by a surgical procedure from the body (35).

Today, as a result of scientific and technological developments, it can be said that the most widely used polymers in each field are the most exposed to radiation and radiation effects. In particular, in the case of disposable medical devices, it is essential that such products are made entirely of polymeric materials and that their properties and their interaction with radiation are well known (36).

The main materials used in medical devices are usually natural or artificial polymers, so the effects of gamma rays on the different kinds of polymers are evaluated after irradiation process. Different chemical and physical changes can be investigated in polymers after radiation sterilization. These polymers based on the irradiation dose and the present circumstances of the materials may exhibit degradation. Sterilization can cause the production of different amounts of free radicals, chain breaks, cross-linking and oxidation. As a result of the oxidation, hydroperoxides can appear. Moreover, ketones, alcohols and carboxylic acids can come off. In polymer molecules, the formation of free radicals make cross-linking connections in amorphous phase in which carbone-carbone distance is enough. So the free radicals which are formed in crystal region will be captivated between amorphous and crystal phase. This can be a start for some irriversible effects of radiations, like fragility (6,18,35,37,38).

Grafts that are used in periodontology are generally collagen based polymers. They can be obtained from human sources, or animal sources like bovine or horse.

Also they can be categorized as dermal or bone. Dermal ones are used for the gum repairment and regeneration and the bone ones are used for the regeneration of the bone part of the teeth. Collagen is a simple protein, composed of three α-helixes gathering together in a triple helix. Collagen might cause a partially loss functionality after exposing to ionizing radiation. Some studies reported that radiation sterilization of the collagen based materials can cause degradation followed by the degeneracy of mechanical properties, increase in sensitivity to enzymatic digestion and dissolving in neutral and acidic media (39).

Radiation for sterilization can cause some changes for biomedical polymers in some biomaterials, like color changes, odor formation, increase in rigidity, softening, change in the melting temperature. Among these changes, chain- breaking and cross-linking are more important than the others. Cross-linking occurs by the formation of double bonds in saturated polymers or by hydrogen deprivation. In some polymers, cross-linking causes chain breaks, but not in the others. While radiation can cause these changes in some materials, on the contrary, it has no effect on the others (6,18,35,37,38).

Chain-breaking in molecules can cause a decrease in the degrading resistance and mechanical characterstics, besides a decrease in molecular weights. When irradiation process was done in an inert environment, the possibility of cross-linking reaction is more than chain breaking. As a result, doing sterilization with gamma radiation in an inert environment can be more advantageous (40). The results of the chain-breaking will be the loss in molecular weight and so molecules will be without bonds and it leads to gas production. Moreover, it can be considered that one of the effects of chain-breaking could be fragility increase in polymers. The possibility of these kinds of degradations can increase in the enviromental higher oxygen concentration. As a result, more chain-breaking process, crystallinity change and brittleness can happen. After these occurance, the oxidative degradation possibility increases by the formation of carbonyl structures. On the other hand, polymer cross-linking can change the physical characteristics of molecules and can attain them a three dimentional structure (6,18,35,37,38).

Based on the radiation dose the mechanical characteristics of the molecules, like tensile strength, stretching rate, strike power can be changed. Chemical and physical stabilization can happen immediately after sterilization, after days, months or even years by chain-breaking or cross-linking reactions (18). In some cases, ionizing radiation can also be used as a unique method for modifying polymer properties, both chemically and thermally (6,18,35,37,38).

Some physical and chemical changes which can be formed by radiation are mentioned below:

- Fragility
- Color
- Odor
- Softness
- Hardness
- Toxicity
- Chemical inertness
- Dispersity
- Chain lenght
- Crystal structure, crystal-amorphous phase rate, crystallization mechanism and kinetics
- Termal features (glass transition temperature, softening temperature, melting temperature, heat of crystallization)
- Optic and mechanic features (color and transparency)
- Viscosity
- Melting point (41).

The Utilization Areas of Gamma Radiation

All the products which are used and consumed by human being, microbiological control is very essential in last decads. New generation foods, pharmaceuticals, cosmetics industry have to ensure a high antimicrobial standarts and conditions to distribute safe and high hygenic products to the public in addition pereventing them from degredation. As a result, from first step of the production

process to the last step before the distribution, the antimicrobial process of production should be under control. Gamma radiation has shown an acceptable effect on the personal care products (42).

The mortal effect of ionizing radiation on the microorganisms has been approved for the last century. So, gamma ray as a radiation with high penetration ability approved as an effective method of sterilization. Sterilization with ^{60}Co is an easy, fast and effective method. This method is acceptable and effective for almost all kinds of materials including different kinds of products of medical devices, cosmetics or pharmaceuticals when the conventional methods could not be enough. The most considerable feature of this method is that it gives the opportunity to irradiate the materials in their final package. Besides that, it will be the best option for the materials that are heat sensitive. Many kinds of different polymers (polyethylene, polyester, polystyrene, polysulphur and polycarbonate) are appropriate for radiation sterilization. On the other side, gamma radiation can not be appropriate for radiosensitive materials like Fluoropolymers (43).

One of the most important aspect of the gamma radiation sterilization is irradiation dose, because even for the standart dose of 25 kGy, some physical and chemichal changes can be observed after irradiation. So determining of lower doses that can make the material sterile can be an important step in minimizing the adverse effects of gamma radiation on the materials. For pharmaceuticals the radiation dose, radiosensitivity, microbiological burden and the chemical structure of the materials have important roles (44). Gamma radiation can also cause cross-linking of bonds in the polymers which is accepted as an advantageous for this method so it is used oftenly for the sterilization of polymers over 50 years.

General usage of the gamma radiation includes (18,36);

- Medical devices sterilization
- Agriculture and breeding researches and applications
- Domestic, industrial and especially hospital wastes
- Microbial decontamination of spices, herbs and herbal kinds
- Foods and food products pereservation

- Encolouring of gemstones
- Polymer modification and developing of new materials (hydrogel).

Products that can be sterilized by gamma radiation includes (18);

- Antibiotics
- Powders
- Containers and the caps
- Contraceptives
- Orthopedic implants
- Perfusion sets
- Pharmaceutical raw materials
- Surgical blades
- Surgical threads
- Implants

2.3.2. Sterilization with Microwave

Ionizing radiation consists electromagnetic waves (ultraviolet (UV) ray, X-ray, gamma-ray), and subatomic particles (α particle, β particle, neutron) which have enough energy to ionize the atoms and molecules of the materials that are exposed to. So, besides gamma ray, UV ray can also be used for disinfection and sterilization of biomaterials and medical devices (45). Microwave radiation, in contrast with gamma, X-ray and UV radiations, falls at the longer wavelength end of the electro-magnetic spectrum. It has wavelengths of approximately 1 mm to 1 m, a range that includes television and police radar wavelengths. Electromagnetic energy in the microwave region (225 MHz to 100 GHz, typically 2,450 MHz) (46) is studied and considered as an alternative disinfection and sterilization technology method for getting rid of bacteria. Microwave application for controlling of bacterial reproduction is particularly appealing for the biomedical industry because of its effectiveness and low cost. Many researchers approved that microwave irradiation can extent the pereservation of tissue by reducing microbial burden (47,48). Microwave treatment is known to inactivate many microorganisms, such as *Burkholderia cepacia, Clostridium perfringens, Escherichia coli, Photobacterium leiognathii, Streptococcus faecalis,*

Staphylococcus aureus, Salmonella and Listeria spp (49,50,51). Sensitivity of bacterial and mould spores to the microwave are also approved (52).

The Mechanism of Microwave Sterilization

The principle of microwave irradiation in sterilization is like other heating methods of sterilization with a significant difference in which this method is applying its effect by an internal heating source unlike the other conventional heating methods with an external heating source. Therefore, the heating rate can penetrate more rapid deep inside and effective than other methods. The electromagnetic field strength and the exposure time are two important factors that can affect the efficiency of microwave sterilization. So, it can be said that the electromagnetic energy is expressed generally in two forms: (i) the factors that depend on the dielectric properties of the dipole molecules of the irradiated materials in the form of heat (thermal effect) and (ii) the factors that do not depend on the dipole molecules in the form of a direct effect of the radiofrequency (nonthermal effect) (46).

Although, microwave sterilization is normally known as a method that applies its effect in thermal way, there are some studies that claim that the non-thermal effect of microwave also plays an important role in inactivation and the heat generated from microwave is inadequate for inactivation (53,54).

On the other hand, some believe that thermal effect of the microwave alone can have the bactericidal and sporicidal effect specially in the liquid systems (55,56). Vela and Wu believe that dry or lyophilized microorganisms are not capable of absorbing microwave energy so, in this condition it can not have a bactericidal effect on the material (57).

Chipley and co-workers on the other hand, think that the thermal and electromagnetic (nonthermal) function of microwave on the dry spores are interdependent (58). Although, using of microwave for sterilization in dry state for medical use is suggested by many investigators (59 ,60) it was approved that, the exact mechanism of the sterilization is still controvertial about how thermal or nonthermal effects in dry or wet environment has fatal effect.

As a result, it is important to study the microwave-irradiated and non-microwave-irradiated samples simultaneously and under the same temperature profiles. Recently, microwave radiation is becoming popular as an alternative for gamma radiation sterilization. The penetration ability of microwave is much lesser than gamma radiation but microwave radiation can generate both thermal and non-thermal effects on the biological system. As it was mentioned above, the thermal effect is shown by rising the temperature of the irradiated system. Besides, physiological responses are based on the intensity and duration of the electromagnetic field. The non-thermal effect shows itself by making changes in the cellular metabolism by inducing both resonance absorption and electromagnetic field which can cause destructive effects on the microorganisms (49).

Advantages of Microwave Sterilization

Microwave, as a novel radiation sterilization method, has gained the interest of scientists in last years. Microwave sterilization has lower costs and lower effects on the structure of biomaterials which is an essential issue. Microwave radiation is known to inactivate many microorganisms such as; *Burkholderia cepacia, Clostridium perfringens, Escherichia coli, Photobacterium leiognathid, Streptococcus faecalis, Staphylococcus aureus, Salmonella and Listeria spp*. Bacteria and spores were also reported to be sensitive to microwave radiation (50,51,61). Singh and Singh (62) approved that even only two min of microwave irradiation with 2450 MHz and 900 W power can inactivate most of Gram-negative and positive bacteria (Gram-positive bacteria: Bacillus subtilis, Corynebacterium, Staphylococcus aureus and three Gram-negative bacteria: Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginae) and some viruses. Based on the results, there was no growth of organisms in the contaminated bone samples after 2 min exposure to microwave radiation.

In a work done in 2005, (63) microwave as a developed heating method was used for bone allograft sterilization. The purpose of this study was to develop a disinfection method for large bone allografts by microwave radiation application with a microwave apparatus. Heating is an effective way of bacterial disinfection or viruses inactivation in bone allografts. However, the size of bone allograft is limited. Large and small samples including a femoral head bone and a metatarsal bone were harvested

from a bovine femur. The influence of size and the electrical or thermal characteristics of the samples were assessed regarding with the temperature distribution after microwave irradiation. Besides, the effect of hot air was also assessed. According to this study, microwave irradiation with a hot-air supply was made on a uniform distribution of temperature at 83.0 ± 0.4°C in the metatarsal bone within 15 min and it supplied a quick heating for disinfection of large bone allografts (64).

Gamma irradiation was noted for effective neutralization of pathogens by its deep penetration ability (65). On the other hand, DNA, as the most important structure of the cells, is the target of gamma radiation exposure. While the focus is on DNA of pathogen, the destructive power of gamma irradiation is always inevitable besides some disadvantages and the high cost of this method. Therefore, microwave irradiation can be a reliable alternative way by just a few minutes of exposure and its cost effectiveness and less side effects and adverse effects on the materials.

Another study investigating the actual effect of microwave was done by David et al (46).This group of researcher have an investigation on the thermal and nonthermal mechanisms of microwave starilization in dry state and they have claimed that the mechanism of sporicidal action of the microwaves was caused solely by thermal effect. The non-thermal effect was not considerably significant and more than 45 min was required to sterilize 105 inoculated spores in dry glass vials at 137°C.

2.4. Materials Used in Periodontology

Grafts that are used in this work are in medical device category.Therefore, some information about medical devices have been given as following;

Medical device: A medical device is any apparatus, appliance, software, material, or other article—whether used alone or in combination, including the software intended by its manufacturer to be used specifically for diagnostic and/or therapeutic purposes and necessary for its proper application—intended by the manufacturer to be used for human beings for the purpose of diagnosis, prevention, monitoring, treatment or alleviation of disease; or compensation for an injury or handicap; investigation, replacement, or modification of the anatomy or of a

physiological process; control of conception; and which does not achieve its principal intended action in or on the human body by pharmacological, immunological, or metabolic means, but which may be assisted in its function by such means (66,67,68).

The regulatory authorities recognize different classes of medical devices based on their design complexity, characteristics, and their risk potential. Each country or region defines these categories in different ways. The authorities also recognize that some devices are provided in combination with drugs, and regulation of these combination products takes this factor into consideration.

The classification of medical devices in the European Union is outlined in Article IX of the Council Directive 93/42/EEC. There are basically four classes, ranging from low risk to high risk as:

1) Class I
2) Class IIa
3) Class IIb
4) Class III

According to this directive, periodontological grafts are included in the Class IIb (68).

Periodontium is a monolith structure from gingiva, alveole bone, periodontal ligament and cement that supports the tooth. Diseases that affect this complex structure are called “periodontal diseases”. Periodontitis, which is an inflammation of the periodontium can cause the loss of the tooth by destroying the supportive structures of it in the first place. The treatment of periodontium diseases has a considerable importance because in the other way, it can cause both functional and esthetics problems. For many years beside conventional methods and treatments, different kinds of regenerative methods have been progressed in the way of controlling periodontological diseases (69.70). Generally, the aim of periodontological treatment is accepted as repairment of the damaged constituted in periodontal structure. However, the main goal of this process is not just repairment of the tissue but also, the regeneration of the damaged tissues that have the potential of regeneration has more importance. Consequently, periodental materials that are used in this thesis are the

grafts that are using in periodontology for regenerating the damaged tissue in gingiva or bone supporting tooth.

Guided tissue regeneration is the periodontal regenerative method that is used for the regeneration of the lost part of periodontal at the present time. Guided bone regeneration (GBR) and guided tissue regeneration (GTR) are dental surgical procedures that use barrier membranes to direct the growth of new bone and gingival tissue at sites with insufficient volumes or dimensions of bone or gingiva for proper function, esthetics or prosthetic restoration (71,72). Recently, a significant increase in this regenerative treatment causes new investigations and works done by researchers in this subject.

2.4.1.Grafts

Grafting refers to a surgical procedure to move tissue from one site to another in the body, or from another creature, without bringing its own blood supply and instead a new blood supplies growing in after it is placed. A similar technique is valid when tissue is transferred with the blood supply intact, it is called a flap. In some instances a graft can be an artificially manufactured device (1).

The aim of using grafts is to find a skeleton for the regeneration (osteoconduction) of lost cells or to speed the act of formation of new bones by helping the inducer protoiens in the graft materials (osteoinduction) for bone grafts and the same for dermal grafts. These grafts can be obtained from human or animal sources besides being syntetic.

In last 50 years, biocompatiable materials like bioceramics and bioglasses as alumina, zirconia, hydroxyapatite, tricalcium phosphates and bioactive glasses gain a significant importance in modern health industry in increasing the life quality of many people in the world. These ceramics are from the kind that can be placed in the body without any rejection and infection for body. As a result, due to some features like a high bone biocompatibility, low density, chemical stability, appropriate mechnical characteristics, high tolerance to erosion and high similarity with the mineral phase of bone for calcium phosphates can be used for bone tissue or bone defects in biomechanic engineering (73, 74).

It took a short time for biomedicals to have a significant progress in their way in healthcare industry and have found different and lots of applications in replacement of hips, teeth, knees, tendons and ligaments and treatment for periodontal diseases, maxillofacial reconstruction, augmentation and stabilization of the jawbone and spinal fusion. The contiribution of calcium phosphates among all different bioceramics in both dentistry and medicine is significant (75).

Bone tissue consists of organic or inorganic mineralize matrix. 70% of bone is made of carbonated calcium phosphate which controls the mineral function of the bone. Organic matrix is mainly made of type I collagen fiber which has supportive duty. The remaining 10% of the bone consists of glycoproteins, glycosaminoglycans, fats, peptides and enzymes. Collagen is accepted as an anisotropic material because of consisiting various crystallinity. Water is also an important component of bone and it affects thermal, electrical and mechanical characteristics of bones. As a result, collagen and water make a system which designate the physical and chemical features of the bone. Moreover, fluoride, chloride, phosphate and magnesium can also be found in the mineral part. Nowadays, gamma radiation is the first option pereferred for sterilization of the bone by bone tissue saving banks. The suggested dose to bone banks for gamma radiation sterilization is 25-35 kGy for the inactivation of bacteria and viruses, however for some viruses more than this dose which is about 100-200 kGy is needed. Generally, structural, physical and chemical changes which can be formed by gamma irradiation to bones are related to collagen destruction and cross-linking of bonds. The irradiation changes the resolution of the collagen and the mechanical, electrical and thermal properties of the bones. Collagen irradiation can lead to the disruption of the hydrogen bridges of peptide bonds in the cross-links in the polypeptide chain, as the collagen loses nitrogen and amino acids (76).

Glass-ceramics and bioactive glasses have the ability to make direct bond with bone, so researchs about these materials have got more importance. In last years, different kinds of bioceramics have progressed for the treatment of damaged or unhealthy bones. These bioceramics can be generally classified as bioinert (eg; Al_2O_3 and ZrO_2), bioactive (eg; biochem and HAp) or biodegradable ceramics (eg; TCP and bone cement).

The reason why biocides and glass ceramics are so widely used is due to some qualities they possess which includes;

- Fast surface reactions leading to chemical bond to the bone,
- Low softening temperatures which allow for curing to bond to ceramics particles, their use as an aid to solidification and filling properties of the micropores in the solidification process,
- Preparing them in suitable properties especially for clinical applications, such as being able to control the binding rates as desired.

On the other hand, dermal graft Regenerative Tissue Matrix (RTM) has been a widely accepted Acellular Dermal Matrix (ADM) for soft tissue applications since its introduction to dentistry in 1997. Dermal graft supports tissue regeneration by allowing rapid revascularization, with cell migration and cell population - ultimately being transformed into host tissue for a strong, natural repair. The field of dermal substitute engineering includes both material science and cell biology. The term tissue engineering refers to the development and application of materials which maintains, restores or improves tissue function (77). These biomaterials may be naturally occurring or synthetic materials. Naturally occurring materials have a number of advantages in skin engineering applications, as we have learned from the success of cadaveric allograft dermal transplants. Unfortunately, the supply of allograft (human source) materials can be limited. On the other hand, use of xenograft (non-human source) as biological materials gives a greater availability and frequent homology with human tissues. Multiple approaches have been used to replace lost, damaged or diseased gingival tissues. One challenge for the periodontist is the coverage of exposed root surfaces associated with gingival recession. Application of dermal grafts in dentistry can include root coverage, gingival augmentation, soft tissue ridge augmentation and soft tissue augmentation around implants. Dermal grafts are the best option to help gingiva regeneration. Root coverage is indicated to cover unesthetic and/or painful exposed root surfaces. Historically, limited success has been achieved especially in deep-wide gingival recession defects. They provide a skeleton for cells regeneration.

2.5. Studies on Sterilization of Grafts Used in Periodontology by Gamma Radiation and Microwave

As it is mentioned before, the sterility of medical devices gained an importance in last decades due to varrious infection and disease transmission from the grafts donor with human or animal source to the recipient, induces researchers to investigate on materials sterilization by different sterilization methods and evaluating their effectivness.

In a study performed by Alaney and co-workers, the effect of high dose gamma irradiation at 50 kGy on fusion rate in rat spinal fusion model of Demineralized Bone Matrix (DBM) was evaluated. In this study, eighty mature athymic nude female rats were used for this study, which formed 10 equal groups. Human DBM exposed to hydrogen peroxide for different time periods (0, 1, 6, and 24 hours) were divided into two major subgroups. One group was further treated with controlled high-dose radiation by using radioprotectants (radiation treated), whereas the other group was frozen immediately without specific treatment (no radiation treated). Increasing the time period of hydrogen peroxide (0, 1, 6, or 24 hours) exposure for preparation of DBM from bone allograft did not affect the fusion rates significantly and there was a trend towards decreasing fusion rates with longer exposure times. The findings in this study demonstrated that radiation exposure up to 50 kGy under protection of clearant processing did not negatively affect the ability of DBM for healing the spinal fusion model, because there were no statistically significant differences between the fusion rates with or without the clearant process (78).

Grieb and co-workers have investigated human bone allograft inactivation by high dose gamma irradiation (50 kGy). According to this study, the safety of allografts may vary and a dose of 25 kGy, the upper limit used by tissue banks in the United States, provided a SAL of 10^{-6} for most bacteria but it is insufficient for the inactivation of HIV, other radioresistant viruses and some bacterial spores need a dose greater than 50 kGy to inactivate HIV. Although, the actual incidences of viral transmissions of HIV from tissue have been notably rare, Buck and colleagues confirmed that HIV could be recovered in culture from bones and tendons of persons with AIDS (79).

In another study, Turker et al have evaluated the effects of irradiation on dental graft materials. Physicochemical analysis [organoleptic control, Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and Scanning Electron Microscope (SEM)] of grafts are followed by microbiological tests including determination of SAL levels, sterility and pyrogen tests were applied. All irradiations were performed under normal conditions (25^0C, 60% relative humidity) in dark using a ^{60}Co cell supplying a dose rate of 1.28 kGy h^{-1} as an ionizing radiation source. All investigations including (organoleptic control, FTIR, DSC, TGA, SEM, ESR, and microbiological analysis) were performed on samples (G1, G2, G3) irradiated at four different dose levels (5 kGy, 10 kGy, 25 kGy, 50 kGy). Unirradiated samples were used as controls to detect physicochemical and antimicrobial activity changes resulting from the application of ionizing radiation on studied samples. According to the results of radiation sterilization of samples, gamma radiation was decided as the most proper sterilization method in comparison with EtO and a significant change that can affect the materials structure and effectiveness were not detected (80).

Singh and Singh investigated microwave radiation for the sterilization of bone allografts comparing with gamma radiation sterilization. Bone allografts were processed from femoral heads were obtained from living donors. The effect of microwave and gamma radiation on the bacteria isolated from bone allografts was evaluated. The microwave radiation treatment was performed at 2450 MHz (frequency) for varying lengths of time at maximum power 900 Watts (W). The sterility test of microwave and gamma irradiated bone allografts was carried out in accordance with International Organization for Standardization (ISO) 11737-2 standarts. This study was undertaken with the aim of exploring the use of microwave radiation for the sterilization of bone allografts and to compare with gamma radiation sterilization. Bone allografts were processed from femoral heads obtained from living donors. The effect of microwave and gamma radiation on the bacteria isolated from bone allograft was evaluated. The microwave radiation treatment was performed at 2450 MHz (frequency) for varying time lengths at maximum power of 900 Watts (W). The sterility test of microwave and gamma irradiated bone allograft was carried out in accordance with ISO 11737-2(81). The study has shown that the sterilization of contaminated femoral head bone allografts can be achieved by short exposure of 2 min to 2450 MHz and 900 W microwave radiation (62).

3.EXPERIMENTAL

3.1. Materials and Instruments Used

All the materials and instruments used in this research have been listed in the following tables (Table 3.1 , 3.2).

Table.3.1. Materials

Cortical Bone	Osteobiol, Italy
Collagenated Bone	Osteobiol, Italy
Collagen	Botiss, Germany
Collagen Matrix	Biohorizane, USA
Soybean Casein Digest Medium	Oxoid, United Kingdom
Fluid Thioglycolate Medium	Oxoid, United Kingdom
^{60}Co Source	Hungary (4523 Ci)

Table.3.2. Instruments

^{60}Co Irradiator	Hungary
Dosimeter	Gammex, Switzerland
ESR Spectrophotometer	Varian X-bant E-L9 Spectrophotometer, USA
	Bruker EMX 113 ESR Spectrophotometer , Germany
Melting Flow Rate Determination Device	Melt Flow TQ, Italy
FTIR System, Spectrum BX	Perkin Elmer, USA
X-Ray Diffractometer	INEL Z.A.C.D. 405-45410-Artenay, France
Microwave oven HF24G541 25 Lt 900 W	Siemens, Germany
TGA / DTA Spectrophotometer	DTG-60H Shimadzu, Japan
SEM	Zeiss EVO ve JEOL SEM 8100, Germany
Gammex	Germany

3.2. Sterilized Grafts

Grafts provided for the analyses were coded according to the origin of the grafts in order to shorten their long names. These codes will be used throughout the thesis. In this research, seven different bone and dermal grafts have been used in which some of them were obtained from animal sources (bovine and horse) and some of them were obtained from human sources. Grafts used in this thesis are coded as PBG1, HBG1, HL1, PDG1, MBG3, MDG2, PDG3. The composition of the grafts are respectively, 100% collagenated cortico-cancelouse porcine bone, collagenated cortico-cancelouse

horse bone, 100 % cortical horse bone, 100% porcine derma, minerelized collagen human bone, 100% human dermis and porcine collagen tissue matrix. Therefore, in Table 3.3, some general information about grafts has been given.

Table 3.3. Information about grafts.

NO	Trade Name	Component	Company	Code
1	DERMA	Porcine Dermal Collagen	Osteobiol	PDG1
2	SP-BLOCK	Horse Bone Collagen	Osteobiol	HBG1
3	DUAL-BLOCK	Porcine Bone Collagen	Osteobiol	PBG1
4	LAMINA	Horse Bone Collagen	Osteobiol	HL1
5	ALLODERM	Human Dermal Collagen	Biohorizan	MDG2
6	MOCUDERM	Porcine Dermal Dermal	Botiss	PDG3
7	MAXGRAFT	Human Bone Collagen	Botiss	MBG3

3.3. Sterilization Methods

Gamma radiation sterilization and microwave sterilization as two different sterilization processes were applied to the grafts.

3.3.1. Gamma Radiation Sterilization

Gamma radiation sterilization was performed in Turkish Atomic Energy Authority (TAEK) Sarayköy gamma radiation center with ^{60}Co γ-source at room temperature. Time was calculated by dosimetric process in order to achieve the required doses. Beside 25 kGy which is the suggested dose by pharmacopeia (14,16), 5, 10 and 50 kGy doses were applied to the materials.

Validation process was performed dosimetrically and biologically.

3.3.2. Microwave Sterilization

Microwave sterilization process was done in the lab. of Hacettepe Univ. Faculty of Pharmacy Dep. of Radiopharmacy by Simens Microwave oven in 900 w, 2450 GHz at 4 different times as 1, 2, 3 and 4 min.

3.3.3. Sterilization Process Validation

Process validation was done to evaluate the validity of the sterilization analytical process and for the aim of both sterilization methods. Therefore, biological ve dosimetric validation methods were applied.

Dosimetric Validation

Dosimetric validation was done to evaluate the amount of radiation gotten by the materials in the gamma radiation cell by placing polymethyl methacrylate dosimeters in the farthest point of the place of the samples. After required time for radiation was applied, dosimeters were evaluated by the absorption spectrometer for the evaluation of the amount of the radiation absorbed by the materials. The absorption amount calculated by the computer base by the below mentioned Equation 3.1.

Equation (3.1) (24)

Dose (Mrad)= [-0.2738 + (5.2555 x A) – (6.2628 x A^2) + (11.0302 x A^3)] A: Absorbans

Biological Validation

In biological validation process, *Bacillus pumilus* as the most radioresistant bacteria was used. For this purpose, in gamma radiation and microwave radiation processes, B. pumilus tapes were placed in the points that the efficiency and completion probability of sterilization are lower than others. It can be concluded that the sterilization was effective and valid, if no microorganism growh was observed in control and test groups.

3.4. Study Plan

The study was planed to carry out the whole analyses on the grafts as pre- and post gamma irradiation at different doses. All grafts were irradiated at 2, 4 ,5 ,20 and 25 ,50 kGy and unirradiated samples were used as control group. Physichochemical and microbiological analyses, were reapeted before and after sterilization as it is given below at Table 3.4.

Table 3.4. Study plan of the sterilization process and analyses.

STERILIZATION	ANALYSES
Before Sterilization Process	Physicochemical analyses - Organoleptic analysis - FTIR analysis - TGA analysis - SEM analysis - SAXS analysis Microbiological tests - Sterility test - SAL dose determination - Pyrogen test
Sterilization Process	Gamma radiation sterilization
	Microwave radiation sterilization
After Sterilization Process	Physicochemical analyses - Organoleptic analysis - FTIR analysis - TGA analysis - SEM analysis - SAXS analysis - ESR analysis Microbiological tests - Sterility test - SAL dose determination - Pyrogen test

3.5. Analyses Before Gamma Radiation Sterilization

In this section, analyses performed on the grafts coded as HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 prior to gamma radiation sterilization have been explained.

3.5.1. Physicochemical Analyses

Organoleptic analysis, FTIR, TGA, SEM, SAXS and ESR analyses were performed prior to gamma radiation sterilization of the grafts coded as HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 .

Organoleptic Analysis

In this section, the grafts coded as HBG1, HL1, MBG3, MDG2, PBG1 ,PDG1, PDG3, were examined in terms of organoleptic properties (shape, color, odor and appereance) before gamma irradiation.

FTIR Analysis

The spectra of HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 coded grafts were studied by spectrophotometer by using an Attenuated Total Reflectance (ATR) accessory with zinc selenide (ZnSe) crystal to obtain spectra of the samples before gamma radiation sterilization by FTIR. The frequencies were expressed in wave numbers (cm^{-1}). All spectra were recorded in absorption mode at 4 cm^{-1} intervals and 32 scans.

TGA Analysis

Thermal analysis of the grafts coded as HBG1, HL1, MBG3, MDG2, PDG1, PDG3, PBG1 were examined before gamma radiation sterilization. When material loss (%) in grafts was examined, the temperature values in which 50% substance loss observed in membranes were examined. The thermograms were drawn under a nitrogen atmosphere. The flow rate was 20 $ml.min^{-1}$ and the temperature increase was 10 $°C.min^{-1}$.

SEM Analysis

The samples were coated with Au-Pd at a thickness of 100 Angstroms in the coating device and then placed on metal stubs with double-sided adhesive tape and were evaluated under the microscope at different magnifications as 100, 500, 1000 and 2000X before gamma radiation sterilization.

SAXS Analysis

SAXS analysis of HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 coded samples were carried out at X- Ray labtaroary at physic department at Hacettepe university. SAXS instrument was used in the measurement and analysis of the biological samples before gamma radiation sterilization. CuKα(λ = 1.54 Å) X-rays were used in the system. The radius of gyration of the nanostructures has been calculated according to the following Equation 3.2:

Equation (3.2)

$$R_g^2 = \frac{\int \mathbf{r}^2 \rho(\mathbf{r}) d\mathbf{r}}{\int \rho(\mathbf{r}) d\mathbf{r}}$$

ESR Analysis

ESR studies were held with ESR spectrometer for grafts coded HBG1, HL1, MBG3, MDG2, PDG1, PDG3, PBG1 before gamma radiation sterilization. The adopted ESR spectrometer operating conditions were given in Table 3.5.

Table 3.5. ESR spectrometer operating conditions adopted throughout the experiments.

Center Field	345.0 mT
Sweep Width	15 mT
Microwave Frequency	9.72 GHz
Microwave Power	2 mW
Modulation Frequency	100 kHz
Modulation Amplitude	0.1 mT
Receiver Gain	$6.32x10^4$
Sweep Time	41.94 s
Time Constant	81.92ms
Conversion Time	40.96 ms
Temperature	RoomTemperature

ESR Spectra of Irradiated at Different Doses (2, 4, 5, 10, 25 and 50 kGy) and Unirradiated Samples

The spectra of unirradiated and gamma irradiated grafts were evaluated. To avoid being affected by uncontrollable changes in spectrometric studies, the spectrum of the standard in the first cavity immediately after each operation was recorded and the studies were carried out on a comparative basis. Spectroscopy splitting factors were calculated based on Equation 3.3. The H_s and H_g center field values of the sample and standard ESR spectra were determined and the spectroscopic splitting factor with the help of the following formula, were taken into account by using the value of the standard g = 2.0028.

Equation (3.3.) $G_g = G_s (H_s / H_g)$ (g= sample graft, s=standard).

G_g: Sample graft spectral splitting factor

G_s : Standard spectroscopic splitting factor

H_s: Standard resonance field (Gause)

H_g : Sample graft resonance field (Gause)

The masses of all examined samples were weighed and mass quantities were adjusted to fill a maximum of 2 cm of ESR tubes with an inner diameter of 4 mm and an outer diameter of 5 mm.

Dose-Response Curves

ESR signal intensity plotted versus absorbed doses were generated to draw dose-response curves, using gamma irradiated samples at different dose values to determine the dosimetric potential of the samples.

Long Term Studies

In this study, the investigated samples were stored at room conditions (93% Relative Humidity, 25 ^{0}C) for nearly three months (82 days) and ESR long term decay data of HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 coded samples were recorded at various time intervals. The free radical content decay percentage and also stability of the irradiated samples in the passing span of time were evaluated in this part of study.

3.5.2. Microbiological Tests

Sterility test was done on the grafts which were used as control group. SAL values were determined (82). Pyrojen test was only applied on the grafts representing gamma radiation sterilized sample and microwave sterilized sample.

Sterility Test

The sterility of the grafts irradiated by gamma radiation and control group were evaluated by using SCDM (Soybean Casein Digest Medium) and FTM (Fluid Thioglycolate Medium) (82).

- SCDM TEST:

It was used for the production of anaerob microorganisms and fungi. 30 g SCDM was dissolved in 1000 ml balloon-fed distilled water. The prepared solution was sterilized in autoclave at 121 ° C for 20 min and then maintained to room temperature. After checking whether the final pH is 7.3 ± 0.2, it is divided into sterile glass tubes to give 15 ml. From sterilized samples, 1 ml sowing was carried out beside the burner flame. For every sampel 5 tube has been used. After the seeding process was completed, the tubes were allowed to incubate at 25 ° C for 2 weeks. It was assessed whether there was reproduction in the tubes that were incubated for 14 days.

SCDM content (for 1 L) :

Casein Pancreatic Extract 17 g

Soya Bean Papain Extract 3 g

Sodium Chloride 5 g

Dipotassium Hydrogen Phosphate 2.5 g

Glucose Monohydrate 2.5 g

Water R 1000 ml

- FTM TEST:

It has been used for the production of anaerobic microorganisms. 29.8 g FTM was suspended in 1000 ml balloon-flask distilled water and then FTM dissolved in a 100 ° C water bath. The prepared solution was sterilized in an autoclave at 121 ° C for 20 min and then allowed to cool. After checking whether the final pH is 7.1 ± 0.2, it is

divided into sterile glass tubes to give 15 ml. From sterilized samples, 1 ml sowing was carried out beside the burner flamer. After the seeding process was completed, the tubes were allowed to incubate at 35 ° C for 14 days with the mouth closed. It was assessed whether there was any reproduction in the tubes left to the incubation.

FTM content (for 1 L):

L-Cystine 0.5 g

Agar, Granule 0.75 g

Sodium Chloride 2.5 g

Glucose Monohydrate 5.5 g

Yeast Extract 5.0 g

Casein Pancreatic Extract 15 g

Sodium Thioglycolate 0.5 g

Tyglycolic Acid 0.3 ml

Sodium Rezorsin Solution 1.0 ml

Water R 1000 ml

SAL Determination

Sterility assurance level determination for samples before irradiation was given in the following part;

Preparation of Bacillus Pumilus Spores

B. pumilus was incubated at 37 ° C for 24 hours in Nutrient Broth, and then the culture was incubated for 7 days at 37 ° C in Tryptic Soy Agar (TSA). The samples taken from TSA were stained with gram negative dyse and collected from the surface of agar with sterile water. The resulting suspension was washed 3 times for 15 min at 3000 rpm by centrifugation. Once resuspended with distilled water, the suspension was heated to 80 ° C to inhibit living vegetative bacteria, suddenly cooled, and the number of spores obtained was calculated in milliliters.

Death Rate of Microorganism Determination

To find the radiation dose to provide SAL 10^{-6} for grafts, the specimens were irradiated with pre-determined radiation and control group infected with B. pumilis spores which are the least sensitive to radiation. Samples were seeded on Nutrient Agar Medium with shaking to count the microorganisms, and logarithmic microorganism mortality versus radiation dose was plotted to determine radiation dose to provide SAL 10^{-6} for each sample.

Pyrogen Test

All the grafts have been taken out from their sterile packages and no incubation for pyrogen test was performed. So, pyrogen test for grafts before irradiation has not been applied.

3.6. Analyses after Gamma Radiation Sterilization Process

3.6.1. Physicochemical Analyses

Organoleptic analysis, FTIR, TGA, SEM, SAXS and ESR analyses were performed after gamma radiation sterilization (2, 4, 5, 10, 25 and 50 kGy) of the grafts indicated by HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 codes as it was explained in Section 3.5.1.

3.6.2. Microbiological Tests

Microbiological tests were performed after gamma radiation sterilization (2, 4, 5, 10, 25 and 50 kGy)of the grafts indicated by HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 codes as it was explained in Section 3.5.2.

Sterility Test

Sterility tests were performed after gamma radiation sterilization (2, 4, 5, 10, 25 and 50 kGy) of the grafts indicated by HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 codes as it was explained in Section 3.5.2.

SAL Determination

Sterility assurance level determination for samples after irradiation with 2, 4, 5, 10, 25 and 50 kGy was performed as explained in Section 3.5.2.

Pyrogen Test

Pyrogenity test was done for just on the grafts coded as PDG1, irradiated at the optimum dose for sterilization (5 kGy) due to the limitation in time and budget. The Test for Bacterial Endotoxins (BET) is used to detect or quantify endotoxin from gram-negative bacteria using amoebocyte or lysate from the horseshoe crab. There are three different methods for this test, in our study gel-clot technique which is based on the gel formation has been used (83). Limulus Amebocyte Lysate (LAL) Rapid Endotoxin Detectio Kit was used for this test as it is explained below and showed at Figure 3.1.

LAL is an aqueous extract of blood cells (amebocytes) from the horseshoe crab, Limulus polyphemus. The test is performed by adding 0.5 mL of the test sample to a Single Test Vial (STV) of Pyrosate. After mixing to dissolve the reagent, 0.25 mL is removed from the vial and either discarded or, if PPC vials are being used, added to a PPC vial. The vials are then incubated at 37 ± 1°C for the time specified on the SPL and PPC package labels and the certificate of compliance. At the end of the incubation period, the vials are removed and inverted in one smooth motion. If a gel has formed and remains intact in the bottom of the vial after inversion, the test is positive; the concentration of endotoxin in the SPL vial is greater than or equal to the stated sensitivity of the Pyrosate (provided that the test is valid). Any other state of the mixture constitutes a negative test and indicates an endotoxin concentration less than the stated sensitivity.

A series of standard endotoxin concentrations of 2λ, λ, ½λ and ¼λ (where λ means the labeled lysate sensitivity) using USP Endotoxin RS (Reference Standard) were prepared. Also a series of dilutions of sample and prepare a PPC for the highest concentration to be tested were prepared. The endotoxin standards, a negative control (prepared with LRW) and the sample dilutions and PPC in duplicate, was tested (84) Figure 3.1.

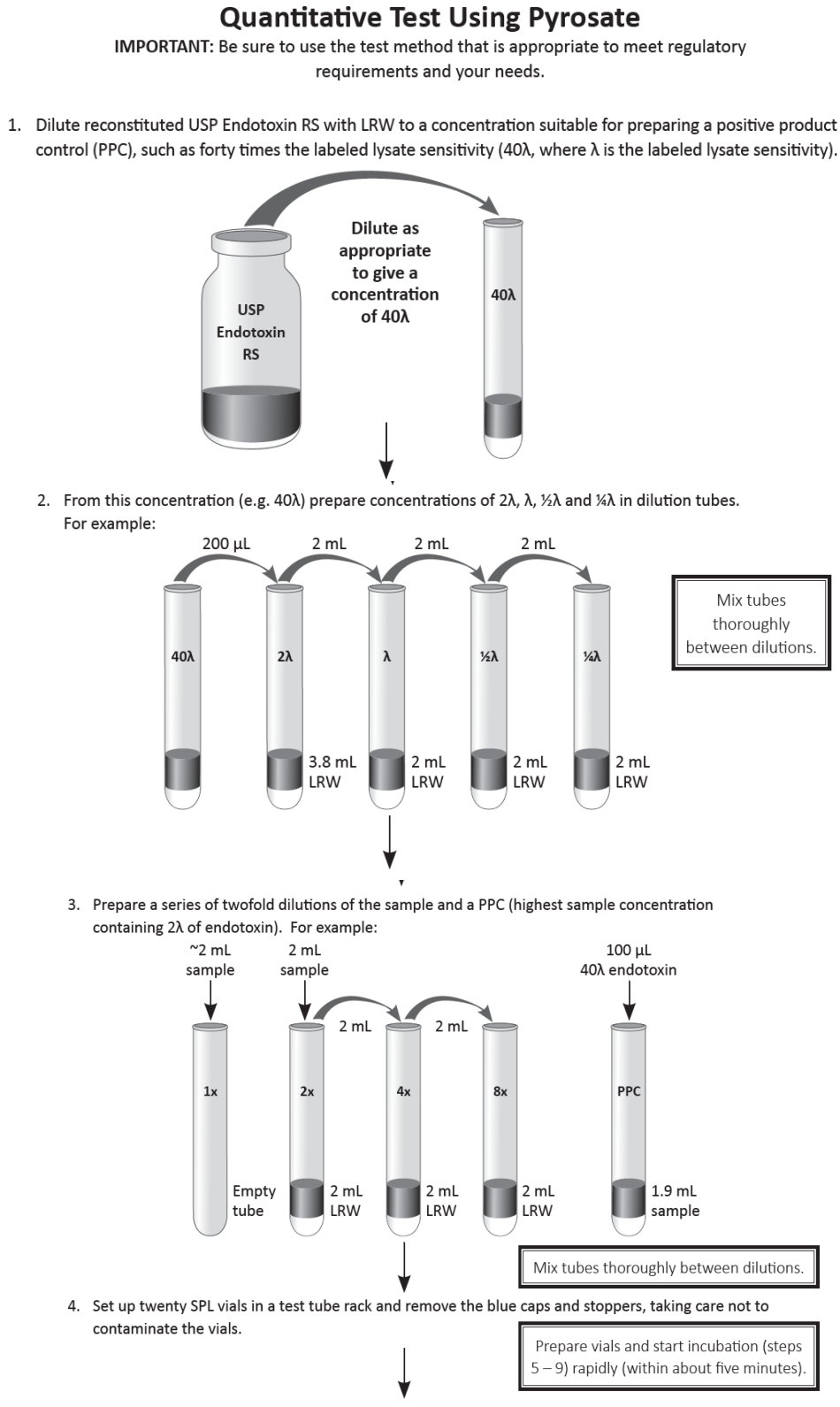

Figure 3.1. Quantitative LAL rapid endotoxin tests process (84).

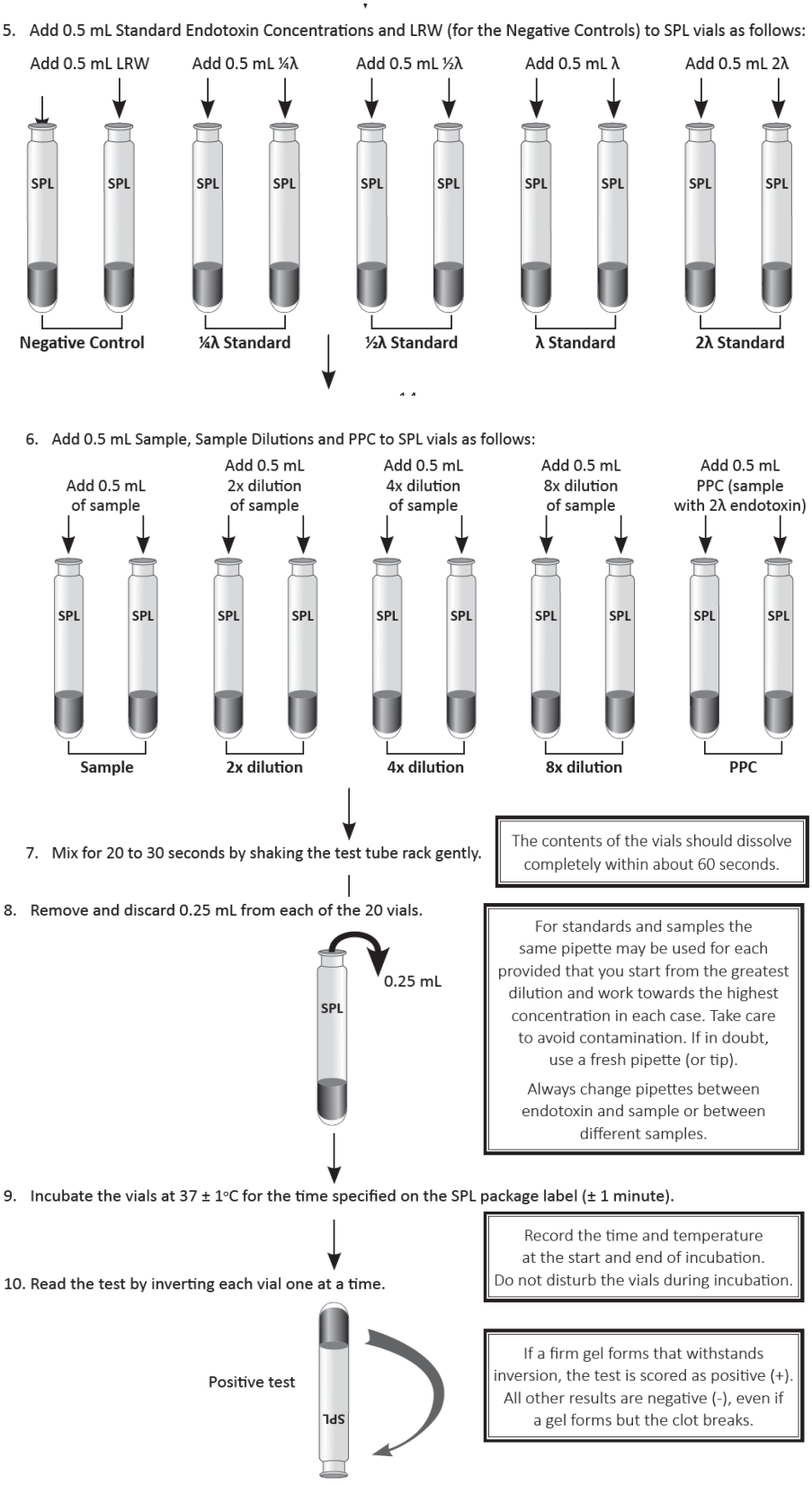

Figure 3.1. Quantitative LAL rapid endotoxin tests process (84). (continued)

3.7. Analyses Pre- and Post- Sterilization with Microwave Radiation in Grafts

Analyses made pre- and post- sterilization with microwave are the same as those performed pre- and post- sterilization with the gamma radiation described in Section 3.5. and 3.6.

3.7.1. Physicochemical Analyses

All analyses including organoleptic analysis, FTIR, TGA, SEM, SAXS and ESR analyses performed before and after sterilization with the gamma radiation described in Sections 3.5 and 3.6 were performed before and after sterilization with microwave irradiation for all grafts too.

- Organoleptic analysis
- FTIR analysis
- TGA analysis
- SEM analysis
- SAXS analysis
- ESR analysis

As it was mentioned above all analyses that were applied for gamma irradiated grafts were done for microwave irradiated too but there was an exeption. This exeption was for SAXS analysis. For every grafts coded as HBG1, HL1, MBG3, MDG2, PBG1,PDG1 and PDG3 optimum one, base on the microbiological results after microwave sterilization, which showed that all the grafts have no microbilogical growth in 3 min of microwave irradiation, were choosen for SAXS analysis, due to the limited time for compeleting analysis.

3.7.2. Microbiological Tests

Microbiological tests as sterility, SAL determination and pyrogen were performed for all grafts after irradiation with microwave like done for gamma irradiated grafts and explained in Section 3.5.2 and 3.6.2.

4. RESULTS

4.1. Validation before sterilization process

Validation process for both gamma and microwave radiation sterilization was done as mentioned in Section 3.3.3, i.e biological and dosimetric validation.

Biological validation process for both gamma and microwave radiation process was done by Bacilus pumulis bacteria. Validation for sterilization processes was given in Table 4.1.

Table 4.1. Validation for sterilization processes.

Validation Method	Gamma Radiation	Microwave Radiation
Biological	B. pumilus (-)	B. pumilus (-)
Dosimetric	Gammex(-)	-

(-) No Microbiological Growth.

The amount of gamma radiation absorbed dose by materials was calculated by the dosimeters placed in the gamma cell next to the other materials during the irradiation process. Absorbed dose of radiation by materials after gamma radiation sterilization was given in Table 4.2.

Table 4.2. Absorbed dose of radiation by materials after gamma radiation sterilization.

Dose (kGy)	**Irradiation Time *(min)**	**Absorbance****	**Absorbed dose (kGy)**
2	94	0.120	2.86
4	188	0.152	4.19
5	235	0.174	5.08
10	470	0.295	10.15
25	1175	0.544	25.08
50	2350	0.764	50.05

*^{60}Co source irradiation rate. For irradiation day, the rate was 1.28 kGy.h^{-1} .

**These amounts were calculated by the equation given in the Section 3.3.3.

4.2. Results of Gamma Radiation Sterilization Pre – and Post Analyses

To compare the results of anlyses obtained pre- and post irradiation have been put together in the same table.

4.2.1.Physicochemical Analysis

The results of the physicochemical analyses of the grafts before and after strilization were given under the headings of organoleptic, FTIR, TGA, SEM, SAXS and ESR.

Organoleptic Analysis

Organoleptic analyses were performed as described in Section 3.5.1. According to the results, a significant difference in shape and color was not detected after sterilization at 2, 4, 5, 10, 25 and 50 kGy gamma irradiation. Results were given at Table 4.3.

Table 4.3. Organoleptic characteristics of garfts before and after gamma radiation sterilization.

Grafts	**Organoleptic Features**						
	Before Radiation	Radiation Dose (kGy)					
		2	4	5	10	25	50
HBG1	White Porous,Fragile	-	-	-	-	-	+
HL1	Cream Elastic	-	-	-	-	+	+
MBG3	Cream Porous,Hardness	-	-	-	-	-	-
MDG2	Cream Elastic	-	-	-	-	+	+
PBG1	Cream Porous,Fragile	-	-	-	-	-	-
PDG1	White Elastic Hardness	-	-	-	-	-	-
PDG3	White Elastic	-	-	-	-	-	+

+ Slightly change - No change

FTIR Analysis Results

FTIR analysis for all unirradiated (0 kGy) and irradiated (2, 4, 5, 10, 25, 50 kGy) grafts were obtained as explained in Section 3.5.1. Results were given in Figure 4.1 to 4.7.

The evaluation of FTIR spectra of all grafts were given below using the abbrevations str: stretching, bend: bending, s: strong, m: medium, w: weak, sh: shoulder.

In the evaluation of HBG1 coded graft as illustrated in Figure 4.1, 3294 (O-H str., N-H str., amide A, hydrogen bonded), 3095, 2915 (C-H str., amide B), 1632 (C=O str., amide I), 1535 (N-H def. + C-N str. amide II), 1416 (C-H bend., C-O str., CO_3^{-2}), 1010, 872 (P-O str., PO_4^{-3}) peaks were observed. Based on these results a significant change before and after irradiation was not detected.

In the evaluation of HL1 coded graft as illustrated in Figure 4.2, 3280 (N-H str., amide A, hydrogen bonded), 3083, 2957 (C-H str., amide B), 1632 (C=O str., amide I), 1538 (N-H def. + C-N str. amide II), 1446, 1337 (C-H bend., C-O str., CO_3^{-2}), 1235 (C-N str., N-H def., amide III), 1078, 1033, 654, 621 (P-O str., PO_4^{-3}) peaks were observed. Based on these results, a significant change before and after irradiation was not detected.

In the evaluation of MBG3 coded graft as illustrated in Figure 4.3, 3276 (N-H str., amide A, hydrogen bonded), 3083, 2925, 2854 (C-H str., amide B), 1633 (C=O str., amide I), 1533 (N-H def. + C-N str. amide II), 1415 (C-H bend., C-O str., CO_3^{-2}), 1003, 872 (P-O str., PO_4^{-3}) peaks were observed. Base on these results a significant change before and after irradiation and was not detected.

In the evaluation of MDG2 coded graft as illustrated in Figure 4.4, 3295 (N-H str., amide A, hydrogen bonded), 3079, 2935, 2880 (C-H str., amide B), 1633 (C=O str., amide I), 1532 (N-H def. + C-N str. amide II), 1444, 1336 (C-H bend., C-O str., CO_3^{-2}), 1228 (C-N str., N-H def., amide III), 1152, 1078, 1025, 653 (P-O str., PO_4^{-3}) peaks were observed. Based on these results a significant change before and after irradiation was not detected.

In the evaluation of PBG1 coded graft as given in Figure 4.5, 3281 (N-H str., amide A, hydrogen bonded), 3087, 2939 (C-H str., amide B), 1636 (C=O str., amide I), 1539 (N-H def. + C-N str. amide II), 1447, 1411 (C-H bend., C-O str., CO_3^{-2}), 1015, 871 (P-O str., PO_4^{-3}) peaks were observed. Based on these results a significant change before and after irradiation was not detected.

In the evaluation of PDG1 coded graft as illustrated in the Figure 4.6, at 3285 (N-H str., amide A, hydrogen bonded), 3087, 2939 (C-H str., amide B), 1633 (C=O str., amide I), 1538 (N-H def. + C-N str. amide II), 1445, 1337 (C-H, bend., C-O str., CO_3^{-2}), 1232 (C-N str., N-H def., amide III), 1077, 1029, 659 (P-O str., PO_4^{-3}) peaks were observed. Based on these results a significant change before and after irradiation was not detected.

In the evaluation of PDG3 coded graft as illustrated in Figure 4.7, 3294 (N-H str., amide A, hydrogen bonded), 3087, 2953, 2880 (C-H str.), 1633 (C=O str., amide I), 1538 (N-H def. + C-N str. amide II), 1445 (C-H bend., 1337 (C-O str., CO_3^{-2}), 1232 (C-N str., amide III), 1078, 1028, 666 (P-O str., PO_4^{-3}) peaks were observed. Base on these results a significant change before and after irradiation was not detected.

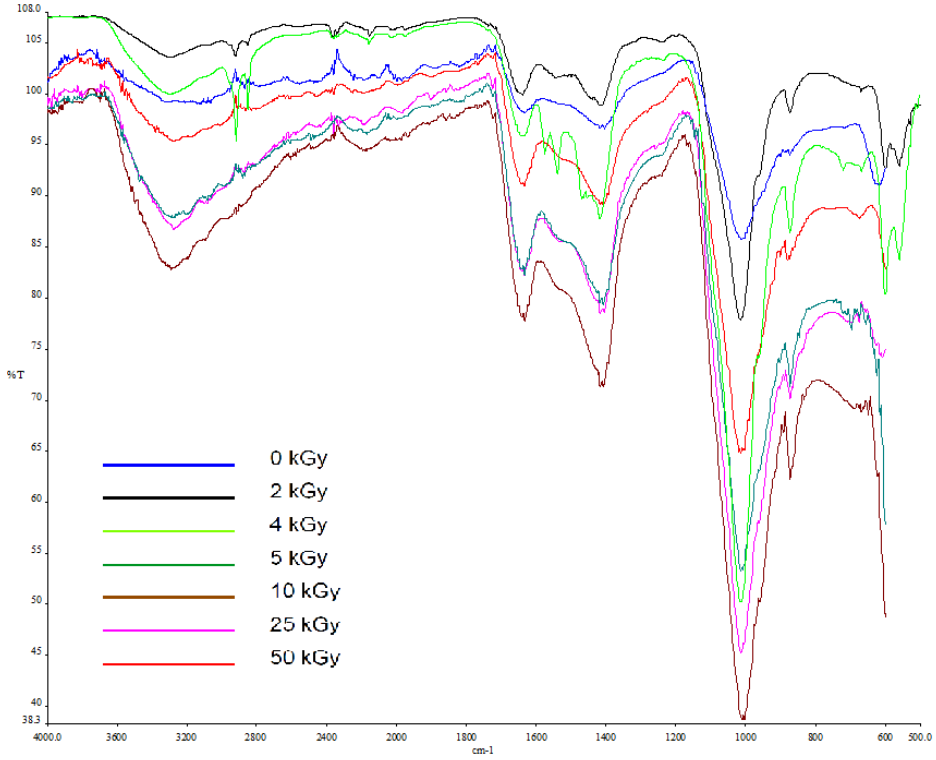

Figure 4.1. FTIR spectra of HBG1 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

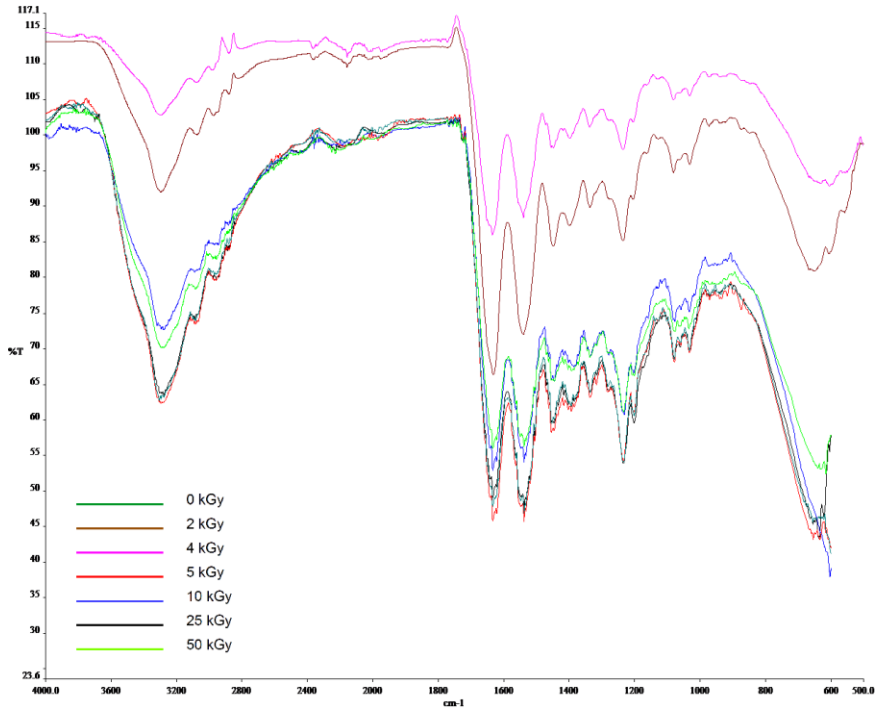

Figure 4.2. FTIR spectra of HL1 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

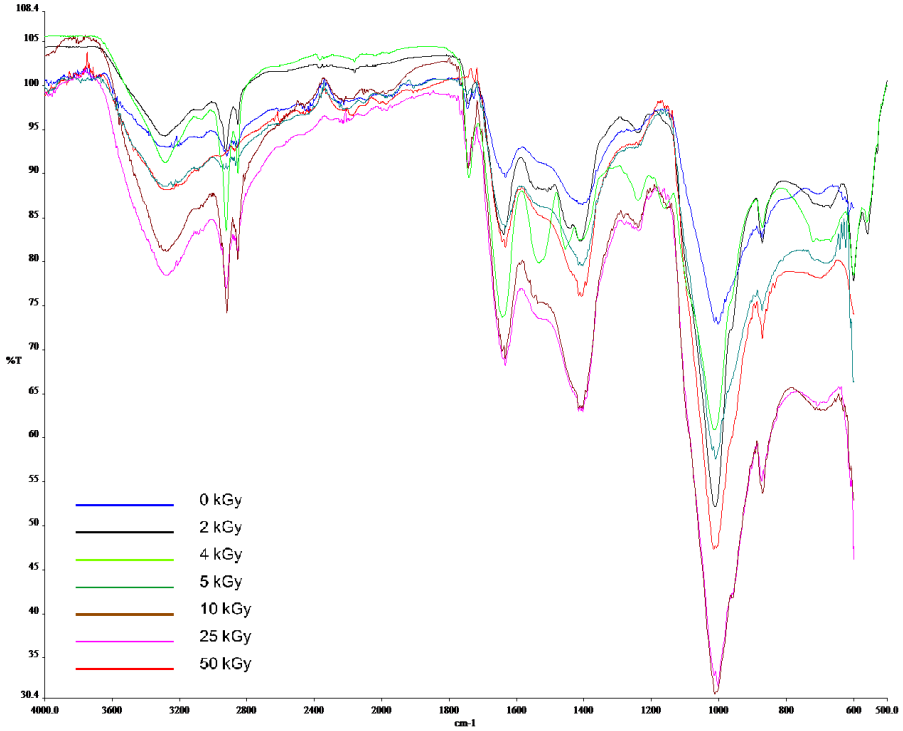

Figure 4.3. FTIR spectra of MBG3 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

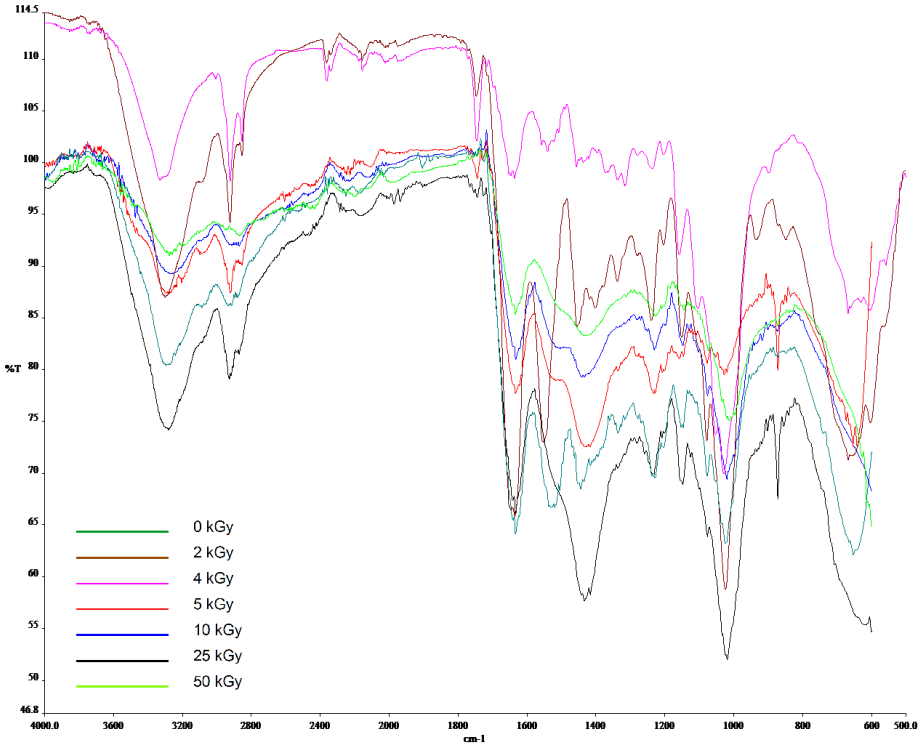

Figure 4.4. FTIR spectra of MDG2 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated .

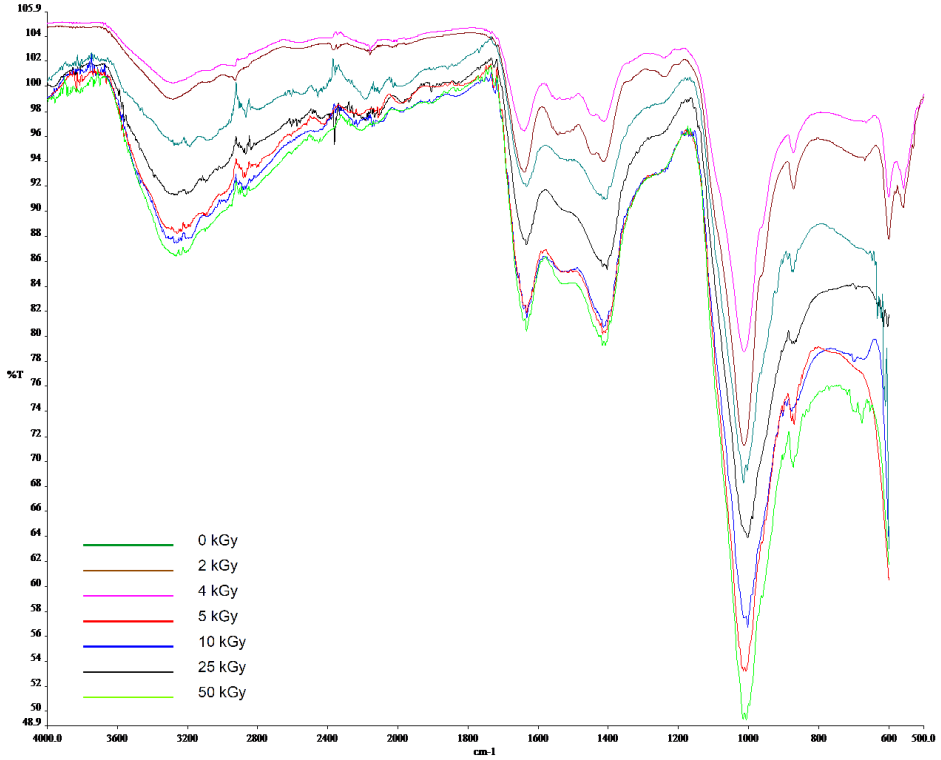

Figure 4.5. FTIR spectra of PBG1 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

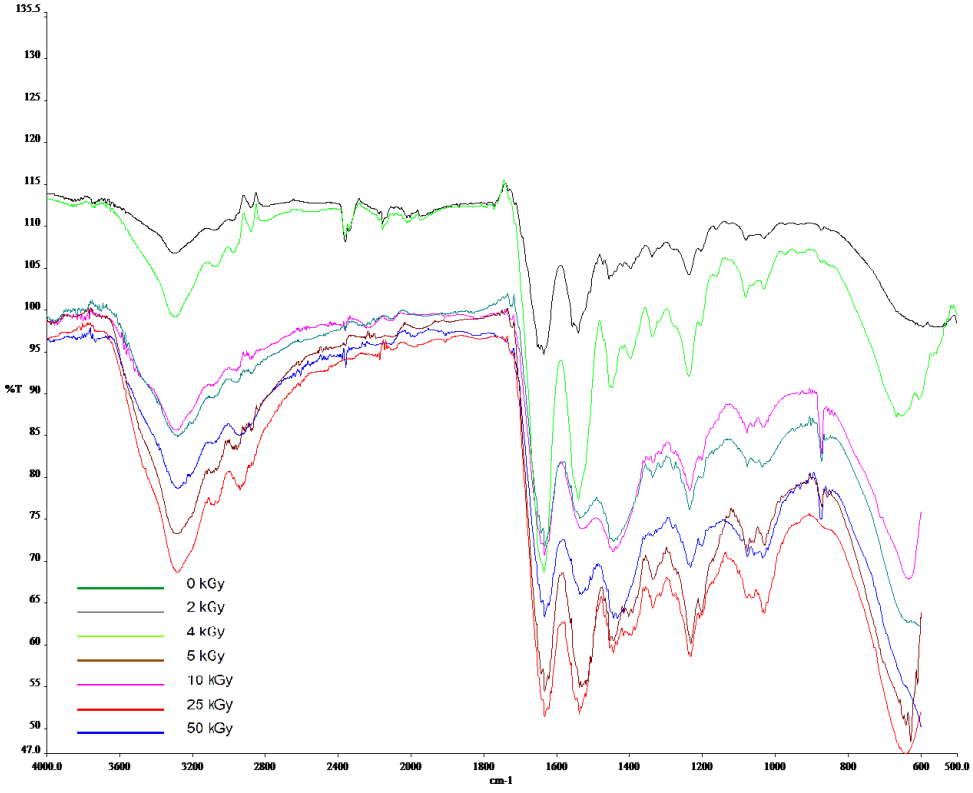

Figure 4.6. FTIR spectra of PDG1 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

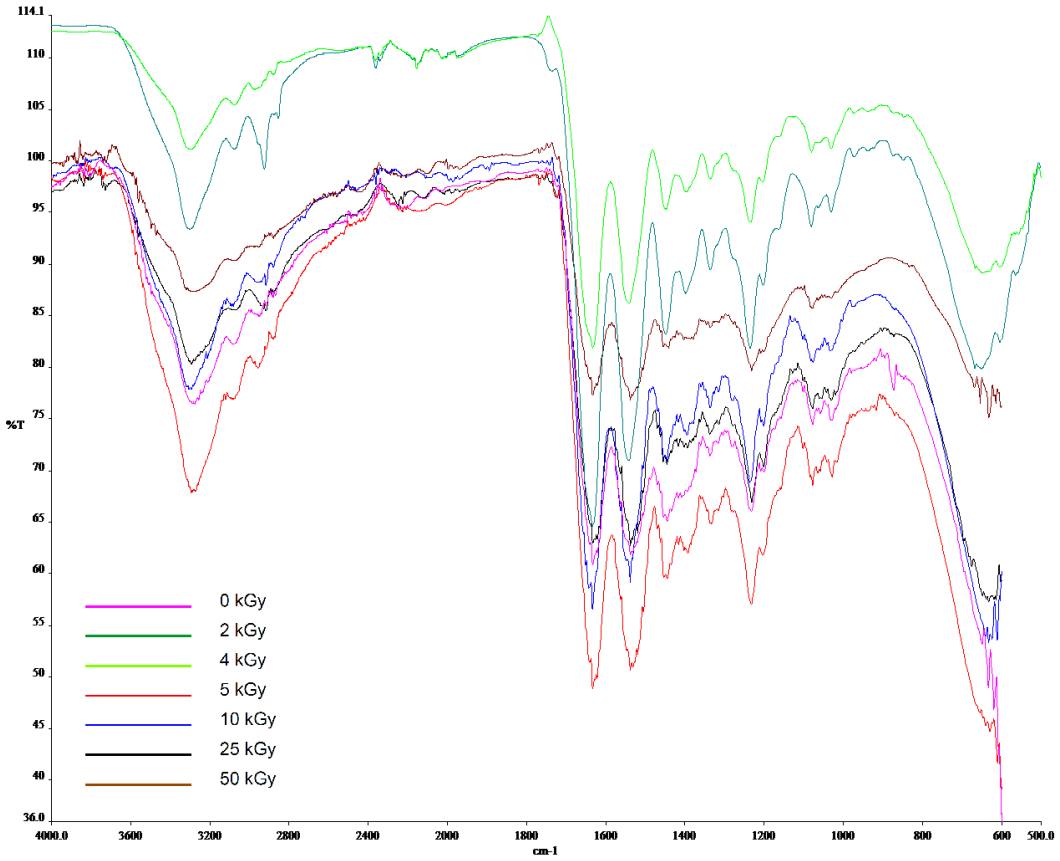

Figure 4.7. FTIR spectra of PDG3 coded grafts irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

Analysis Results

TGA analysis were performed for unirradiated and 2, 4, 5, 10, 25, 50 kGy irradiated grafts by gamma radiation as given in Section 3.5.1. The results of TGA analysis were given in Figure 4.8 to 4.14. To determine the thermal stability of unirradiated and irradiated samples, the temperature for half-life ($T_{1/2}$) were found directly from their dynamic thermograms (Figure 4.8 to 4.14) and were given in Table 4.4. Moreover, grafts residue % (at 550 ^{0}C) for different radiation doses was also determined from TGA thermograms (Table 4.5) and the temperature for maximum weight loss (T_{max}) was determined by first derivative curve of TGA thermograms (Figure 4.15 to 4.21 and Table 4.6).

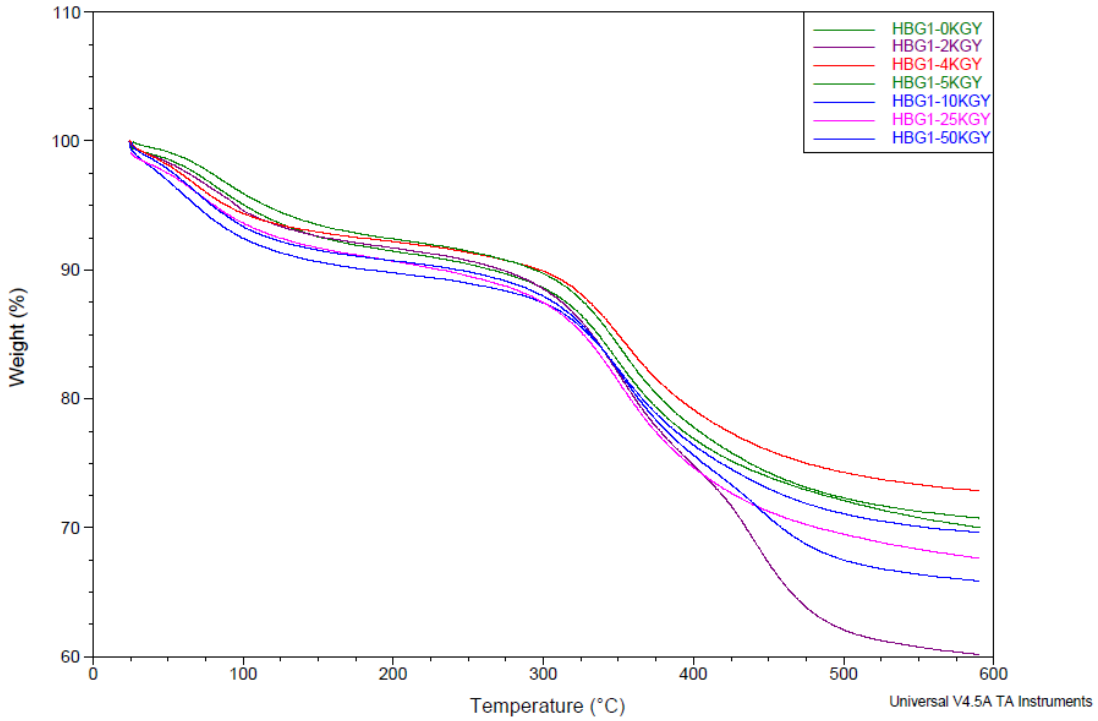

Figure 4.8. TGA thermograms of HBG1 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated .

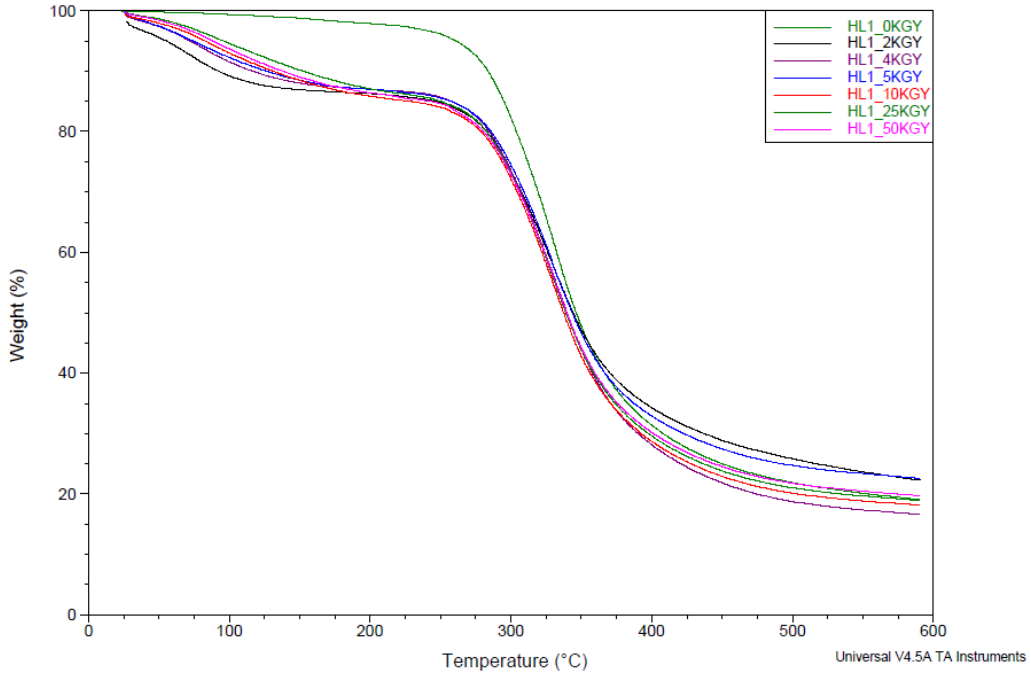

Figure 4.9. TGA thermograms of HL1 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

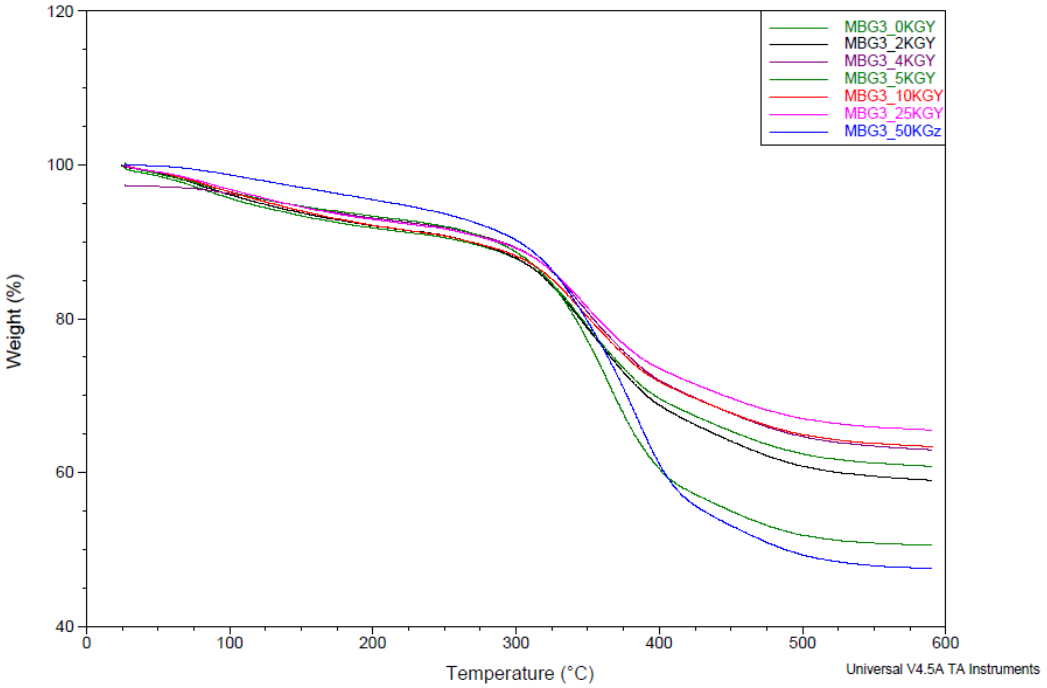

Figure 4.10. TGA thermograms of MBG3 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

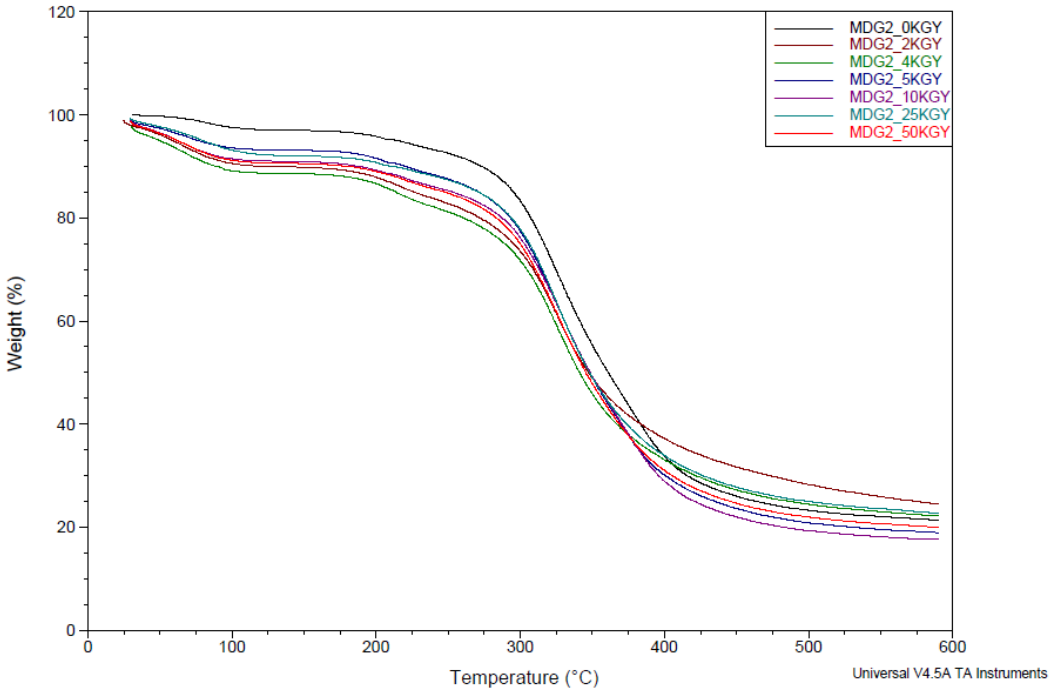

Figure 4.11. TGA thermograms of MDG2 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

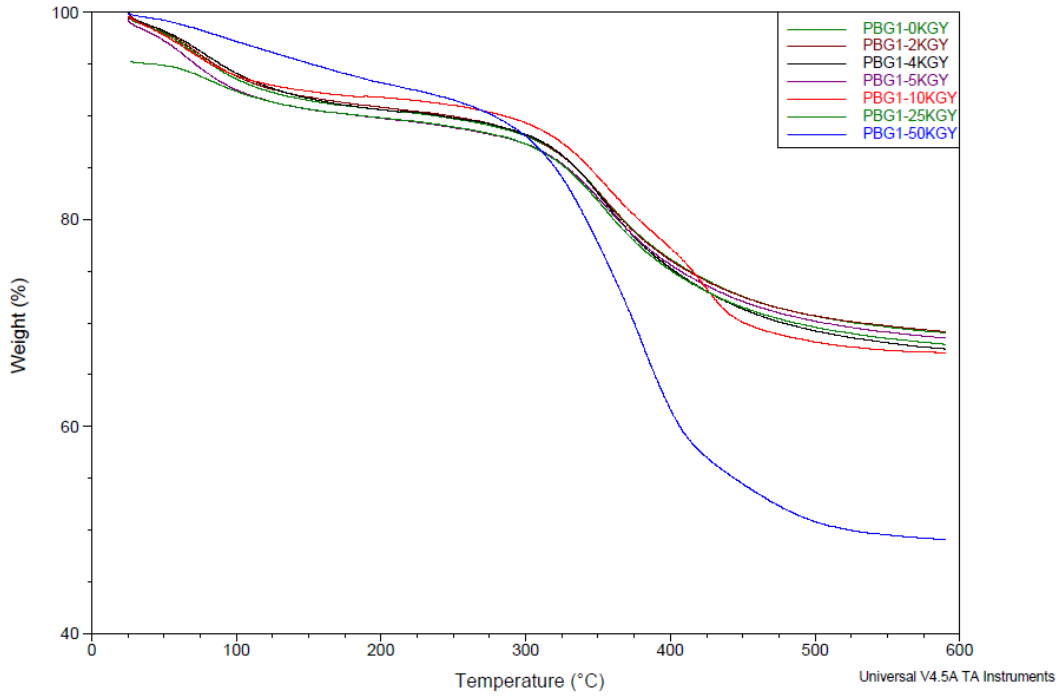

Figure 4.12. TGA thermograms of PBG1 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated .

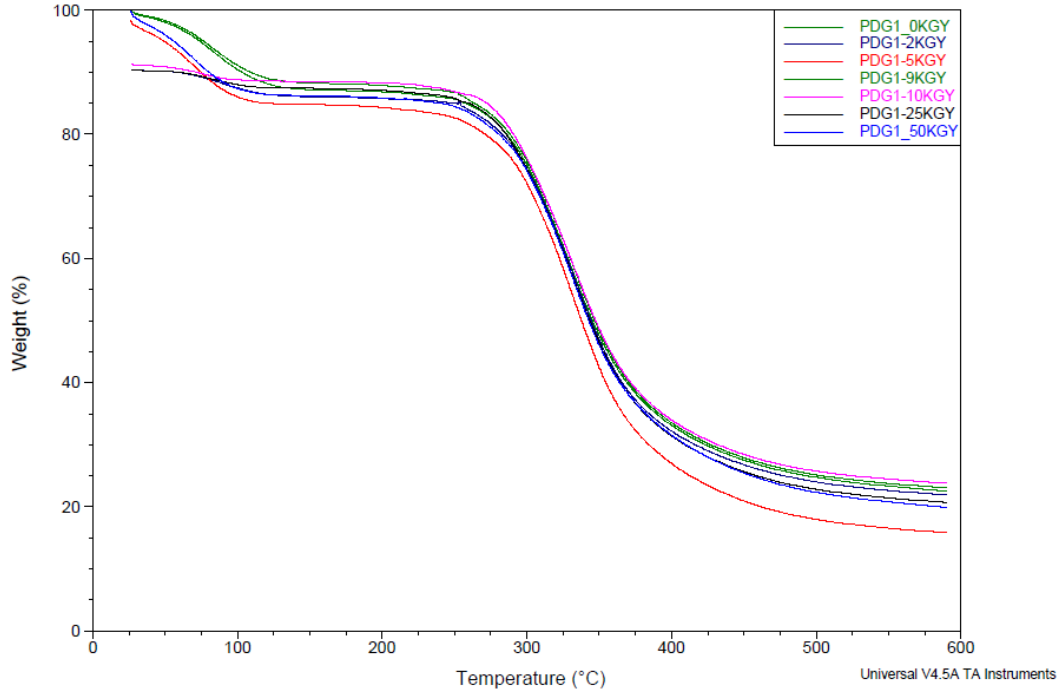

Figure 4.13. TGA thermograms of PDG1 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

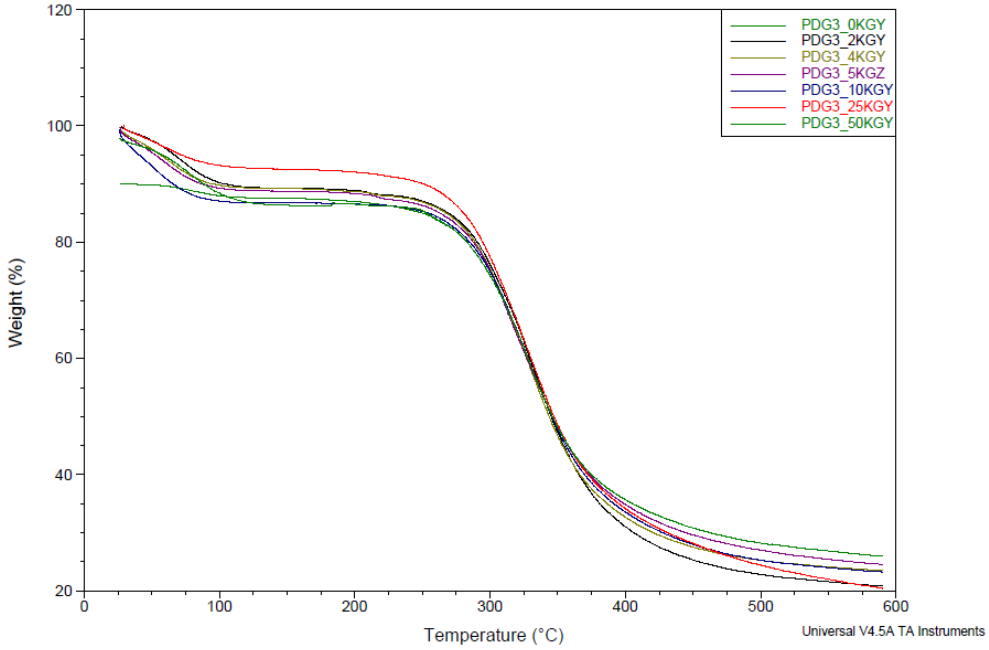

Figure 4.14. TGA thermograms of PDG3 irradiated at 2, 4, 5, 10, 25, 50 kGy and unirradiated.

Table 4.4. The temperatures for half-life $T_{1/2}$ (^{0}C) for different radiation doses.

Sample	The temperature for half-life ($T_{1/2}$) (°C)						
	Irradiation Dose (kGy)						
	0	2	4	5	10	25	50
HBG1	-	-	-	-	-	-	-
HL1	352	349	348	347	350	351	353
MBG3	-	-	-	553	-	-	476
MDG2	358	350	351	349	352	348	347
PBG1	-	-	-	-	-	-	490
PDG1	348	349	349	344	348	346	347
PDG3	348	348	349	347	348	249	349

Table 4.5. Residue percentage at 550 ^{0}C for different radiation doses.

Sample	Residue at 550 °C (%)						
	Irradiation Dose (kGy)						
	0	2	4	5	10	25	50
HBG1	73	72	75	60	69	66	63
HL1	20	23	16	23	20	19	19
MBG3	61	59	60	51	64	65	49
MDG2	22	23	21	20	19	18	19
PBG1	62	63	63	65	66	67	48
PDG1	23	22	21	20	17	18	16
PDG3	24	25	21	23	24	21	22

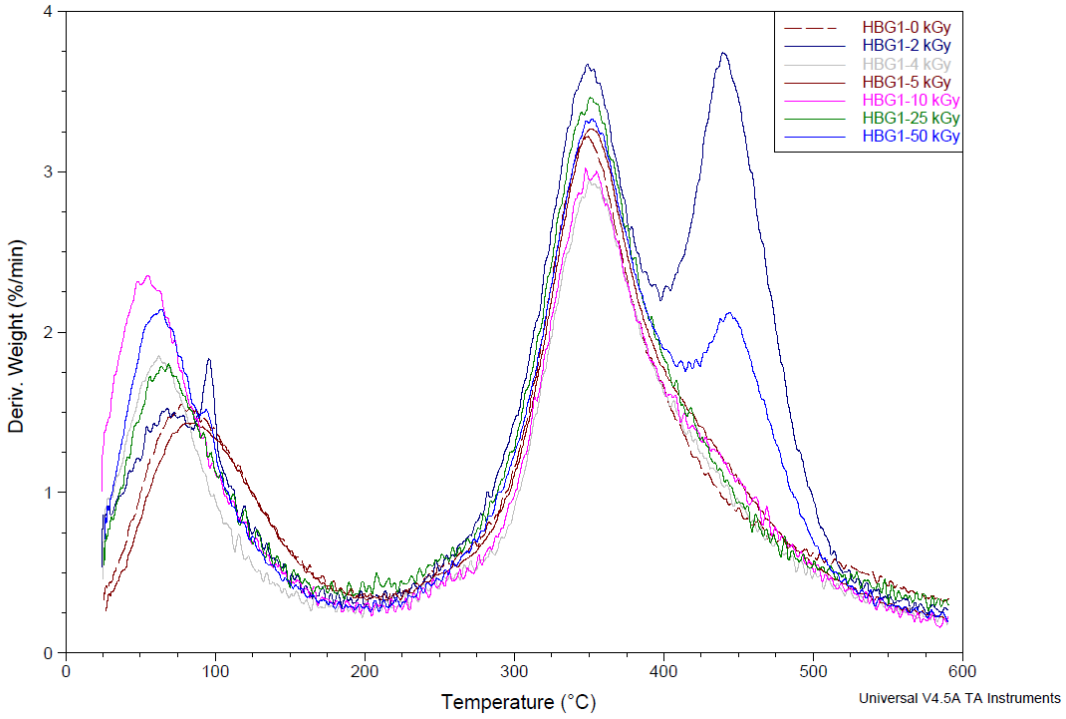

Figure 4.15. First derivative curves of TGA thermograms after irradiation by gamma at different doses for HBG1.

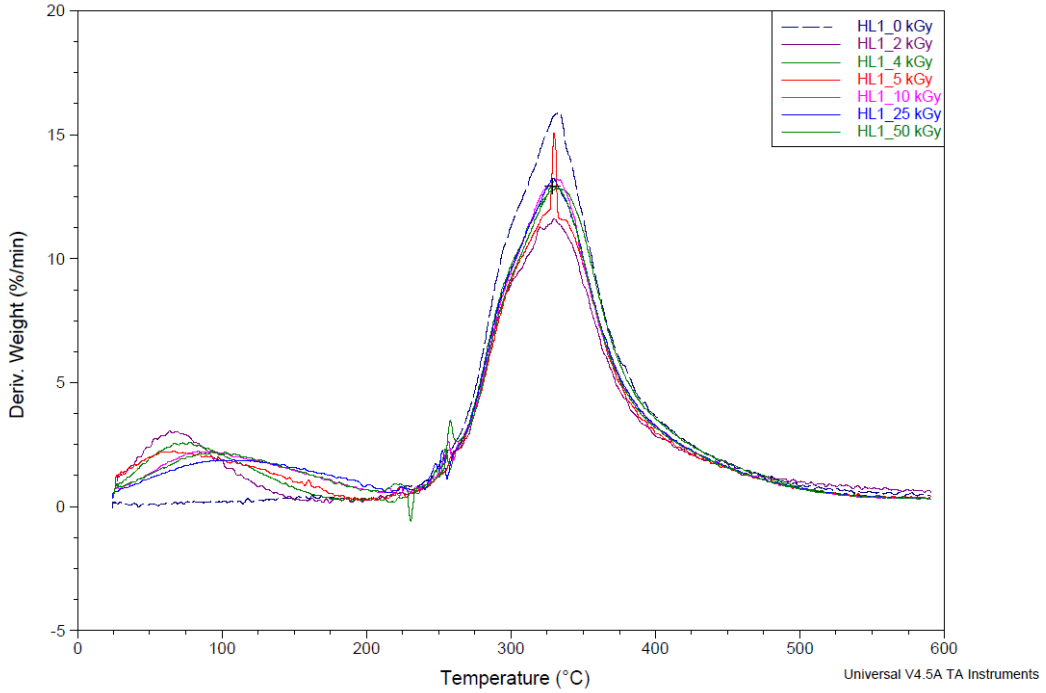

Figure 4.16. First derivative curves of TGA thermograms after irradiation by gamma at different doses for HL1.

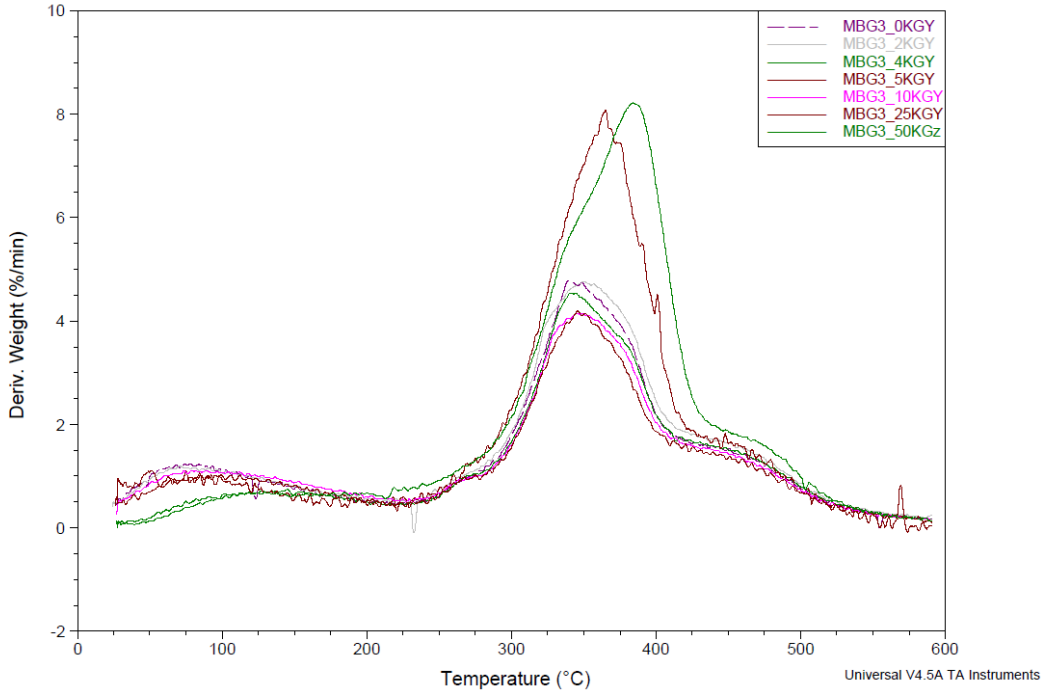

Figure 4.17. First derivative curves of TGA thermograms after irradiation by gamma at different doses for MBG3.

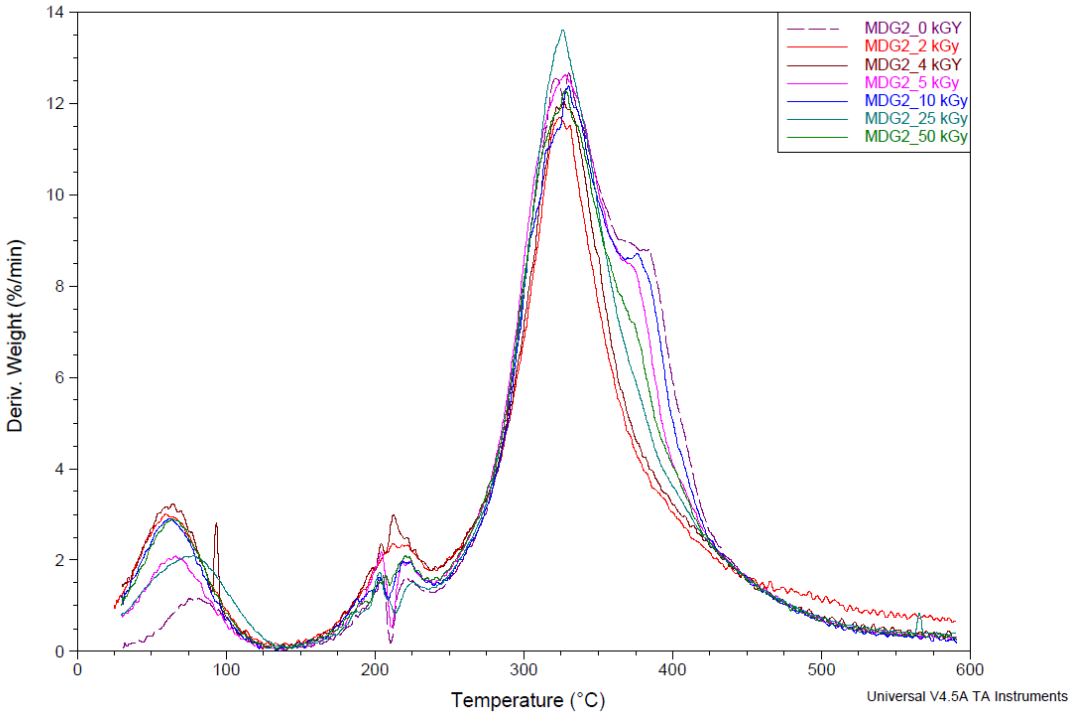

Figure 4.18. First derivative curves of TGA thermograms after irradiation by gamma at different doses for MDG2.

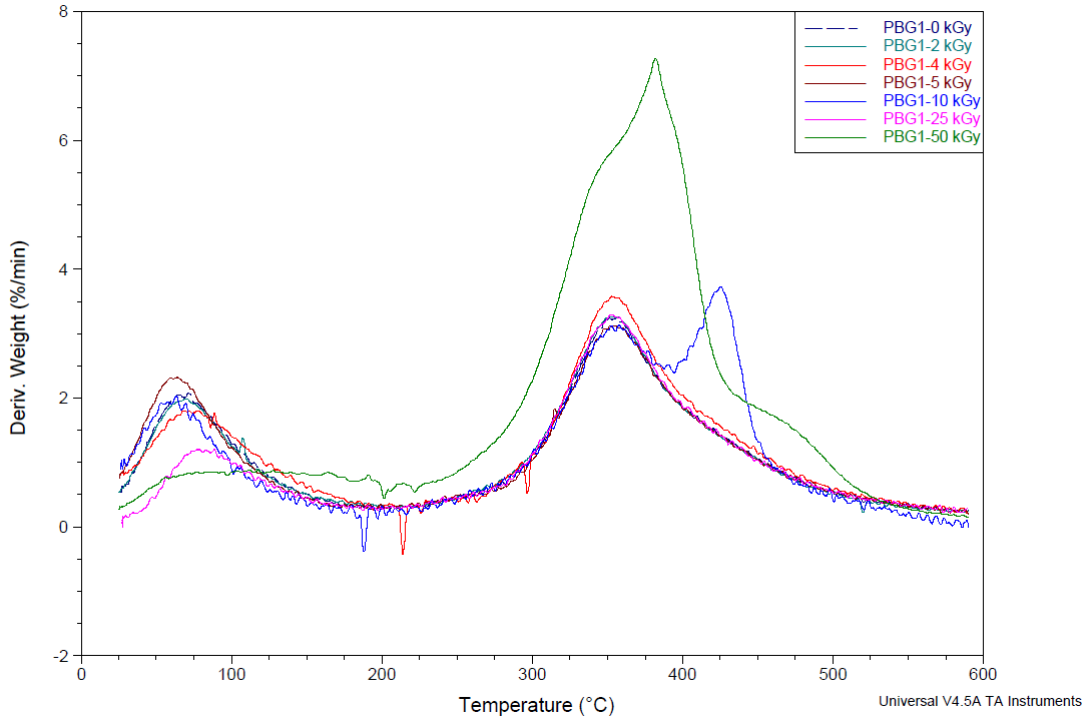

Figure 4.19. First derivative curves of TGA thermograms after irradiation by gamma at different doses for PBG1.

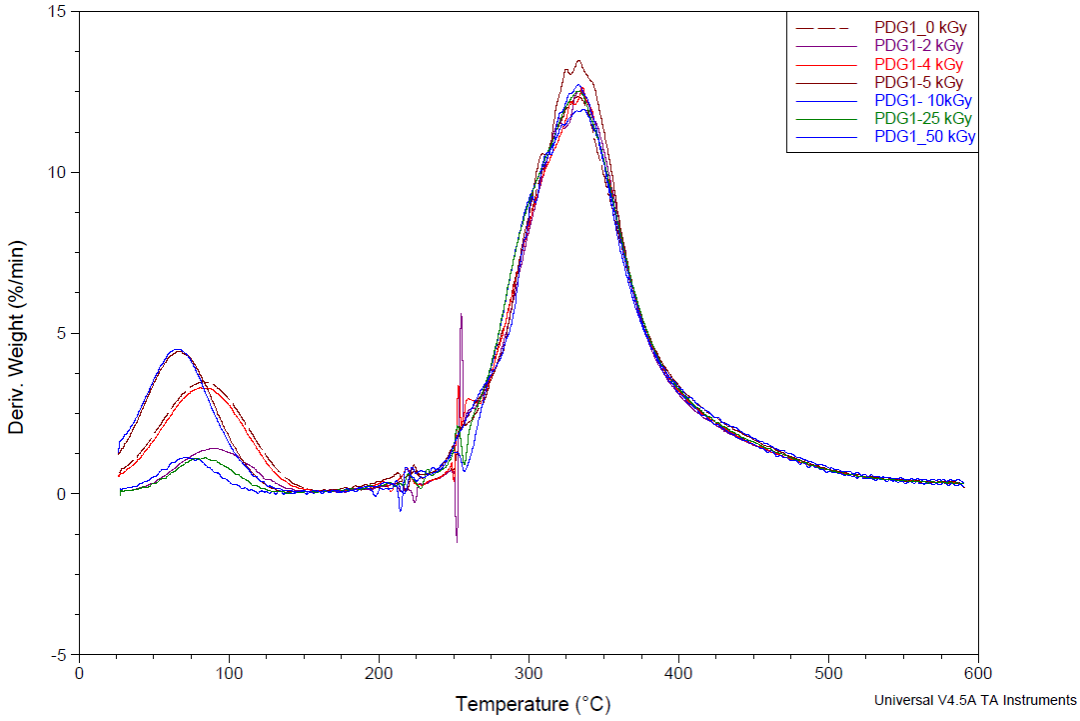

Figure 4.20. First derivative curves of TGA thermograms after irradiation by gamma at different doses for PDG1.

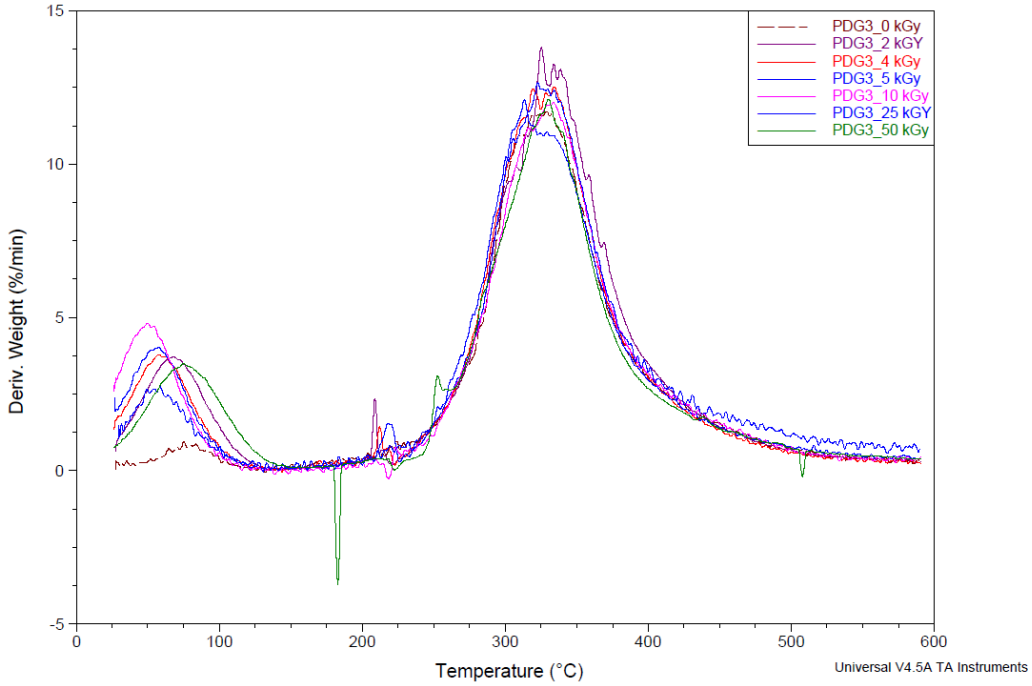

Figure 4.21. First derivative curves of TGA thermograms after irradiation by gamma at different doses for PDG3.

Table 4.6. The temperature for maximum weight loss (T_{max}) (^{0}C) for different irradiation doses .

Sample	The temperature for maximum weight loss (T_{max}) (°C)						
	Irradiation Dose (kGy)						
	0	2	4	5	10	25	50
HBG1	347.96	441.07	354.20	351.07	346.82	353.07	354.74
HL1	332.63	330.93	332.79	330.93	331.50	330.30	328.20
MBG3	339.44	441.07	339.44	364.99	344.55	346.26	383.16
MDG2	330.36	324.11	326.39	327.52	330.36	326.95	328.66
PBG1	353.07	354.77	355.34	356.48	425.17	352.50	381.46
PDG1	334.33	335.47	335.47	334.33	337.74	334.33	333.20
PDG3	329.79	325.25	335.47	314.46	333.20	321.28	329.79

SEM Analysis Results

Before gamma irradiation (0 kGy) and radiation at different doses (2, 4, 5, 10, 25 and 50 kGy), SEM results were shown in Figure 4.22 to 4.28.

HL1 sample has a kind of smooth, non-porous surface with few numbers of not deep holes in the surface. HL1 had no significant change due to sterilization with gamma radiation applied to the surface.

In HBG1 case, when the elemental structure of the material was detected, the porotic properties of the samples was observed in a very stable course. Sterilization with gamma radiation showed, no significant change in the surface and porosity of HBG1.

In the case of MBG3, a strict and rocky view of the surface was observed. After sterilization process in different doses also the same strict view was observed and any significant change was also not detected .

Dermal graft MDG2 , had a fibrillary view in a close watch and it was not porous . After irradiation, some little change was observed in the fibrillar string order shapes. However, it can not be approved that the radiation caused a significant change on it.

PBG1, like other bone grafts, had a porous surface view and the size of pores were different in size and had no a homogenous view. After sterilization also, the elemental structure of the material and the porotic properties were observed stable. So, sterilization with gamma radiation, did not cause any change in the surface and the porosity of PBG1 .

PDG1 had a combination of both globular and fibrillary view. Any significant change after radiation was not detected.

In PDG3, as other dermal graft, a complex fibrillary matrix was observed. After sterilization, especially in high irradiation doses, a slight change in the matrix shape was detected but still no significant change was observed.

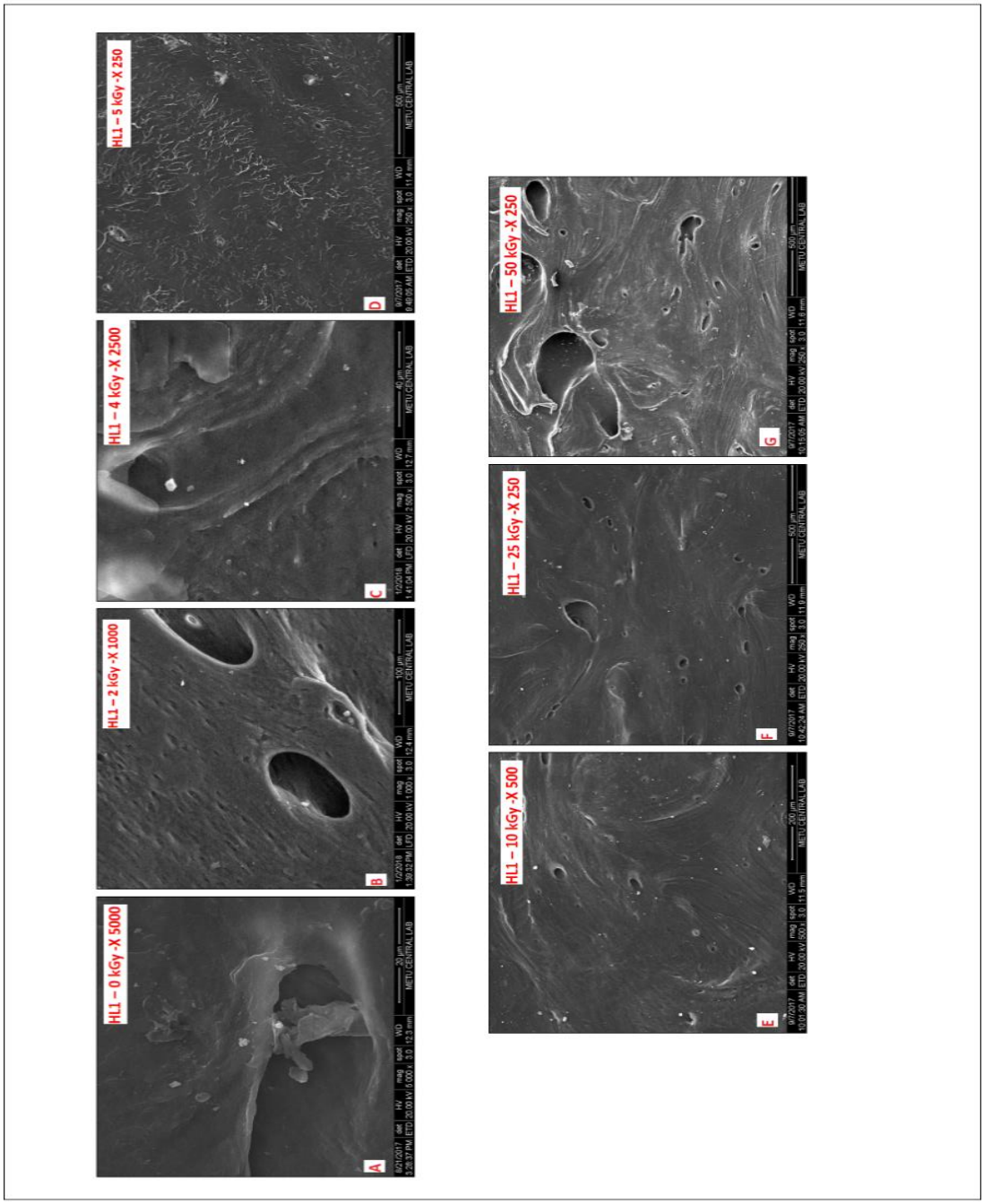

Figure 4.22. SEM images of HL1 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25, 50 kGy) in various magnifications. A)HL1-0 kGy-X5000, B)HL1-2 kGy-X1000, C)HL1-4 kGy-X2500, D)HL1-5 kGy-X250, E)HL1-10kGy-X500, F)HL1-25 kGy-X250, G)HL1-50kGy-X250.

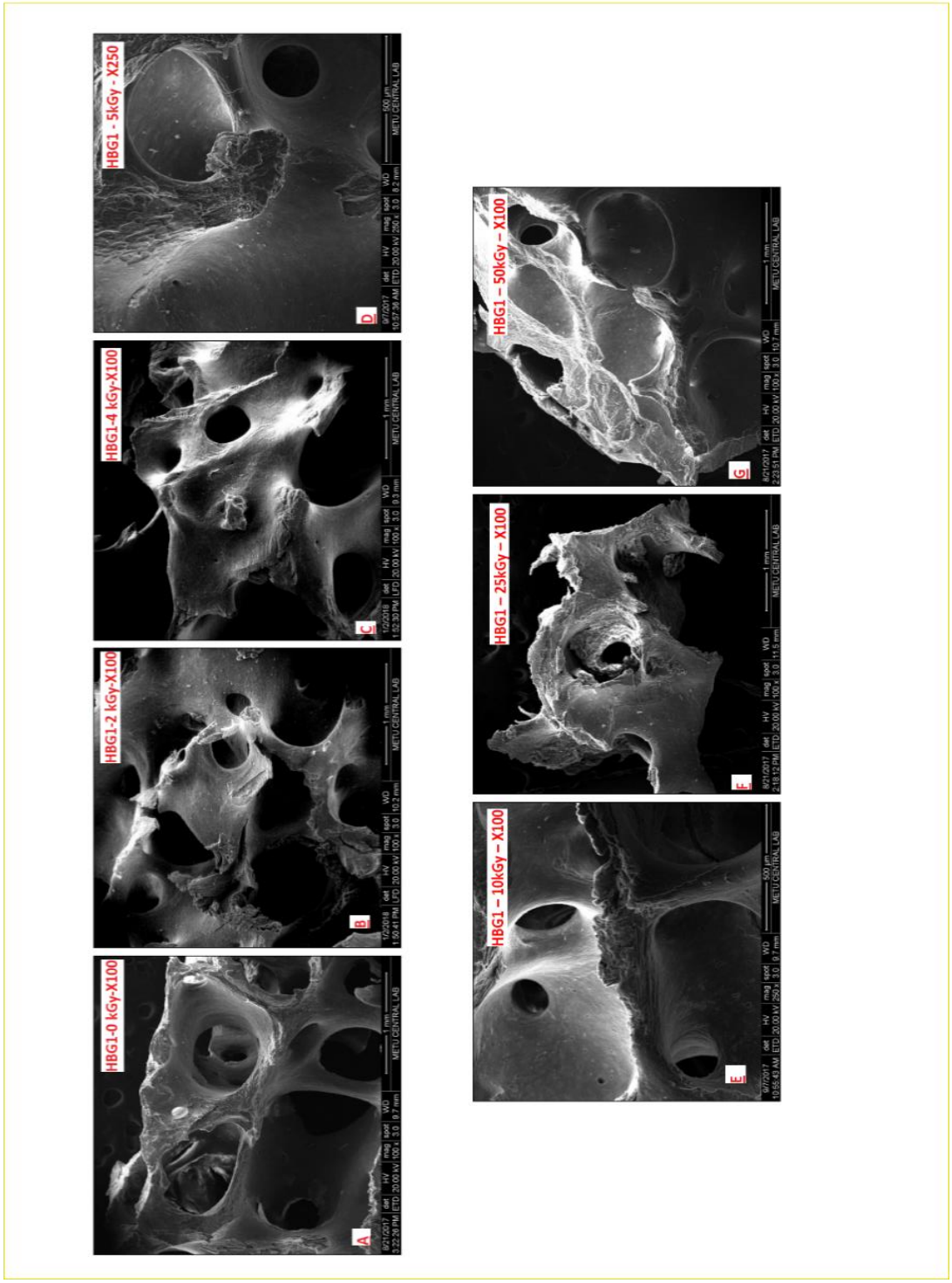

Figure 4.23. SEM images of HBG1 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25, 50 kGy) in various magnifications. A)HBG1-0 kGy-X100, B)HBG1-2 kGy-X100, C)HBG1-4 kGy-X100, D)HBG1-5 kGy-X250, E)HBG1-10kGy-X100, F)HBG1-25 kGy-X100, G)HBG1-50kGy-X100.

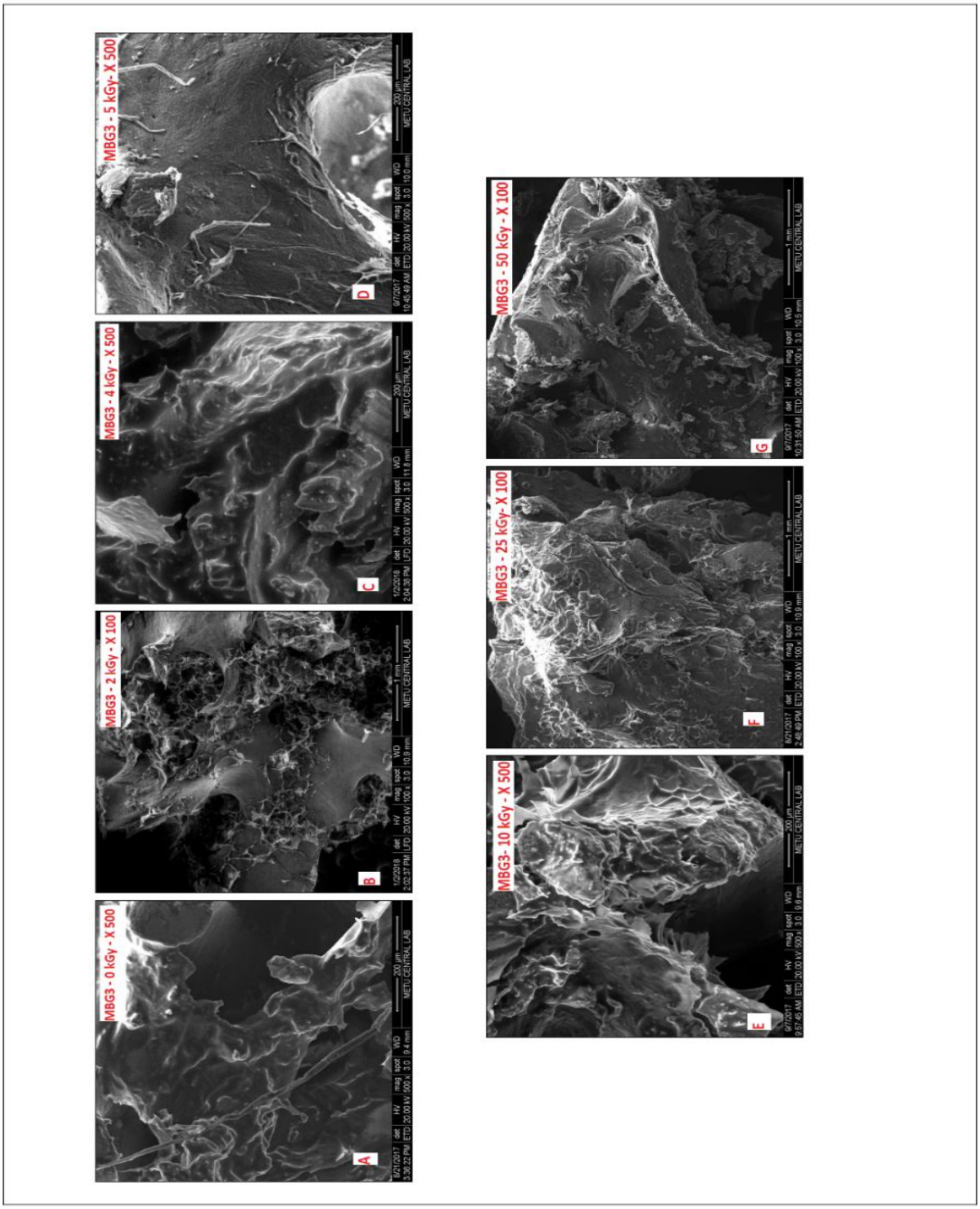

Figure 4.24. SEM images of MBG3 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25, 50 kGy) in various magnifications. A)MBG3-0 kGy-X500, B) MBG3-2 kGy-X100, C) MBG3-4 kGy-X500, D) MBG3-5 kGy-X500, E) MBG3-10kGy-X500, F) MBG3-25 kGy-X100, G) MBG3-50kGy-X100.

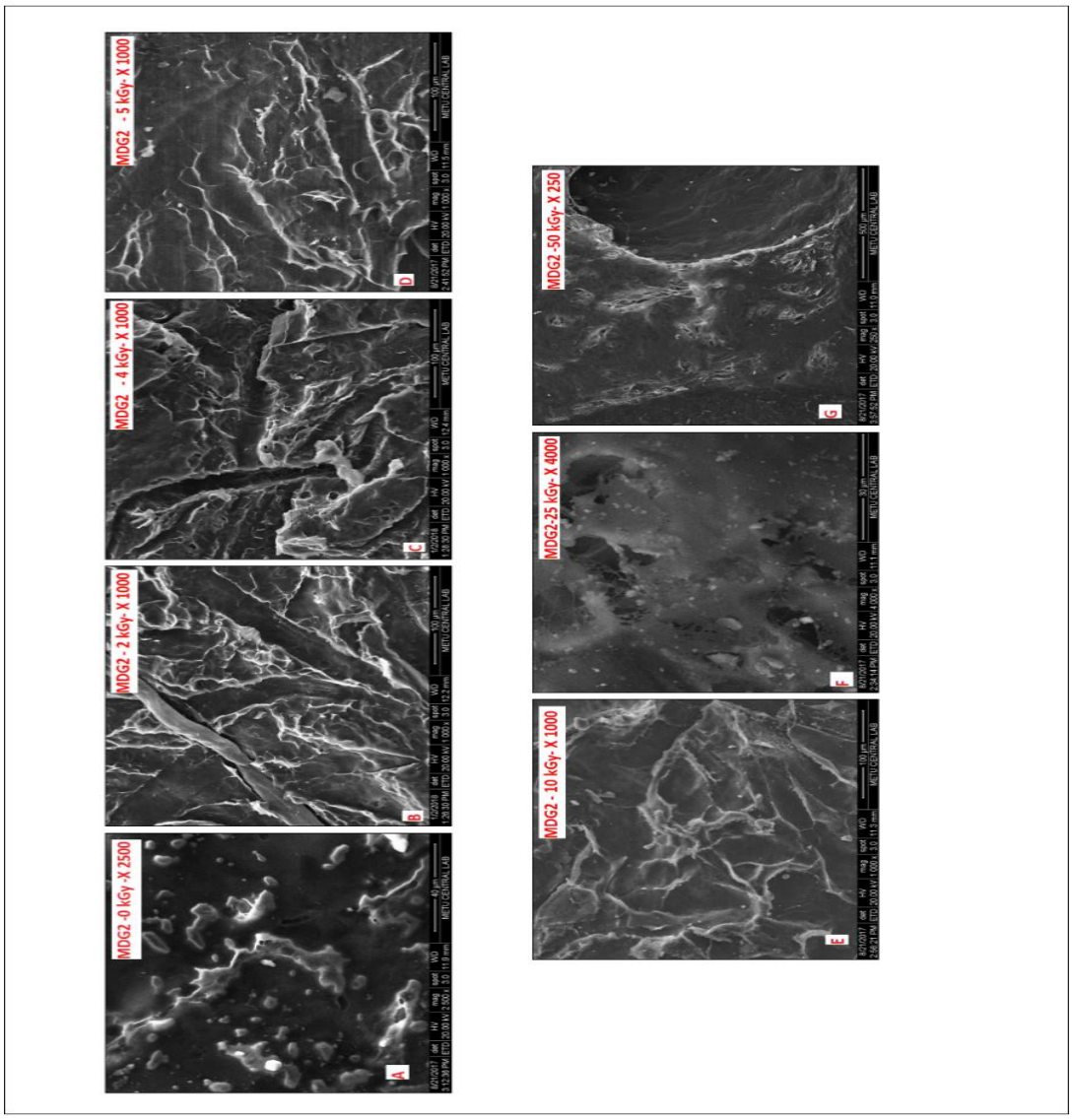

Figure 4.25. SEM images of MDG2 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25 ,50) in various magnifications .A) MDG2 - 0 kGy- X2500 B) MDG2 -2 kGy-X1000 C) MDG2 -4 kGy-X1000 D) MDG2 -5 kGy X1000 E) MDG2 -10kGy-X1000 F) MDG2 -25 kGy-X4000 G) MDG2 -50kGy-X200.

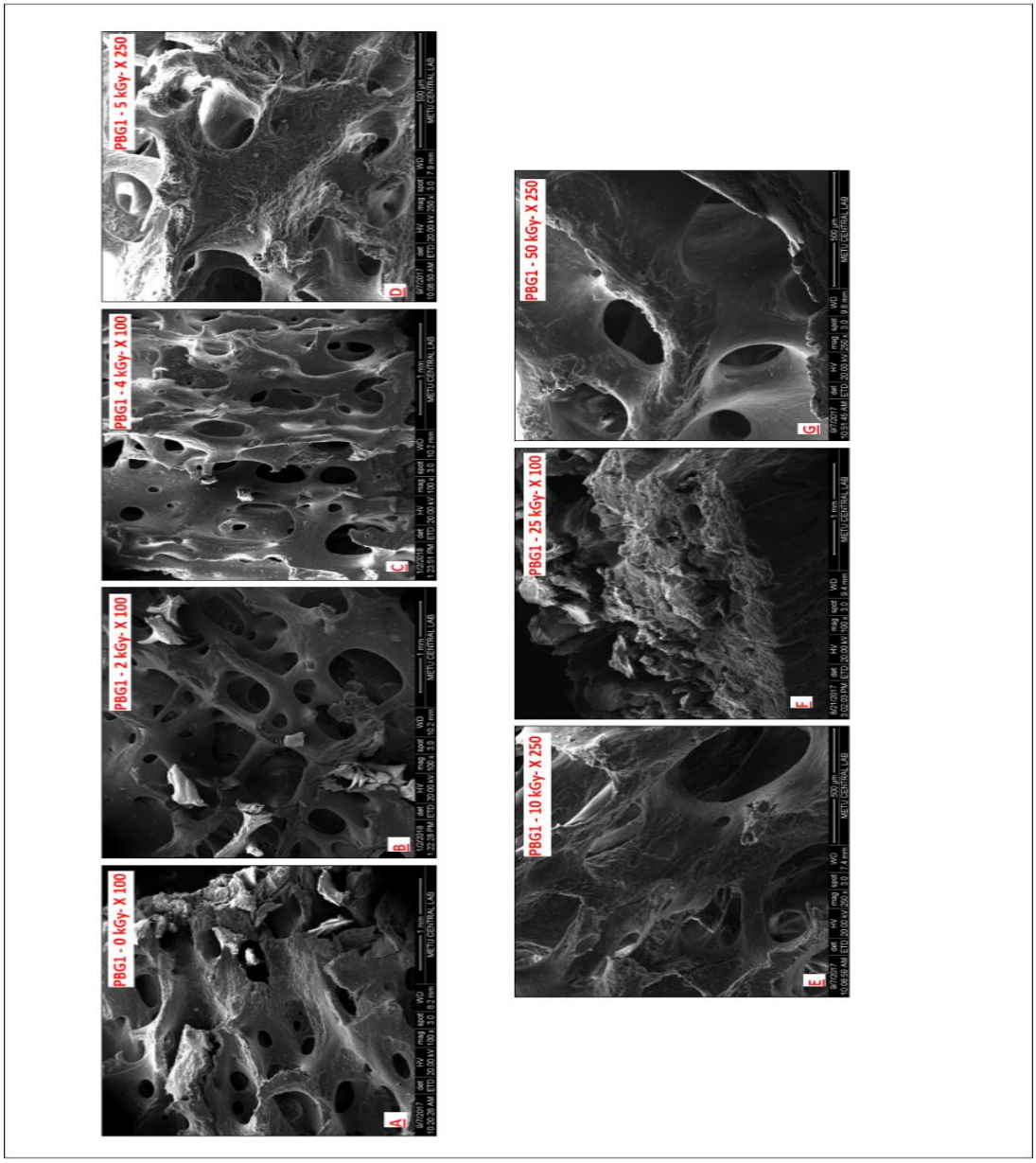

Figure 4.26. SEM images of PBG1 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25, 50 kGy) in various magnifications. A) PBG1- 0 kGy-X100, B) PBG1-2 kGy-X100, C) PBG1-4 kGy-X100, D) PBG1-5 kGy-X250, E) PBG1-10kGy-X250, F) PBG1-25 kGy-X100, G) PBG1-50kGy-X250.

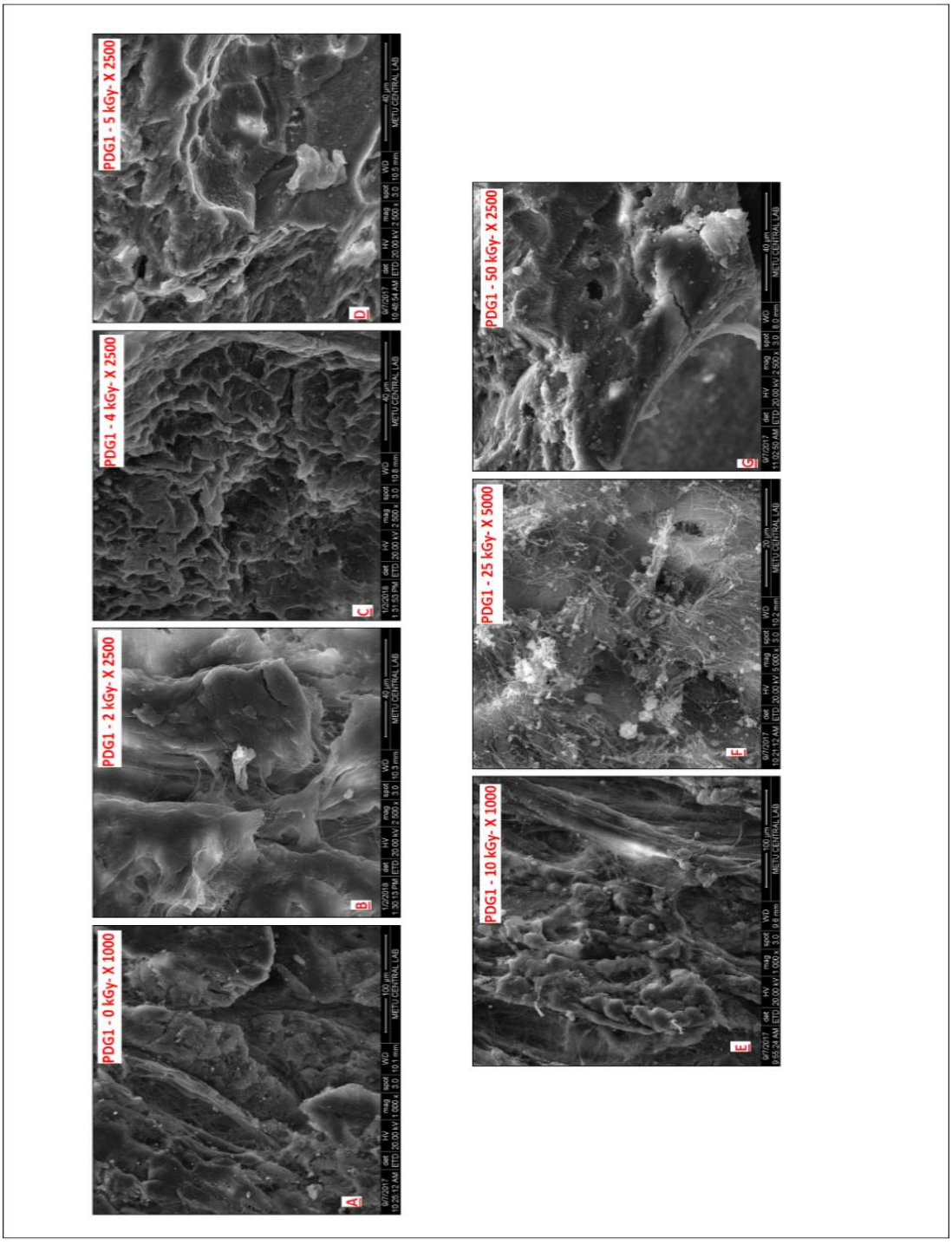

Figure 4.27. SEM images of PDG1 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25, 50 kGy) in various magnifications. A) PDG1- 0 kGy-X1000, B) PDG1-2 kGy-X2500, C) PDG1-4 kGy-X2500, D) PDG1-5 kGy-X2500, E) PDG1-10kGy-X1000, F) PG1-25 kGy-X5000, G) PDG1-50kGy-X2500.

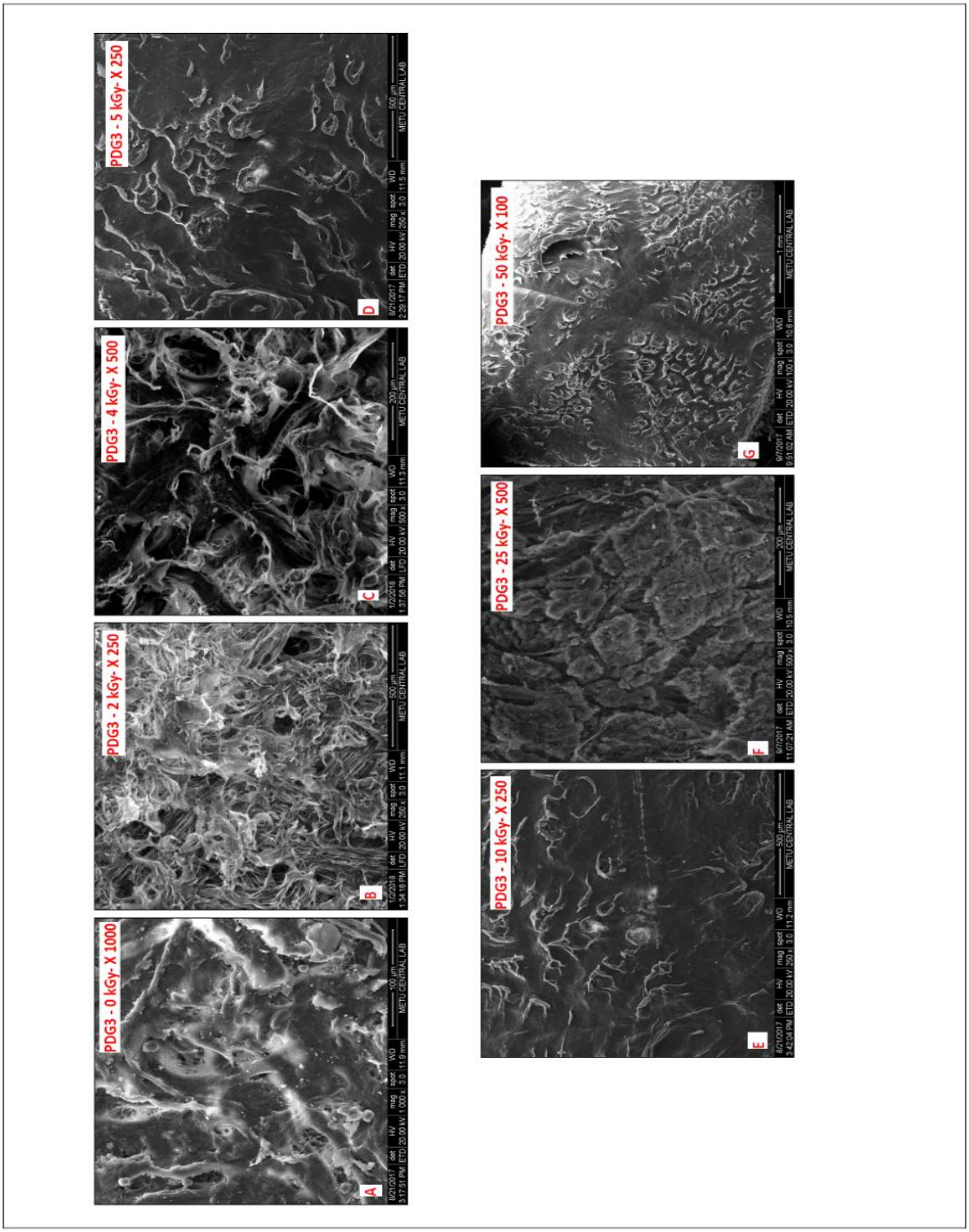

Figure 4.28. SEM images of PDG3 coded grafts unirradiated and irradiated at different doses (2, 4, 5, 10, 25, 50 kGy) in various magnifications. A) PDG3-0 kGy-X1000, B) PDG3-2 kGy-X250, C) PDG3-4 kGy-X500, D) PDG3-5 kGy-X250, E) PDG3-10kGy-X250, F) PDG3-25 kGy-X500, G) PDG3-50kGy-X100.

SAXS Analysis Results

SAXS analysis were performed on all grafts as explained in Section 3.5.1. The graft samples were nanostructurally affected by irradiation. The most possible three dimentional (3D) morphologies and the shapes of the nanostructured aggregations were obtained by using SAXS analysis as described in Section 3.5.1. Besides of the morphologies, distributions of the obtained shaped nanoglobules were also determined by using pair distance distribution (PDDs) function. Compact and densed 3D morphologies are the evidence of stable nanoglobules. Based on the nanoglobules formation results from each graft at all irradiation doses, as the irradiation dose increasing, their densities also increased. For all grafts a firm, dense and desirable nanoglobules were observed in nonostractural results. So, it can be concluded that irradiation did not show adverse and negative effects on the grafts' nanostructure, on the contrary it had a positive effect. Based on the results, HL1 and PDG3 coded grafts were the best groups for the stable structures in nanoscale. While HL1 group has spherical like globules, PDG3 indicate cylindrical/ellipsoid nanoaggregations. PDG1 and HBG1 had the worst nanoglobules. Because, the radiation affected easily the morphologies and caused very different structures. But, 50kGy irradiated HBG1 has small and well shaped spherical nanoglobules. PBG1 sample irradiated at 5 kGy was valuable with rod like nanostructured graft sample. Globular 3D nano-formations of all grafts at different radiation doses and unirradiated ones were given at Figure 4.29 for comparison. Moreover, as it was mentioned before, Double-distant distribution function was considered too, for all grafts at different irradiation doses and unirradiated sample (Figure 4.30). Detailed double-distant distribution function of all grafts were given in Figure 4.31 to Figure 4.37.

Moreover, Radius of gyration of all grafts has been calculated based on the Equation 3.2. after irradiation for different doses and for every grafts the desirable measurement has been given in table 4.7.

Table 4.7. Optimum Radius of gyration calculated for all grafts.

Codes	Irradiation dose	Rg (Radius of gyration)
HBG1	**50**	**18.4 nm**
HL1	**50**	**58.9 nm**
MBG3	**50**	**49.8 nm**
MDG2	**10**	**41.9 nm**
PBG1	**10**	**Cylinder diameter = 3.8 nm, Length = 16.6nm**
PDG1	**5**	**34.0 nm**
PDG3	**25**	**50.8 nm**

Figure 4.29. Global 3-D nano formations of unirradiated and gamma irradiated HBG1, HL1, MBG3, MDG2, PBG1, PDG1 and PDG3 samples analysed by SAXS.

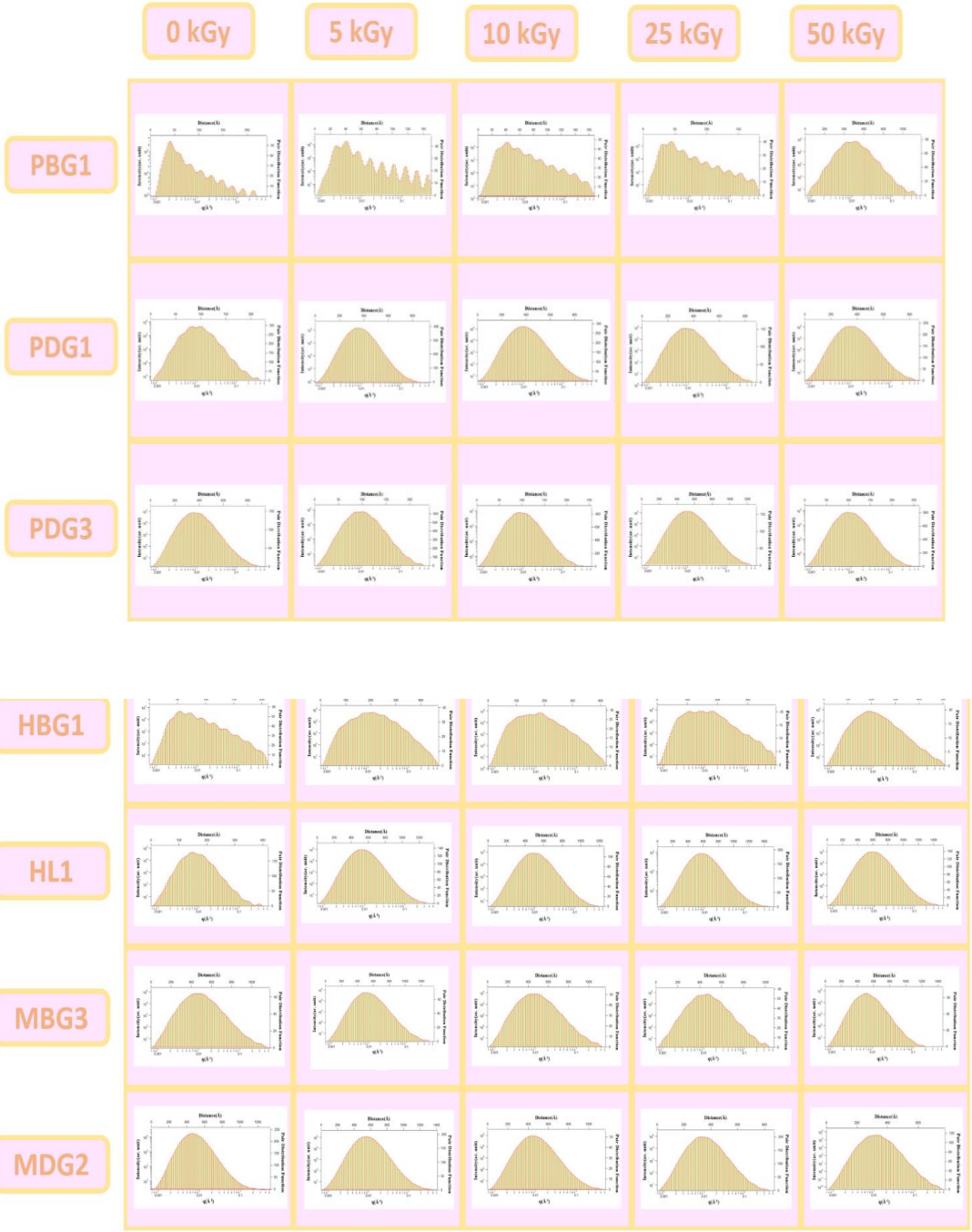

Figure 4.30. Double distant distribution function with SAXS analysis of 5, 10, 25, 50 kGy of gamma irradiated and unirradiated of HBG1, HL1, MBG3, MDG2 , PBG1, PDG1, PDG3 coded materials.

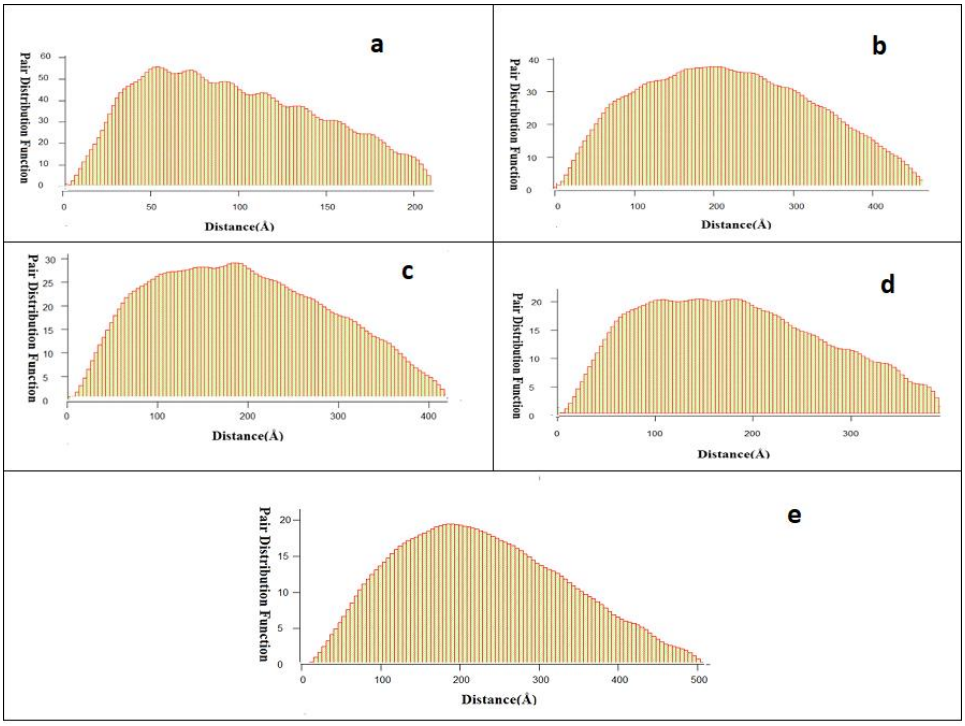

Figure 4.31. Double distant distribution function with SAXS analysis for HBG1 coded graft.

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e) 50 kGy

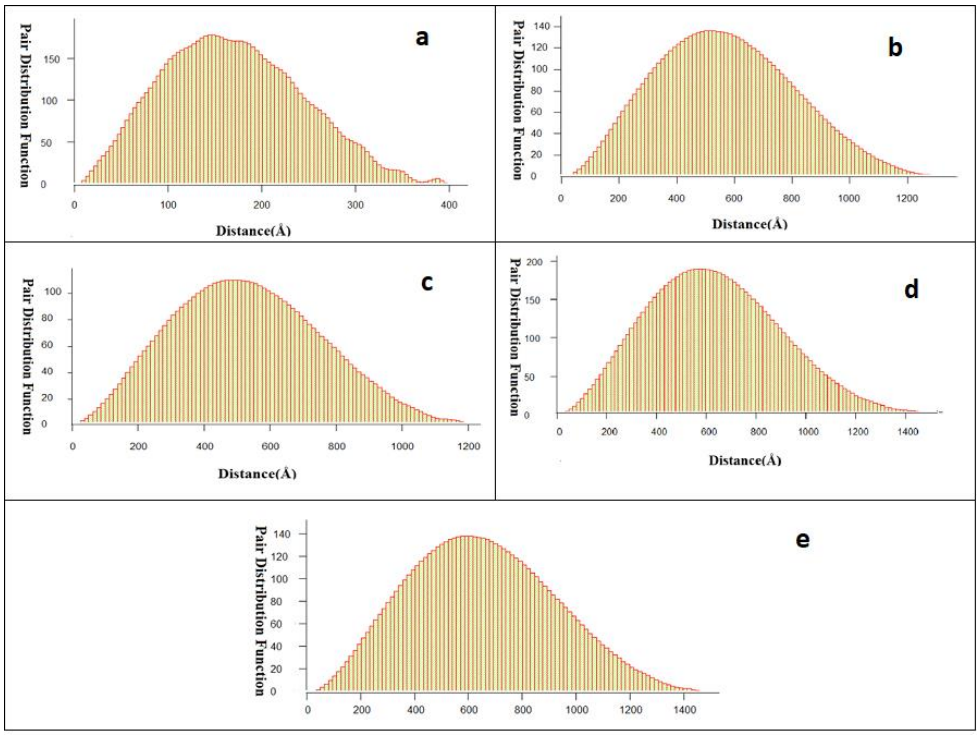

Figure 4.32. Double distant distribution function with SAXS analysis for HL1 coded graft.

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e) 50 kGy

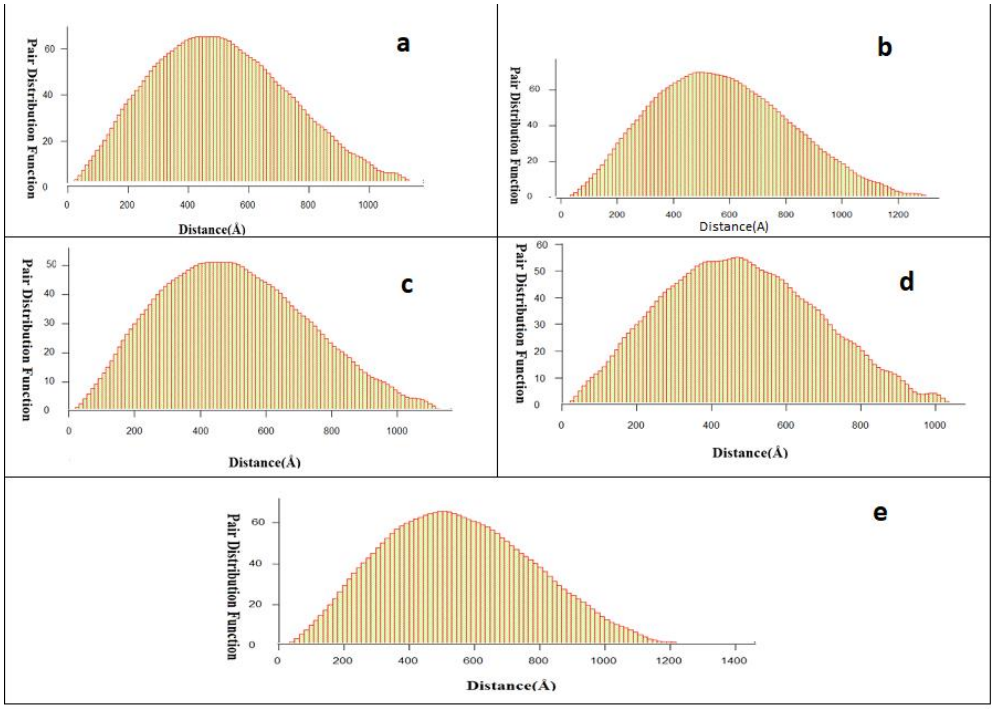

Figure 4.33. Double distant distribution function with SAXS analysis for MBG3 coded graft .

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e)50 kGy

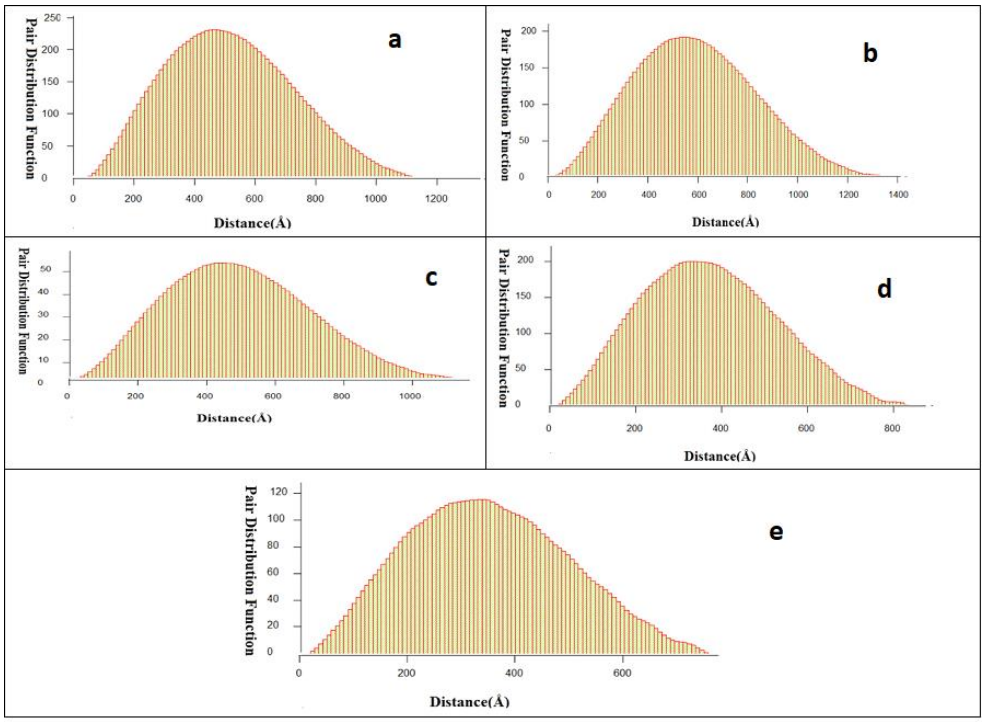

Figure 4.34. Double distant distribution function with SAXS analysis for MDG2 coded graft.

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e) 50 kGy

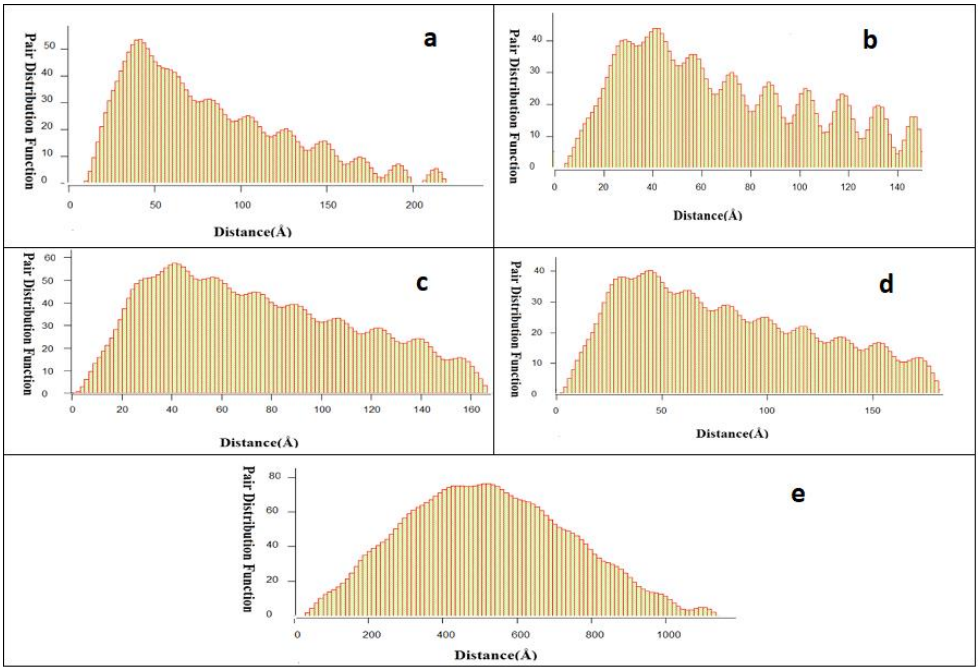

Figure 4.35. Double distant distribution function with SAXS analysis for PBG1 coded graft.

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e) 50 kGy

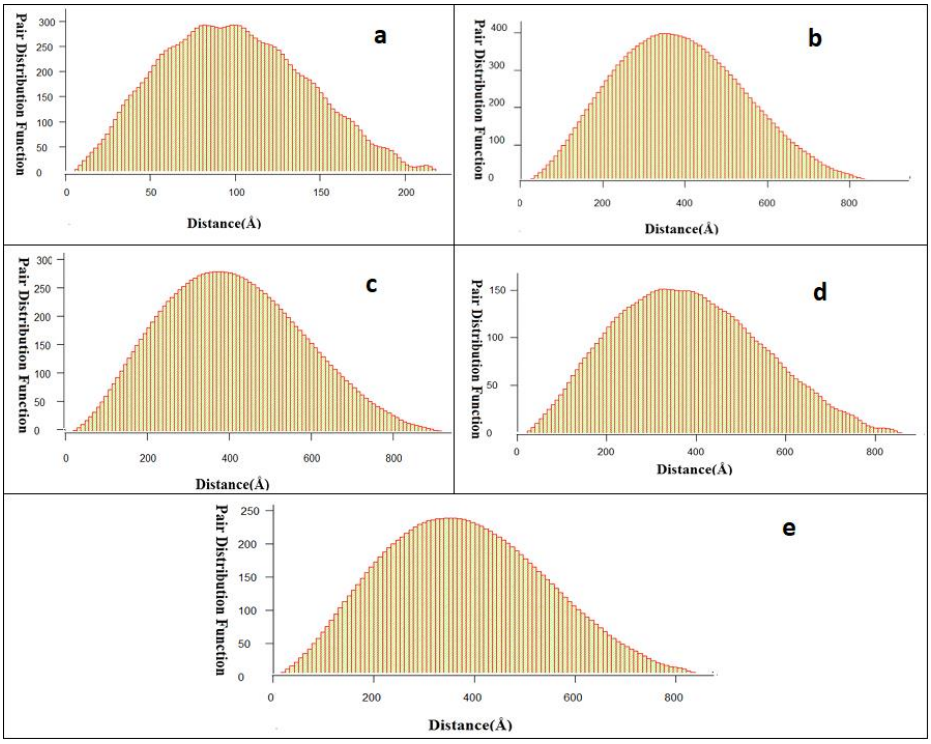

Figure 4.36. Double distant distribution function with SAXS analysis for PDG1 coded graft.

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e) 50 kGy

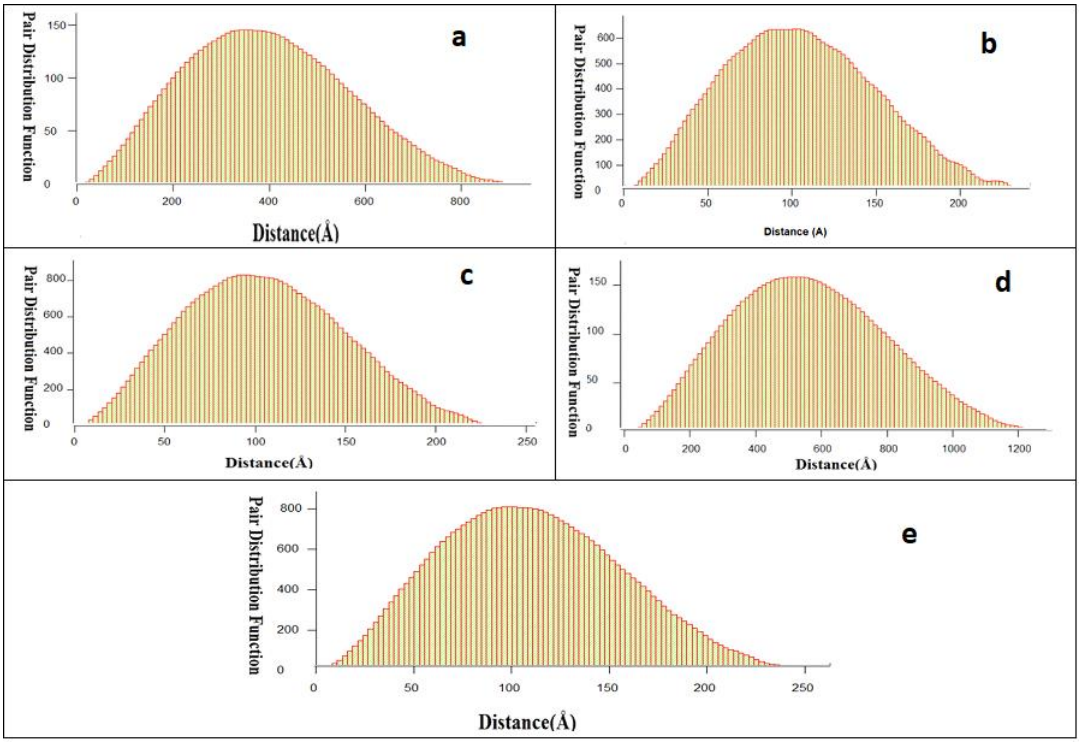

Figure 4.37. Double distant distribution function with SAXS analysis for PDG3 coded graft.

a) Unirradiated, b) 5 kGy, c) 10 kGy, d) 25 kGy, e) 50 kGy.

ESR Analysis Results

Within the scope of the ESR analysis results, all grafts irradiated at different doses (2, 4, 5, 10, 25 and 50 kGy) were evaluated for their :

i. ESR spectra,

ii. Dose-response curves,

ESR Spectra of Unirradiated and Gamma Irradiated Grafts

ESR analysis was performed as explained in Section 3.5.1. ESR spectra results of all grafts unirradiated and gamma irradiated by 5, 10, 25, 50 kGy doses given in Figure 4.38. to 4.44. ESR spectral parameters of investigated grafts were mentioned in Table 4.8.

Table 4.8. ESR spectral parameters of investigated samples.

SAMPLE TYPE	SPECTROSCOPIC SPLITTING FACTOR g_{mid}-value	PEAK-to-PEAK LINE WIDTH ΔH_{pp} (mT)	MAGNETIC FIELD REGION of SPECTRA (mT)
HBG1	2.0020	0.37	4.5
HL1	2.0032	1.10	9.2
MBG3	2.0020	0.38	4.6
MDG2	2.0059	1.22	3.4
PBG1	2.0022	0.35	5.0
PDG1	2.0032	1.10	6.0
PDG3	2.0055	1.10	4.0

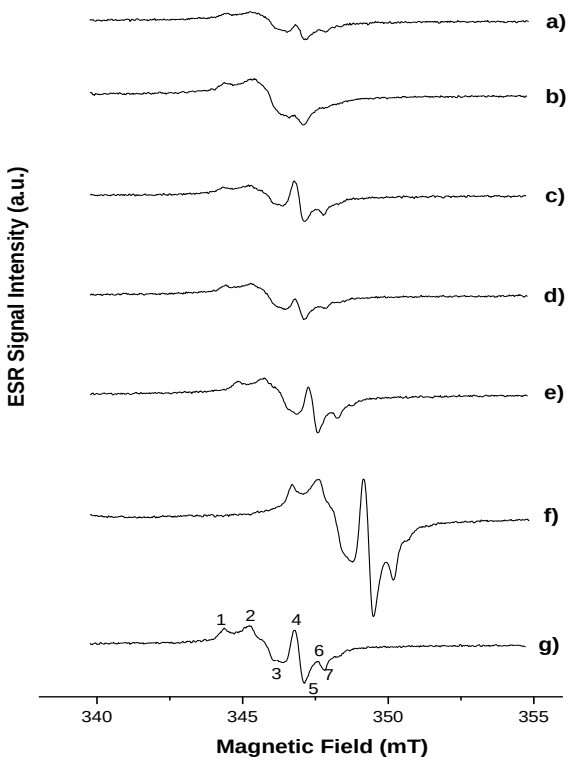

Figure 4.38. ESR spectra of gamma irradiated HBG1 at room temperature.

a) Unirradiated, b) 2 kGy, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

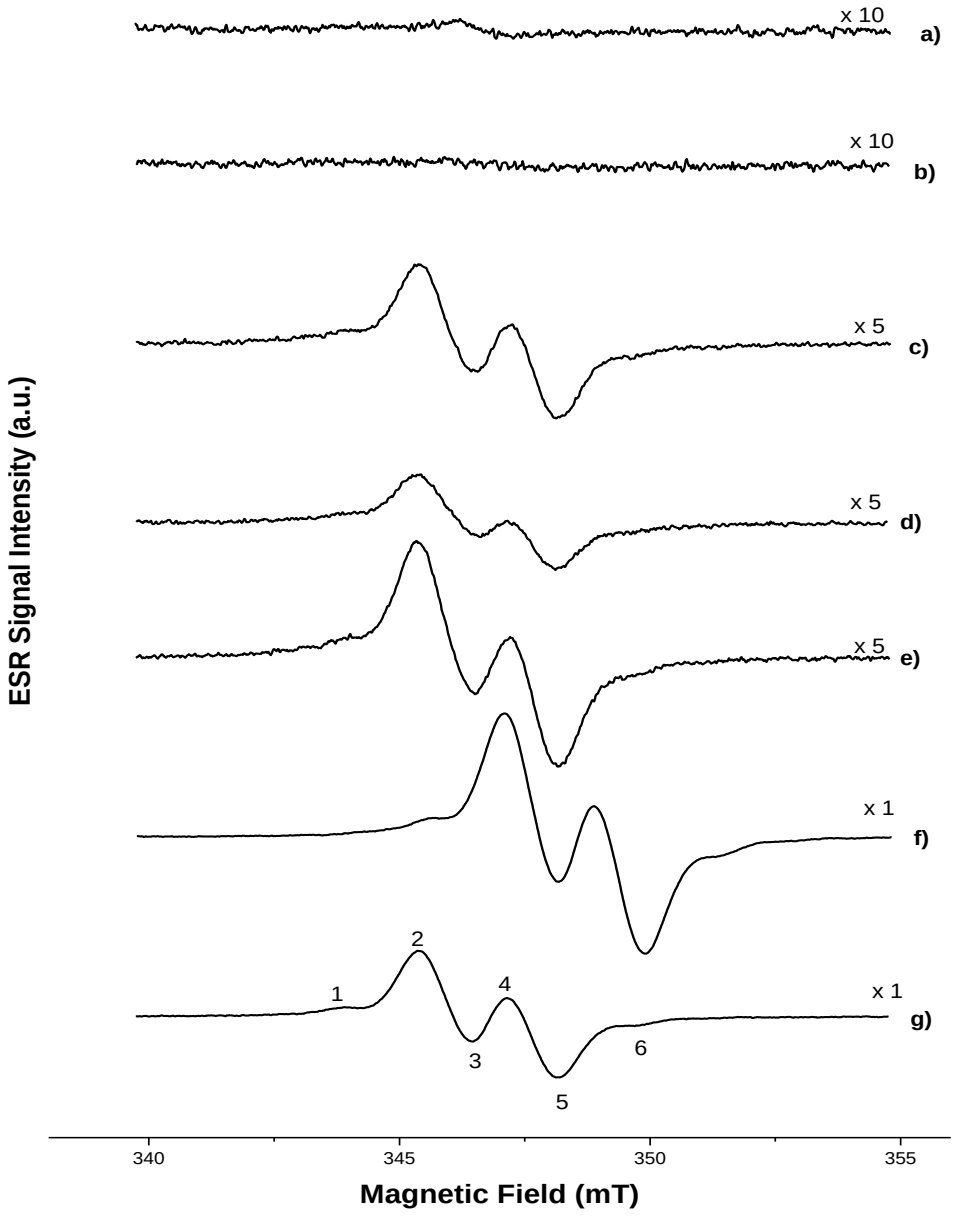

Figure 4.39. ESR spectra of gamma irradiated HL1 at room temperature.

a) Unirradiated, b) 2 kGy, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

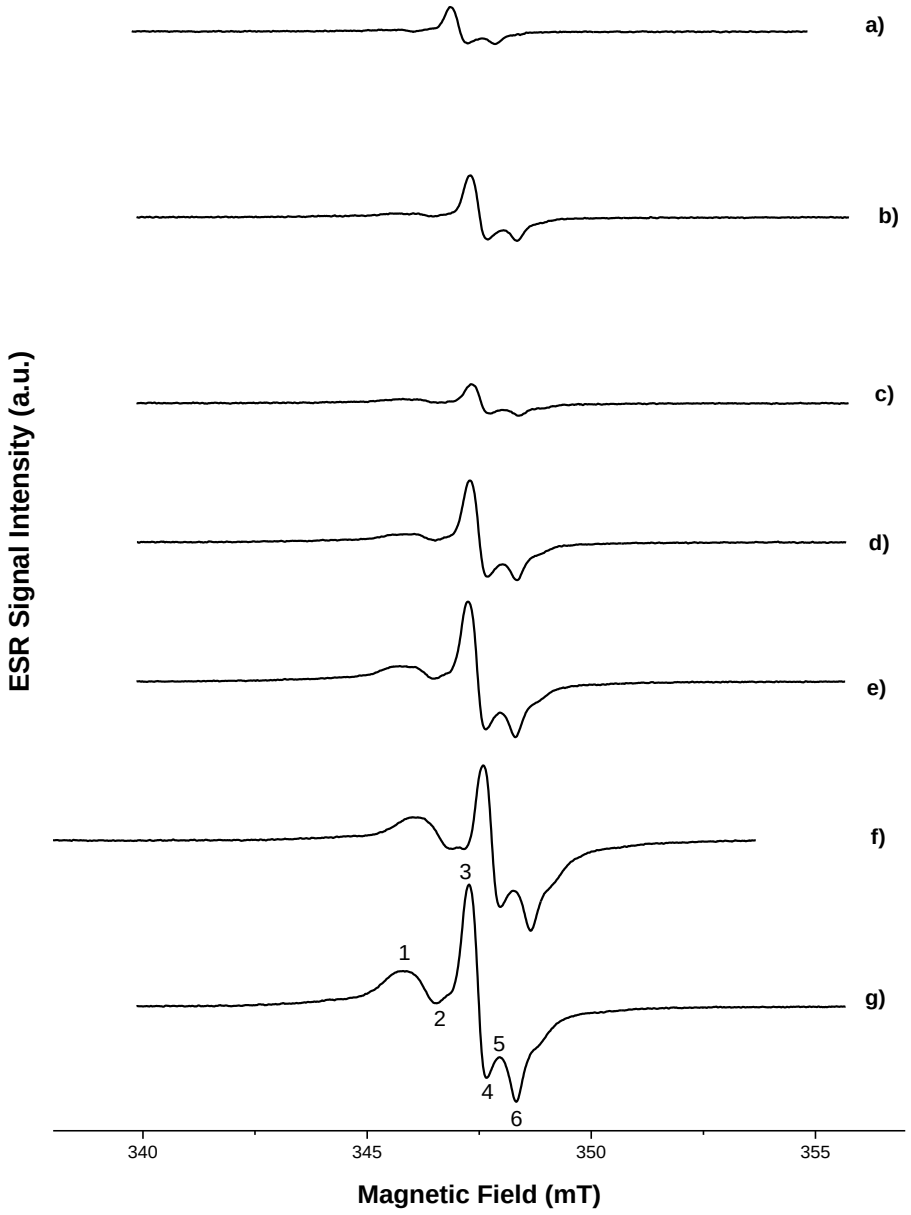

Figure 4.40. ESR spectra of gamma irradiated MBG3 at room temperature.

a) Unirradiated, b) 2 kGy, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

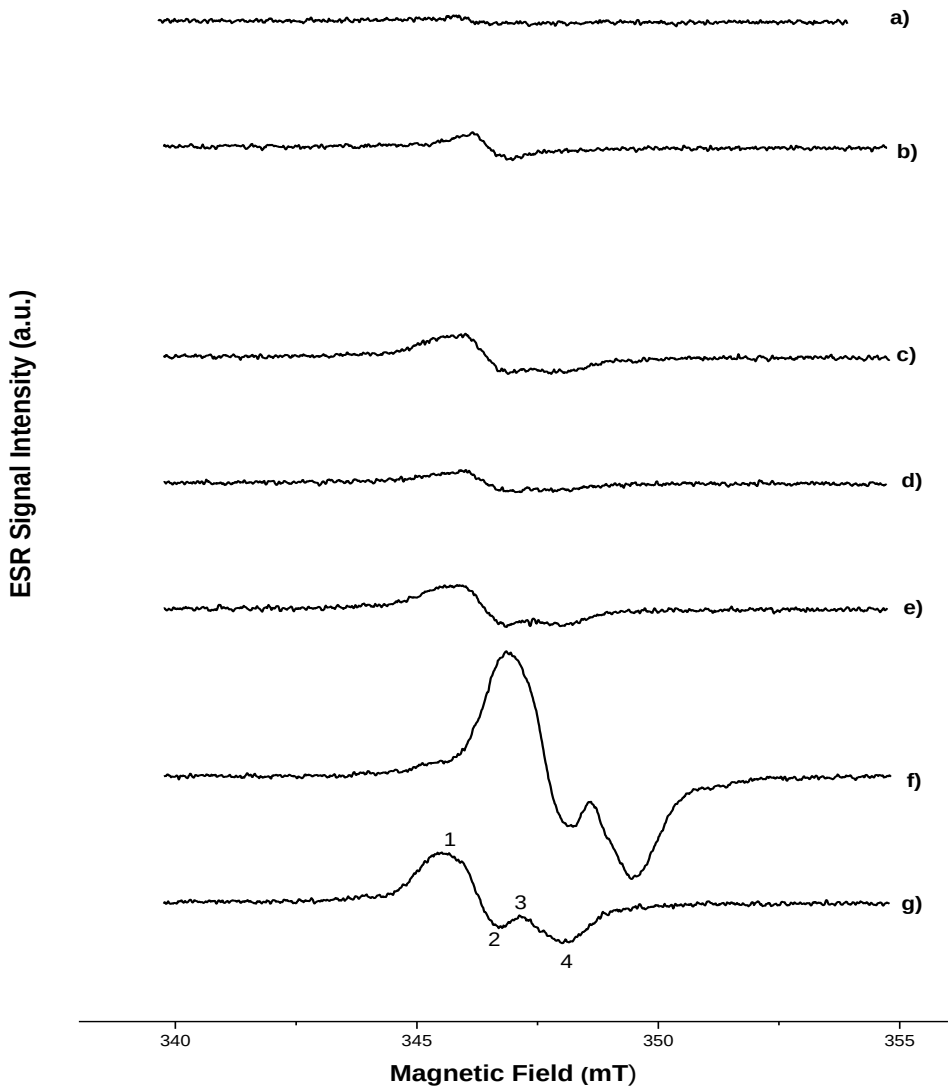

Figure 4.41. ESR spectra of gamma irradiated MDG2 at room temperature.

a) Unirradiated, b) 2 kGy, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

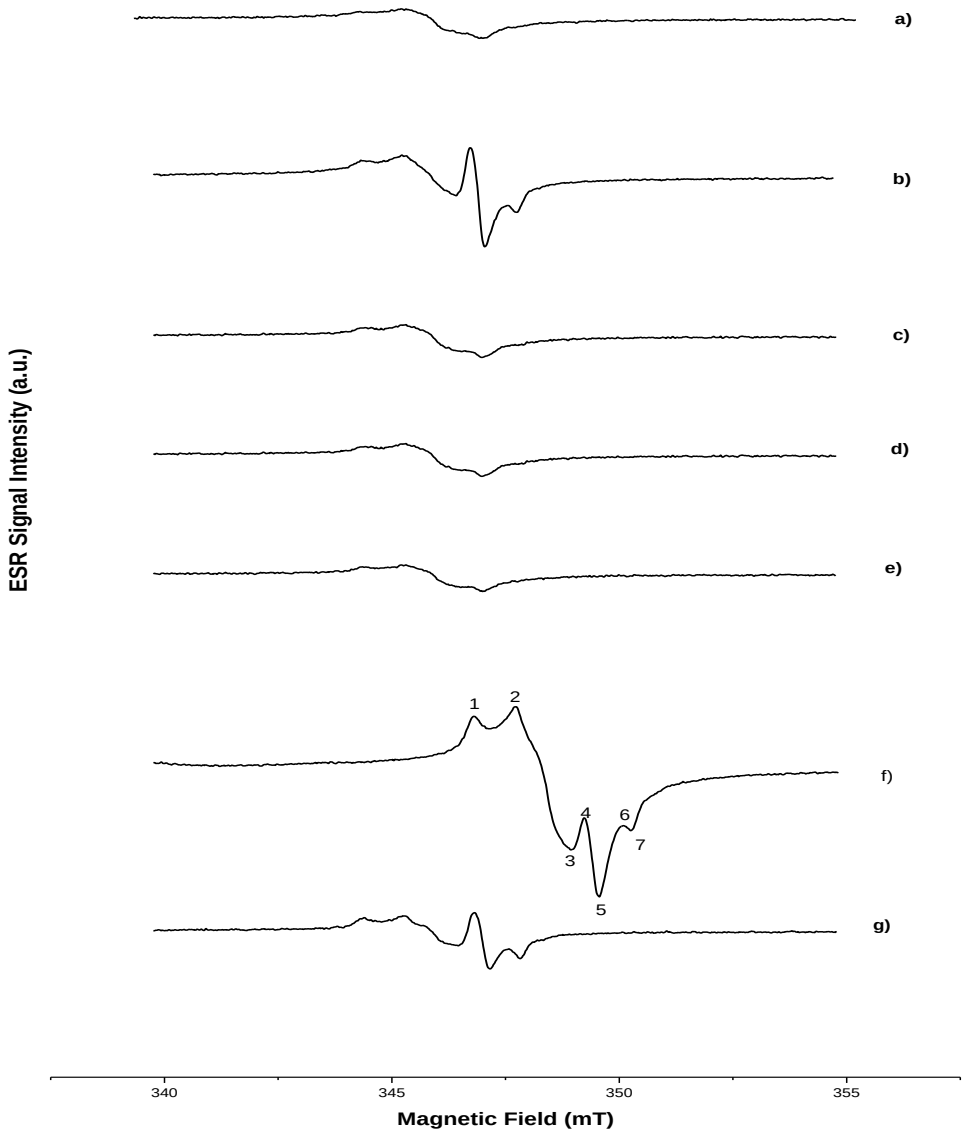

Figure 4.42. ESR spectra of gamma irradiated PBG1 at room temperature.

a) Unirradiated, b) 2 kGy, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

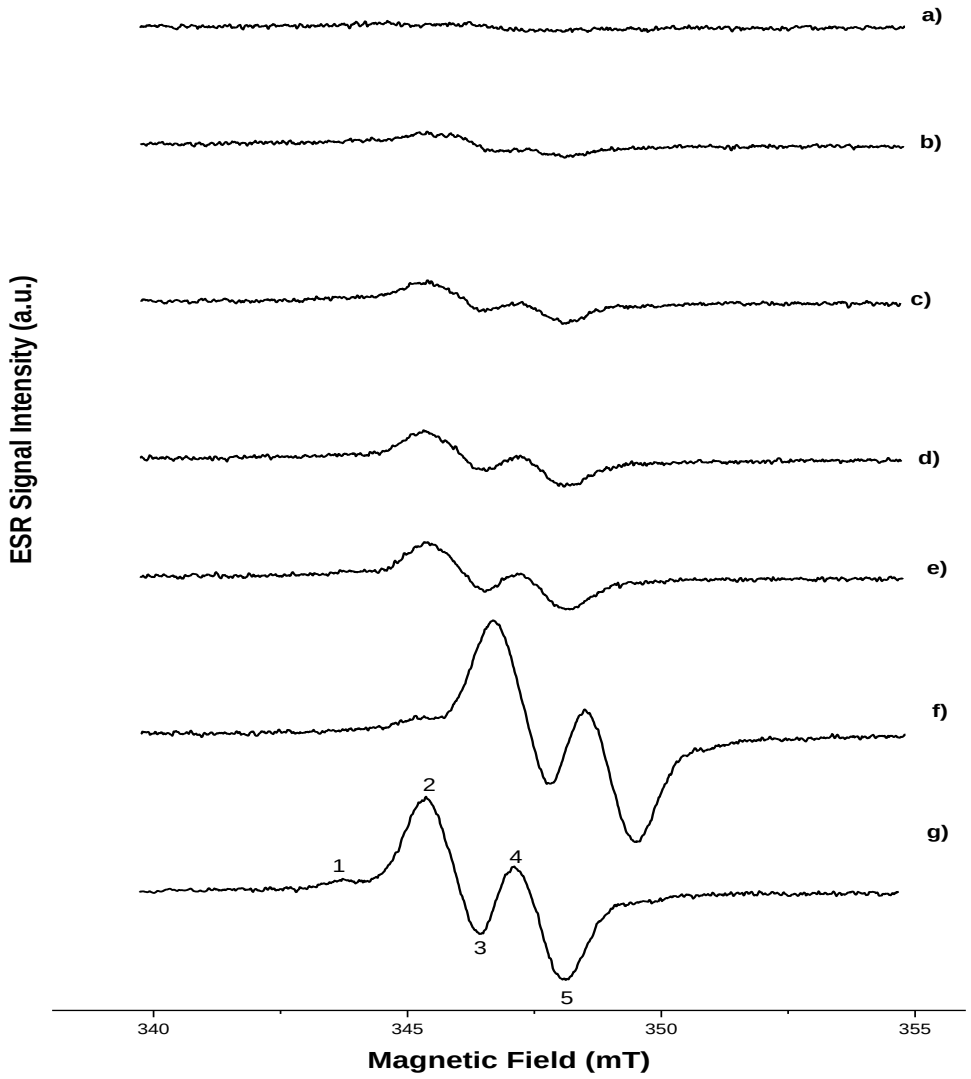

Figure4.43. ESR spectra of gamma irradiated PDG1 at room temperature.

a) Unirradiated, b) 2 kGy, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

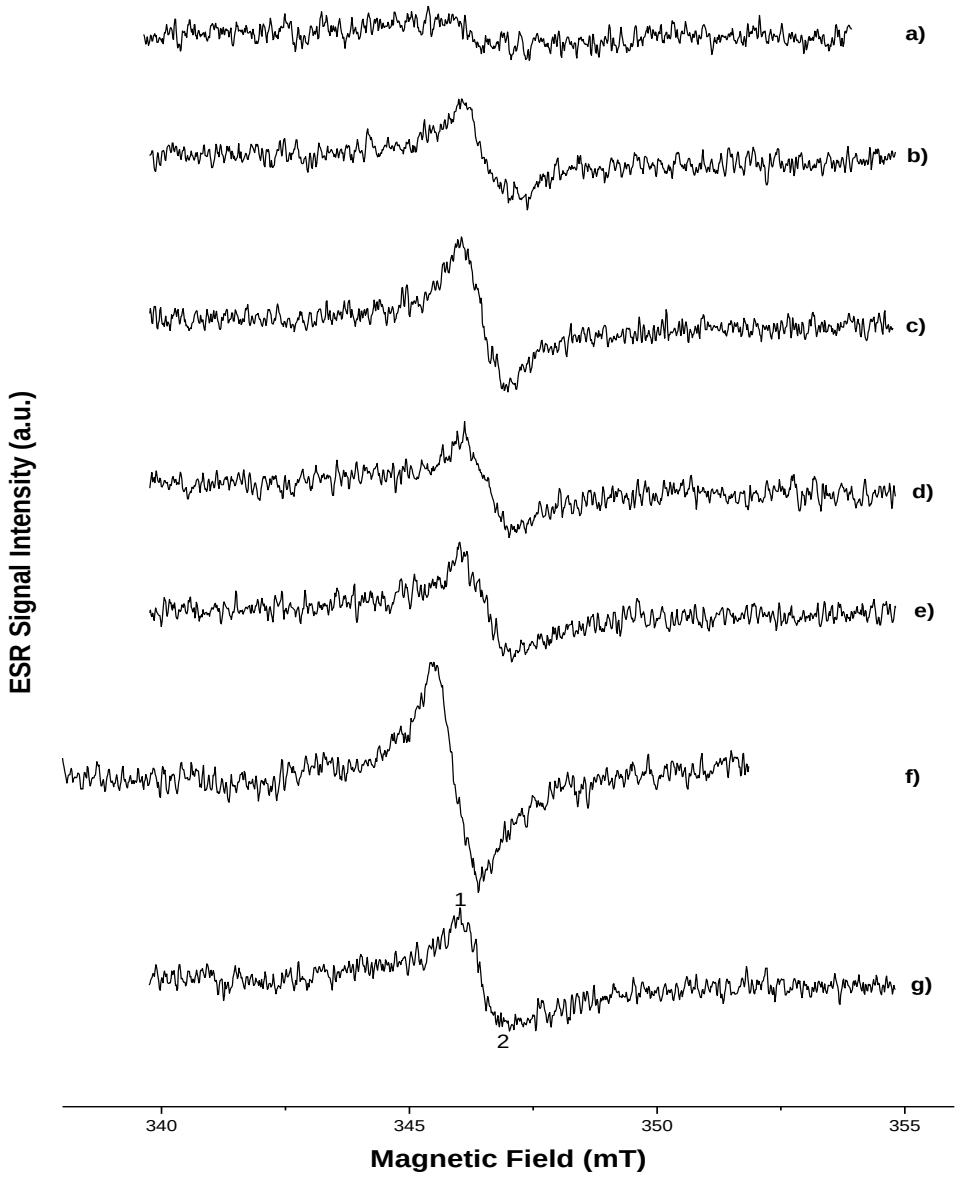

Figure 4.44. ESR spectra of gamma irradiated PDG3 at room temperature.
a) Unirradiated, b) 2 kG, c) 4 kGy, d) 5 kGy, e) 10 kGy, f) 25 kGy, g) 50 kGy

Dose-Response Results

Based on the results of ESR analyses, unirradiated HBG1 samples showed ESR spectra with low intensity resonance lines. ESR intensities of the resonance peaks of HBG1 increased in increasing rate of absorbed dose but, the spectrum pattern remained unchanged even at high absorbed doses (Figure 4.45). Unirradiated HL1 sample indicated ESR spectrum that can hardly be distinguished from noise. ESR

intensities of the resonance peaks of HL1 increased with the absorbed gamma radiation dose but the spectrum pattern of the sample remained unchanged for different absorbed doses (Figure 4.46). Unirradiated MBG3 sample indicated ESR spectrum with low intensity resonance lines. ESR intensities of the resonance peaks of MBG3 increased by increasing the irradiation dose values, but the spectrum pattern did not change during the dose-response studies. MBG3 sample was observed to be a relatively radio-sensitive material (Figure 4.47). Unirradiated MDG2 sample indicated ESR spectrum with very low intensity resonance lines. ESR intensities of the resonance peaks of MDG2 increased by increasing rate of absorbed dose where the spectrum pattern remained the same for even high absorbed doses (Figure 4.48). Unirradiated PBG1 sample showed an ESR spectrum with relatively low intensity. ESR intensities of the resonance peaks of PBG1 increased by the increasing value of irradiation dose where the spectrum pattern remained the same (Figure 4.49). Unirradiated PDG1 sample indicated ESR spectrum with very low intensity resonance lines and hardly recognizable. ESR intensities of the resonance peaks of PDG1 increased by increasing rate of absorbed dose but, the spectrum patterns remained the same at different absorbed doses (Figure 4.50). Unirradiated PDG3 sample showed an ESR spectrum which can be hardly distinguised from noise. ESR intensities of the resonance peaks of PDG3 increased by increasing rate of absorbed dose while spectra patterns remained the same. By referring the dose-response curves of the samples, radiation sensitivity of PDG3 sample was not found high, thus PDG3 can be considered to be a relatively radio-resistant material (Figure 4.51).

Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results for all grafts were also given in Table 4.9 to 4.15.

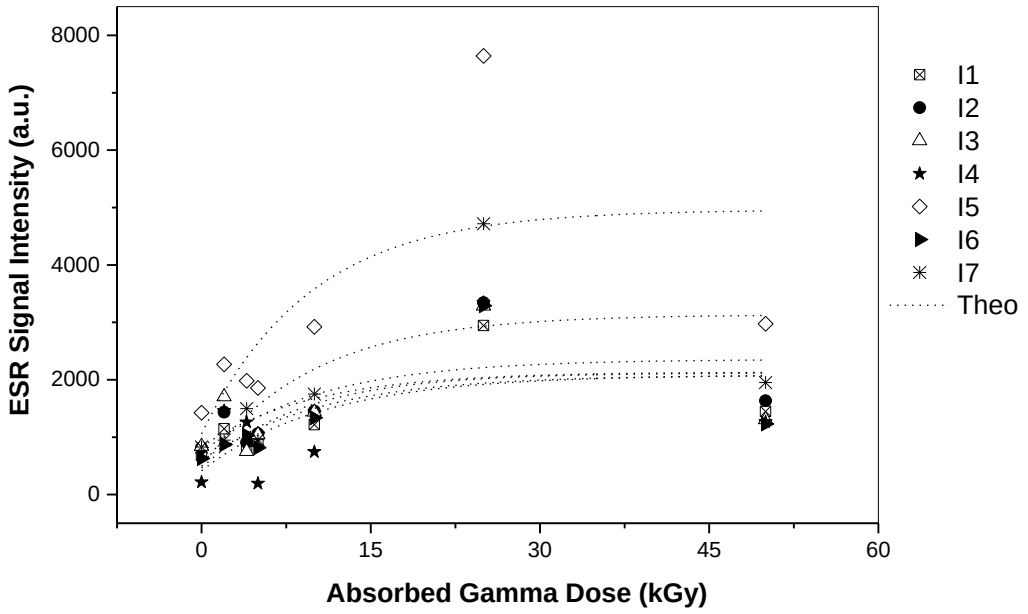

Figure 4.45. Variations of peak heights of HBG1 with absorbed radiation dose. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇); I_6 (▶); I_7 (✳). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.9. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results of HBG1.

Intensity (a.u.)	a	b	c
I_1	2504.06	3627.10	0.89
I_2	16899.18	19133.22	0.93
I_3	6102.05	9056.71	0.90
I_4	4442.06	6585.41	0.91
I_5	15686.88	17787.87	0.92

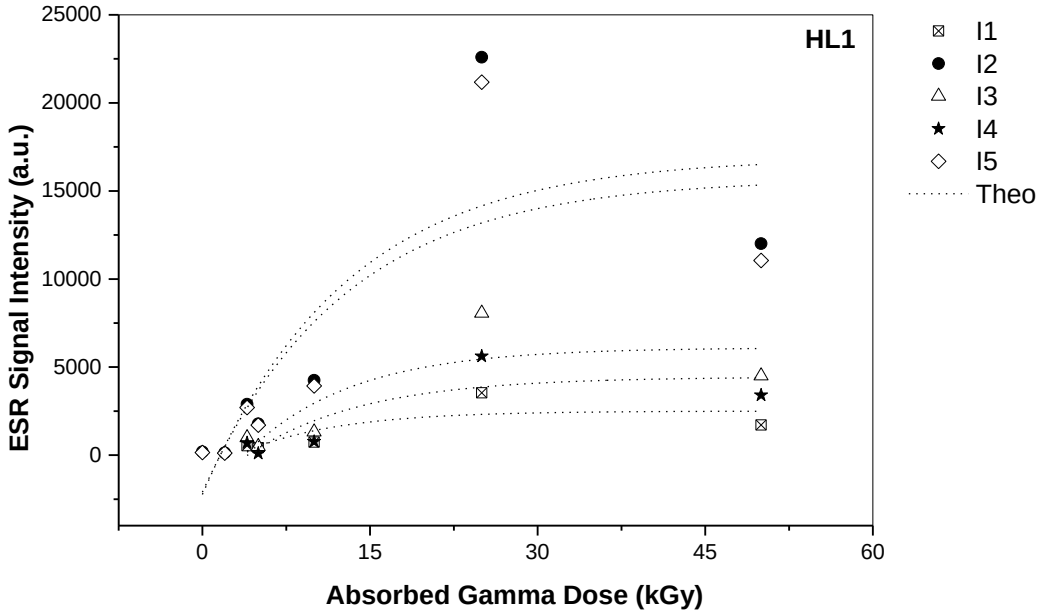

Figure 4.46. Variations of peak heights of HL1 with absorbed radiation dose. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.10. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response resultsof HL1.

Intensity (a.u.)	a	b	c
I_1	2079.93	1496.55	0.91
I_2	2356.56	1625.56	0.91
I_3	2136.31	1305.74	0.90
I_4	2085.61	1669.87	0.91
I_5	4961.78	3904.95	0.90
I_6	2119.73	1657.75	0.89
I_7	3135.86	2042.92	0.90

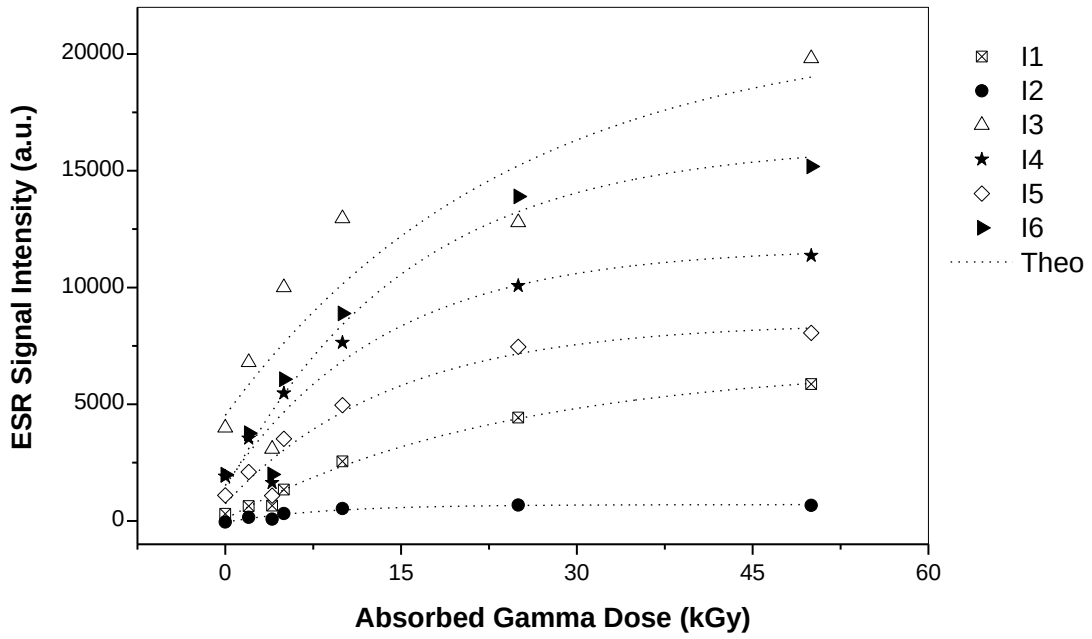

Figure 4.47. Variations of peak heights of MBG3 with absorbed radiation dose. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇); I_6 (▶). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.11. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results of MBG3.

Intensity (a.u.)	a	b	c
I_1	6705.20	6621.19	0.96
I_2	700.13	760.23	0.89
I_3	21099.01	16573.49	0.96
I_4	11709.30	10190.20	0.93
I_5	8491.50	7752.99	0.93
I_6	16157.72	14867.39	0.94

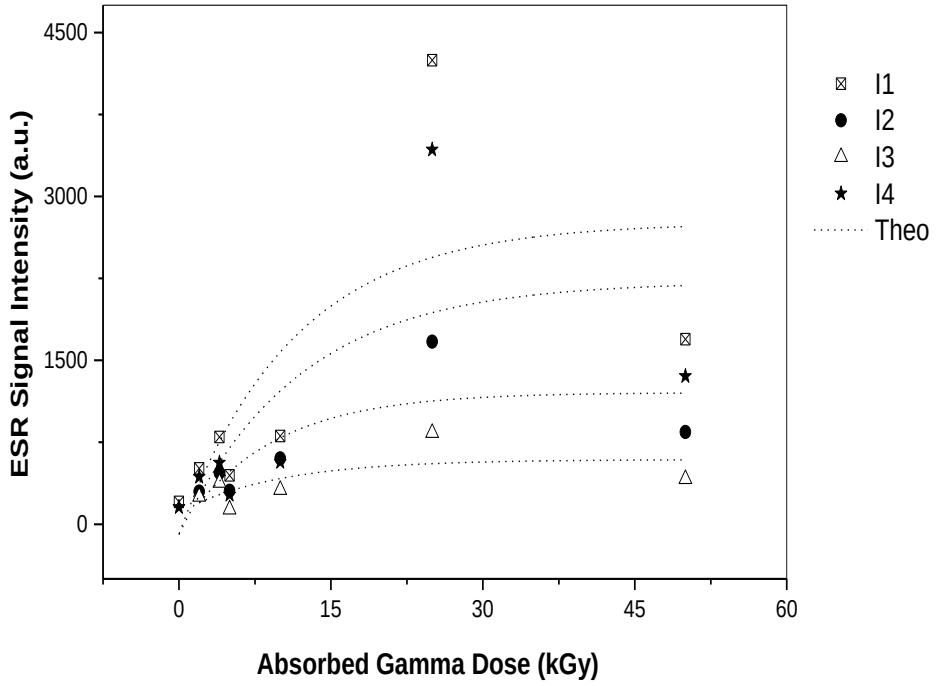

Figure 4.48. Variations of peak heights of MDG2 with absorbed radiation dose. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.12. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results of MDG2.

Intensity (a.u.)	a	b	c
I_1	2759.41	25847.41	0.92
I_2	1205.02	1262.89	0.89
I_3	591.76	481.99	0.90
I_4	2219.56	2314.99	0.92

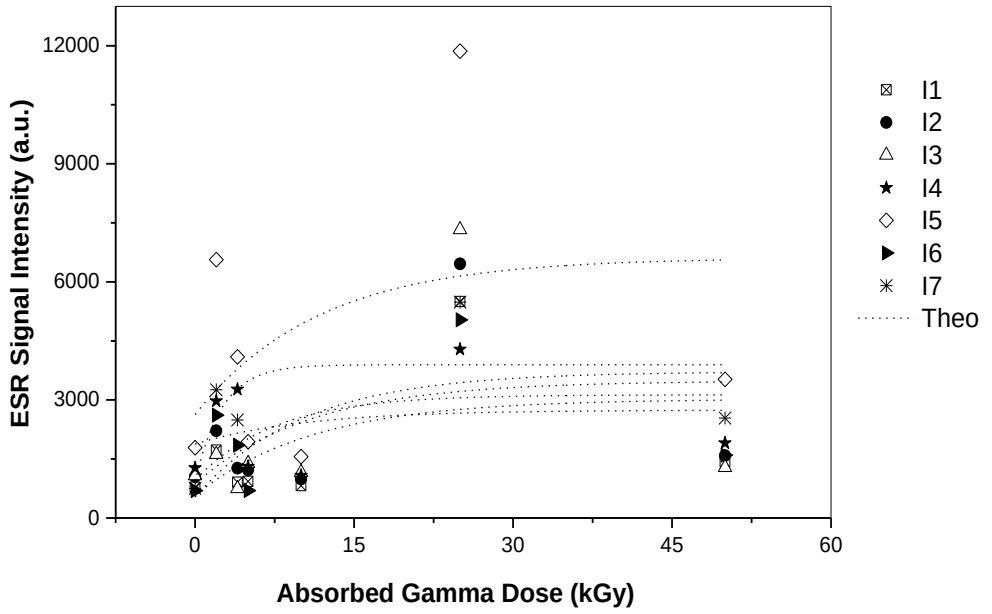

Figure 4.49. Variations of peak heights of PBG1 with absorbed radiation dose. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇); I_6 (►); I_7 (∗). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.13. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results of PBG1.

Intensity (a.u.)	a	b	c
I_1	3023.22	2446.19	0.91
I_2	3484.48	2610.49	0.91
I_3	3718.30	3184.27	0.91
I_4	2741.60	863.02	0.91
I_5	6610.69	3981.46	0.92
I_6	3130.46	2173.12	0.87
I_7	3894.28	2981.69	0.67

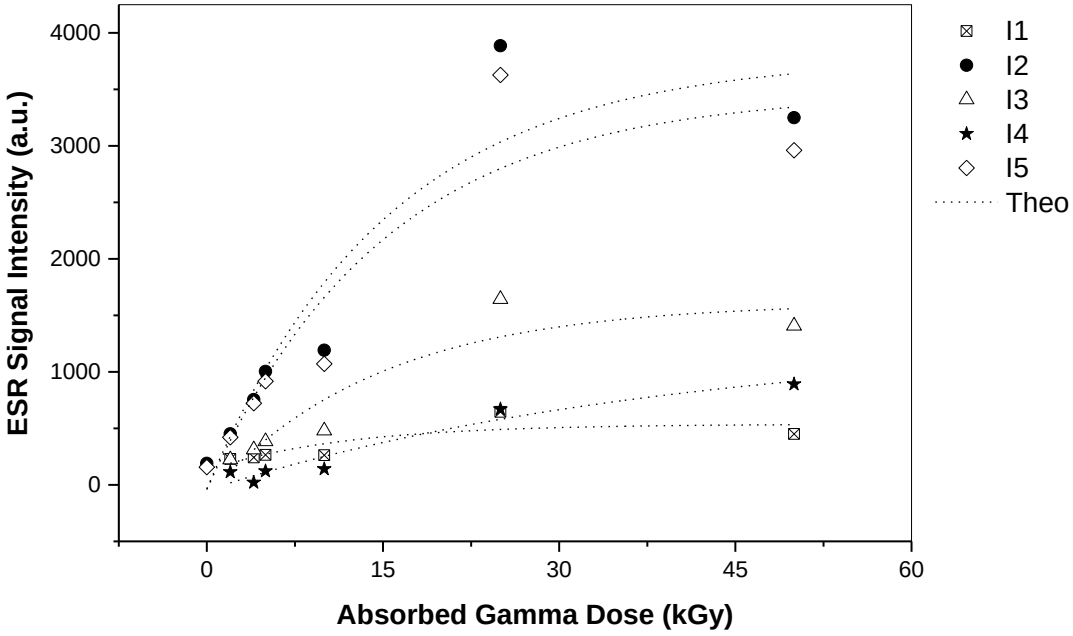

Figure 4.50. Variations of peak heights of PDG1 with absorbed radiation dose. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.14. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results of PDG1.

Intensity (a.u.)	a	b	c
I_1	537.95	411.75	0.92
I_2	3787.52	3822.06	0.94
I_3	1613.33	1718.27	0.93
I_4	1303.44	1350.46	0.98
I_5	3471.77	3512.95	0.94

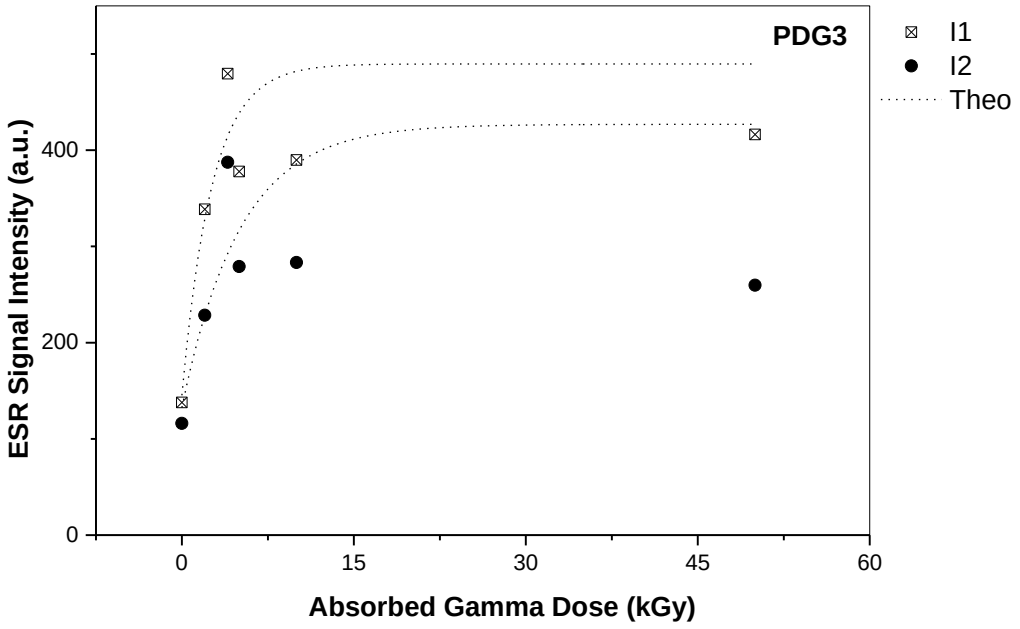

Figure 4.51. Variations of peak heights of PDG3 with absorbed radiation dose. I_1 (☒); I_2 (•). Dashed lines present theoretical curves best fitting to experimental data.

Table 4.15. Theoretical asymptotic function $\left[I = a - b \times c^{(Dose)}\right]$ used in fitting dose-response data and calculated parameters best describing experimental dose-response results of PDG3.

Intensity (a.u.)	a	b	c
I_1	489.63	344.35	0.68
I_2	426.90	294.35	0.82

Long Term Studies of Gamma Irradiation

Long term studies of ESR results of the irradiated samples of all grafts as 25 kGy after 82 days were explained below;

As it was mentioned in Section 3.5.1. , long term studies were held for 25 kGy gamma irradiation for all samples. The investigated samples were stored at room conditions (room temperature (25 ° C) and 93 % relative humidity) for nearly three months (82 days) and ESR long term decay data of HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 coded samples were recorded at various time intervals.

The free radical content of 25 kGy gamma irradiated HBG1 decayed about 33% during its storage period and the sample was concluded to be relatively stable (Figure 4.52). The free radical content of 25 kGy gamma irradiated HL1 decayed about 97% during the storage period, thus the sample was concluded to be unstable. As I_1 and I_4 resonance peaks decayed at a duration time of about 1 week, only the variation of I_2 and I_3 resonance peaks were given in (Figure 4.53). The free radical content of 25 kGy gamma irradiated MBG3 decayed about 66% during its storage period. MBG3 can be considered to be a relatively stable sample (Figure 4.54). The free radical content of 25 kGy gamma irradiated MDG2 decayed about 92% during its storage period. Besides, I_1 and I_4 resonance peaks have decayed in the early storage time studies (about 1 week), thus the free radicals type(s) involved in MDG2 upon irradiation can be concluded to be unstable (Figure 4.55). The free radical content of 25 kGy gamma irradiated PBG1 decayed about 16% during its storage period, thus PBG1 can be considered to be relatively stable graft (Figure 4.56). The free radical content of 25 kGy gamma irradiated PDG3 decayed about 11% during its storage period, so this sample can be concluded to be relatively stable when it was stored under normal conditions (Figure 4.57). The free radical content of 25 kGy gamma irradiated HBG1 decayed about 75% during its storage period. I_1, I_4 and I_5 resonance peaks have already decayed after 1 week storage time. Thus PDG1 sample can be concluded to be an unstable graft (Figure 4.58).

Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism for all grafts also were calcualated as:

$$[\text{as } I = I_0 + a \times \exp(-k \times time)]$$

The results were given at Table 4.16 to 4.22.

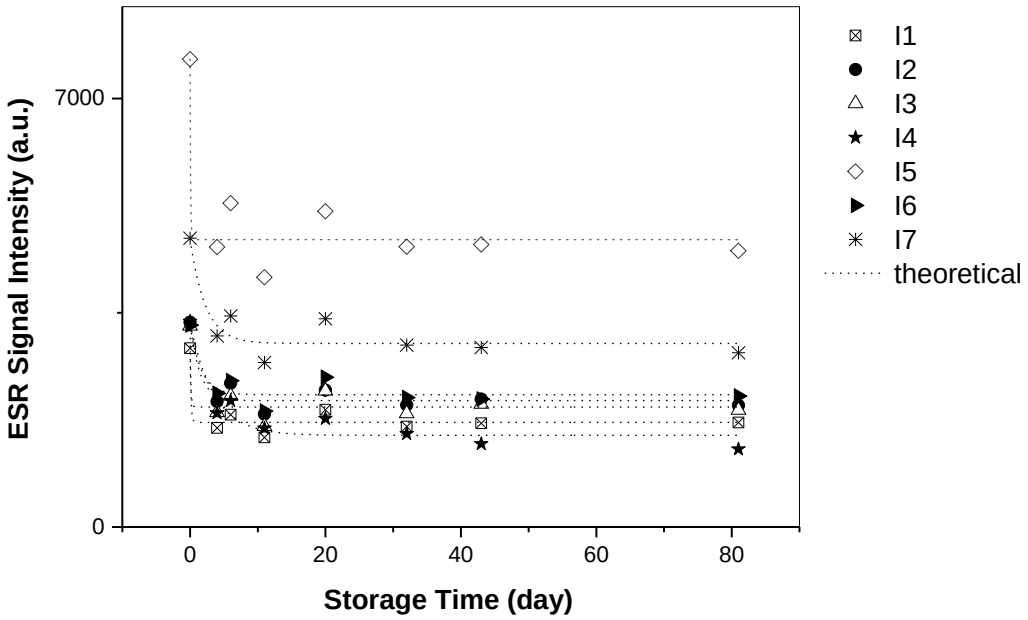

Figure 4.52. Variations of the peak heights of 25 kGy gamma irradiated HBG1 with storage time at normal conditions. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇); I_6 (►); I_7 (✳). Dashed line present theoretical curves best fitting to experimental data.

Table 4.16. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_1	1709.89	1161.10	7.29
I_2	2064.29	1279.24	0.70
I_3	1960.51	1257.27	9.42
I_4	1497.67	1831.25	0.28
I_5	4693.74	2948.42	12.36
I_6	2161.15	1135.53	0.61
I_7	2999.11	1711.19	0.44

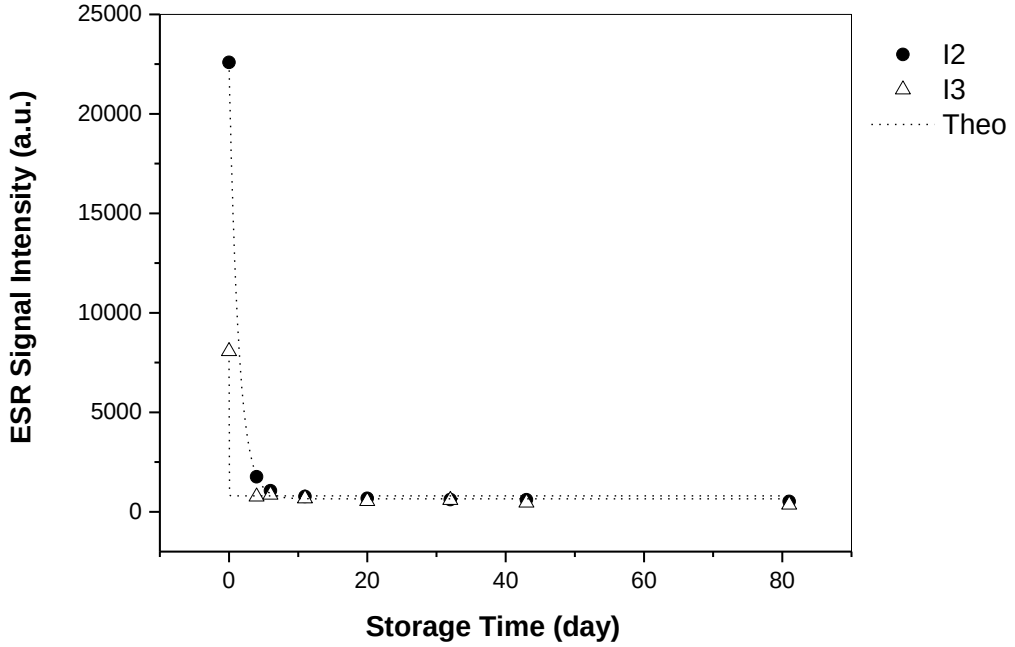

Figure 4.53. Variations of the peak heights of 25 kGy gamma irradiated HL1 with storage time at normal conditions. I_2 (•); I_3 (Δ). Dashed line present theoretical curves best fitting to experimental data.

Table 4.17. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_2	652.97	21937.17	0.736
I_3	798.95	6797.40	63.98

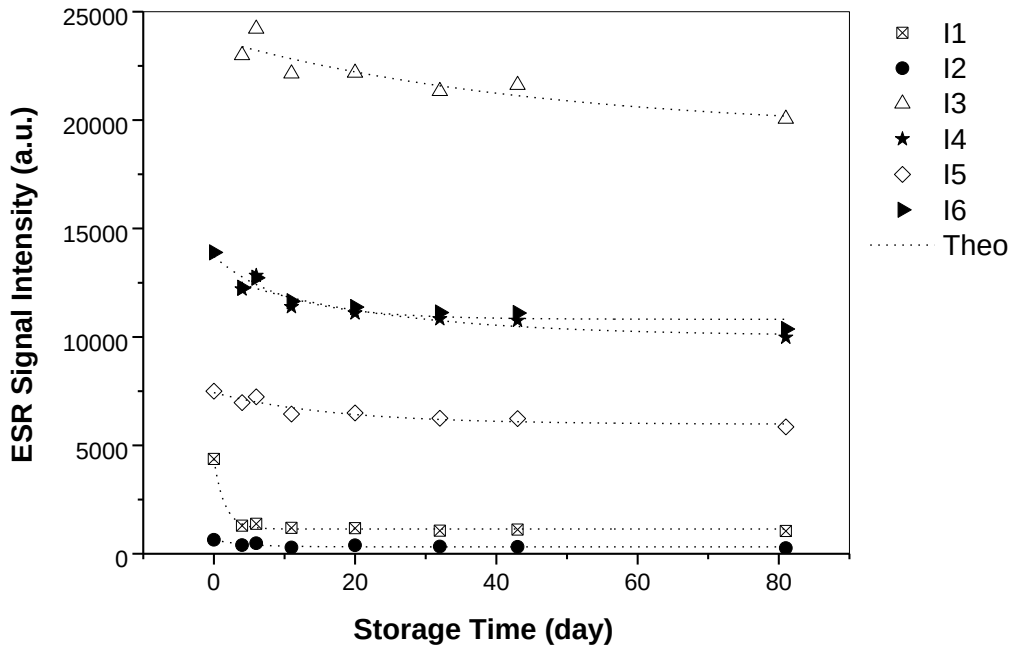

Figure 4.54. Variations of the peak heights of 25 kGy gamma irradiated MBG3 with storage time at normal conditions. I_1 (☒); I_2 (●); I_3 (Δ); I_4 (★); I_5 (◇); I_6 (►). Dashed line present theoretical curves best fitting to experimental data.

Table 4.18. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_1	1139.84	3229.37	0.66
I_2	315.42	323.66	0.22
I_3	19468.36	4279.28	0.02
I_4	10040.98	2841.82	0.04
I_5	5972.58	1461.56	0.05
I_6	10812.51	2907.48	0.09

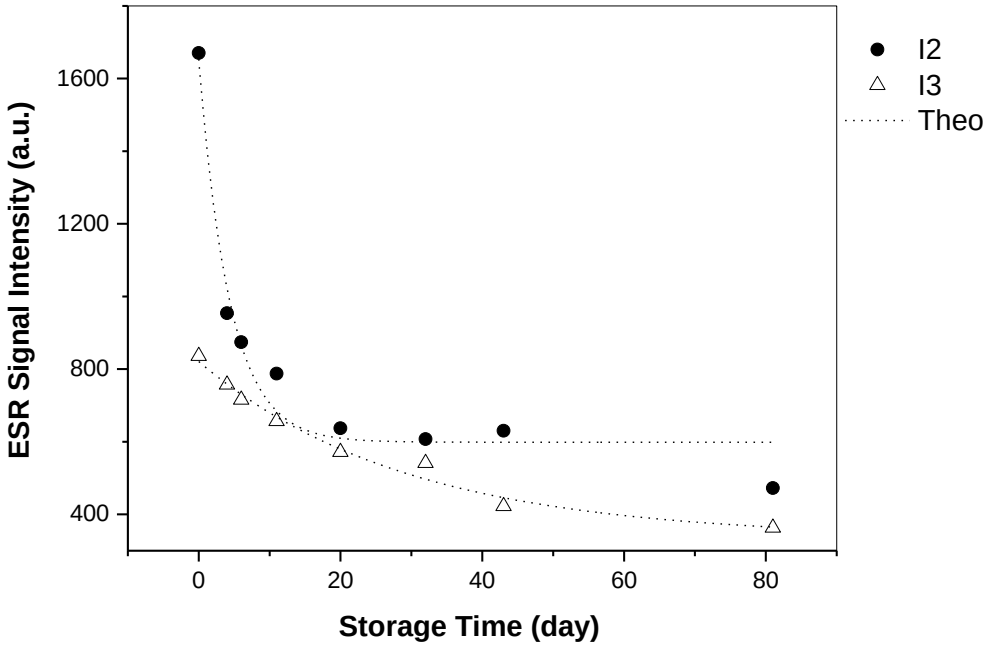

Figure 4.55. Variations of the peak heights of 25 kGy gamma irradiated MDG2 with storage time at normal conditions. I_2 (•); I_3 (Δ). Dashed line present theoretical curves best fitting to experimental data.

Table 4.19. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_2	598.72	1054.79	0.23
I_3	334.84	486.05	0.03

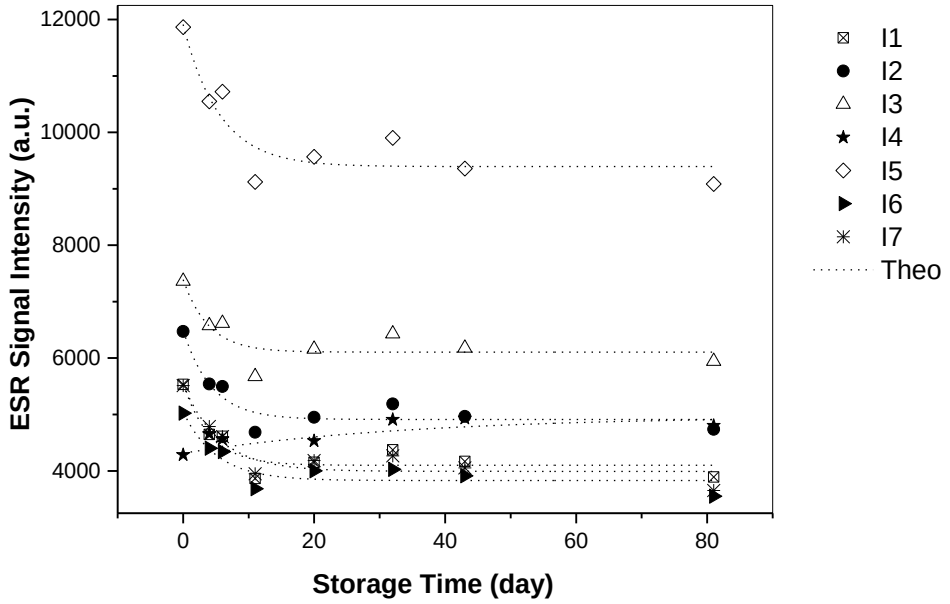

Figure 4.56. Variations of the peak heights of 25 kGy gamma irradiated PBG1 with storage time at normal conditions. I_1 (☒); I_2 (•); I_3 (Δ); I_4 (★); I_5 (◇); I_6 (►); I_7 (✳). Dashed line present theoretical curves best fitting to experimental data.

Table 4.20. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_1	4099.32	1452.11	0.24
I_2	4911.69	1581.51	0.23
I_3	6105.46	1284.89	0.26
I_4	4974.82	661.80	0.03
I_5	9393.81	2517.00	0.18
I_6	3829.49	1216.39	0.19
I_7	3992.24	1542.84	0.18

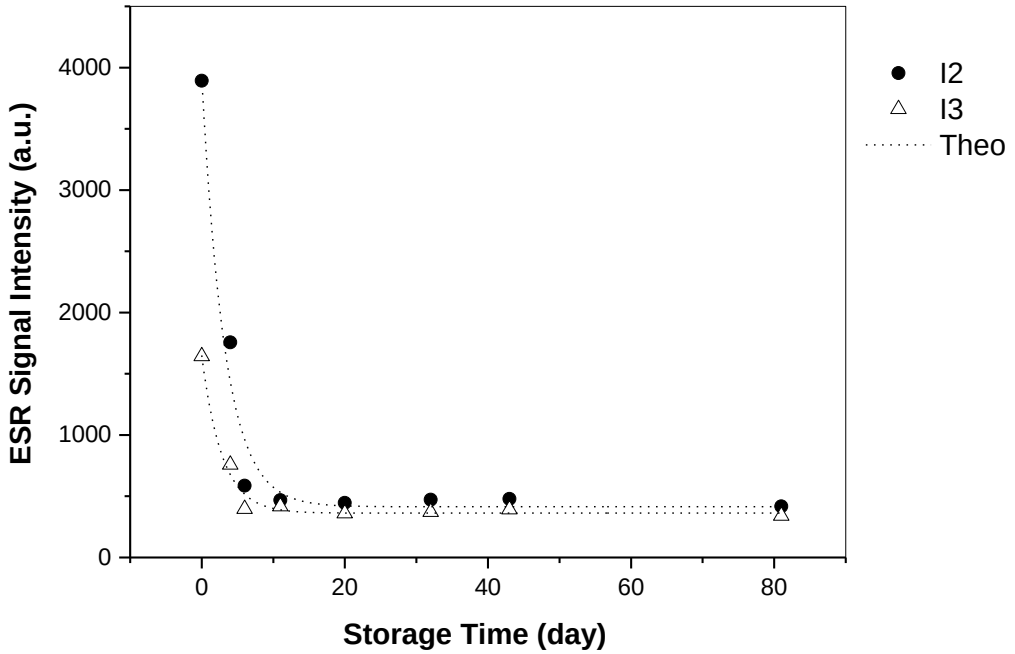

Figure 4.57. Variations of the peak heights of 25 kGy gamma irradiated PDG1 with storage time at normal conditions. I_2 (•); I_3 (Δ). Dashed line present theoretical curves best fitting to experimental data.

Table 4.21. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_2	414.27	3511.26	0.31
I_3	362.29	1287.84	0.35

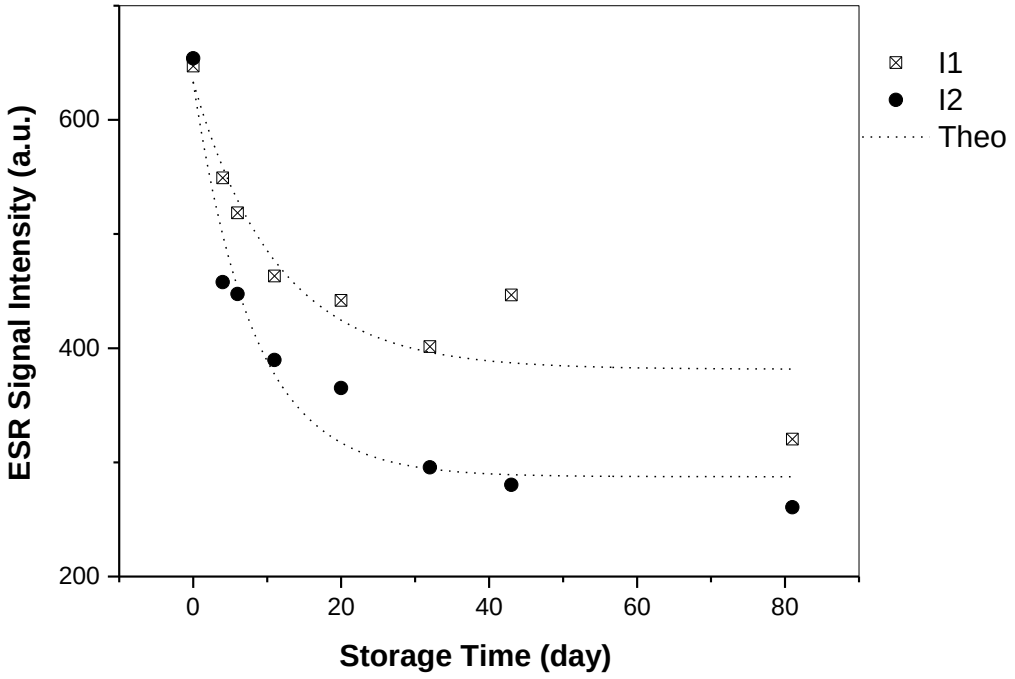

Figure 4.58. Variations of the peak heights of 25 kGy gamma irradiated PDG3 with storage time at normal conditions. I_1 (☒); I_2 (•). Dashed line present theoretical curves best fitting to experimental data.

Table 4.22. Relative weights and decay constants for the contributing radicals calculated from long term signal intensity decay data obtained at normal conditions by fitting to 1st order decay mechanism.

Intensity (a.u.)	I_0 (a.u.)	a (a.u)	Decay constant k (1/day)
I_1	381.57	251.89	0.09
I_2	287.44	345.84	0.12

4.2.2. Microbiological Tests

The sterility and pyrogen tests and the determination of SAL doses were performed as given in Section 3.5.2 as microbilogical tests. The results of the sterility test on grafts after irradiation were given in Table 4.22. No growth was observed in grafts on both media (FTM and SCDM) at 5 kGy and above.

Sterility Test

The sterility tests of all grafts before and after gamma radiation sterilization were performed as given in Section 3.5.2. The results of the sterility test on grafts after irradiation are given in Table 4.23. No microorganism growth was observed in grafts on both media for gamma irradiation with 5 kGy and above doses.

Table 4.23. Sterility test results of grafts after sterilization with gamma radiation.

Grafts	Medium	
	FTM (35 ^{0}C)	SCDM (25 ^{0}C)
HBG1	-	-
HL1	-	-
MBG3	-	-
MDG2	-	-
PBG1	-	-
PDG1	-	-
PDG3	-	-

(-) No Growth.

SAL Determinations Results

The SAL tests of all grafts before and after gamma radiation sterilization were performed as given in Section 3.5.2. Microbial mortality rates for grafts were plotted and SAL values were determined (Log N/N_0 versus Radiation Dose). Microorganism dead graphics of all grafts after irradiation between 0-10 kGy were drawn; due to the findings, no growth of microorganism at 5 kGy and higher doses were observed. All the graphics were given at Figure 4.59 to 4.65.

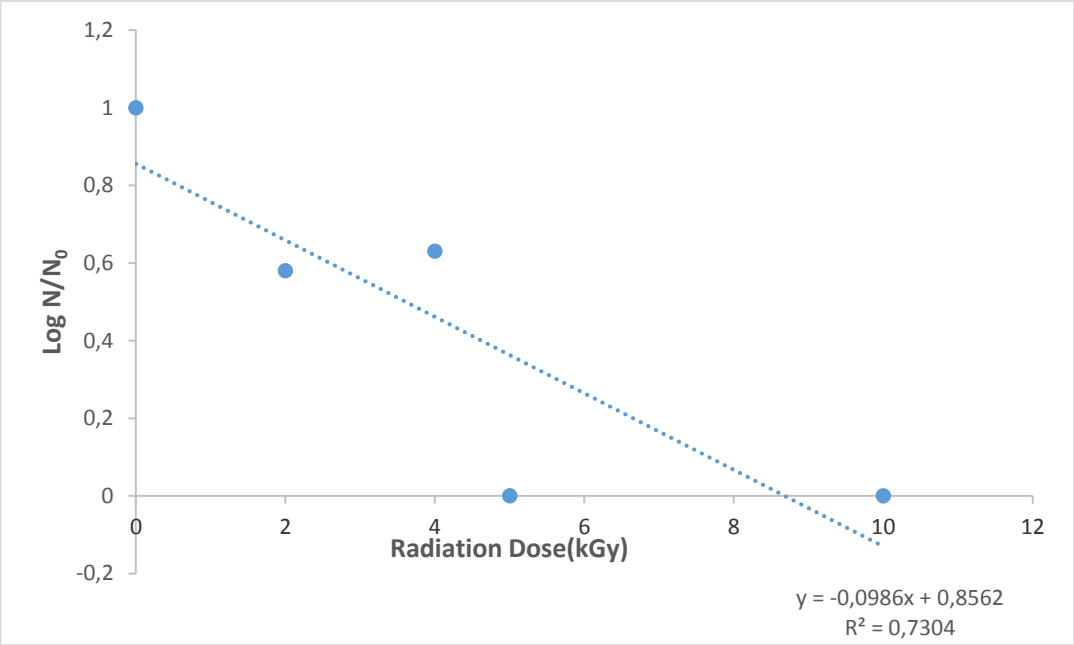

Figure 4.59. Microorganism dead graphic of HBG1 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

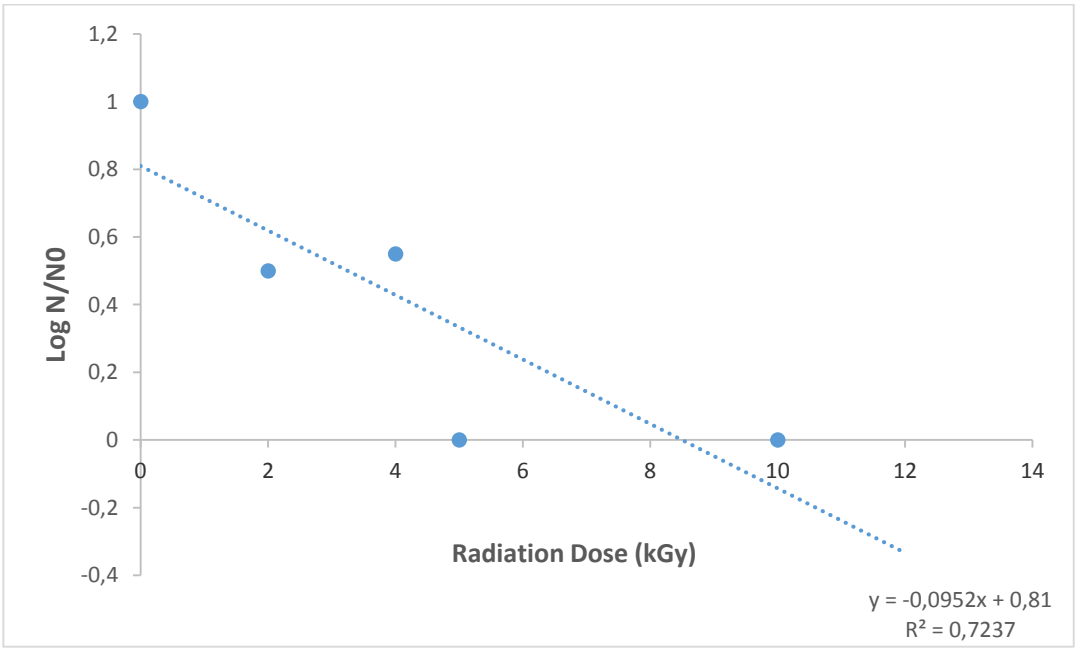

Figure 4.60. Microorganism dead graphic of HL1 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

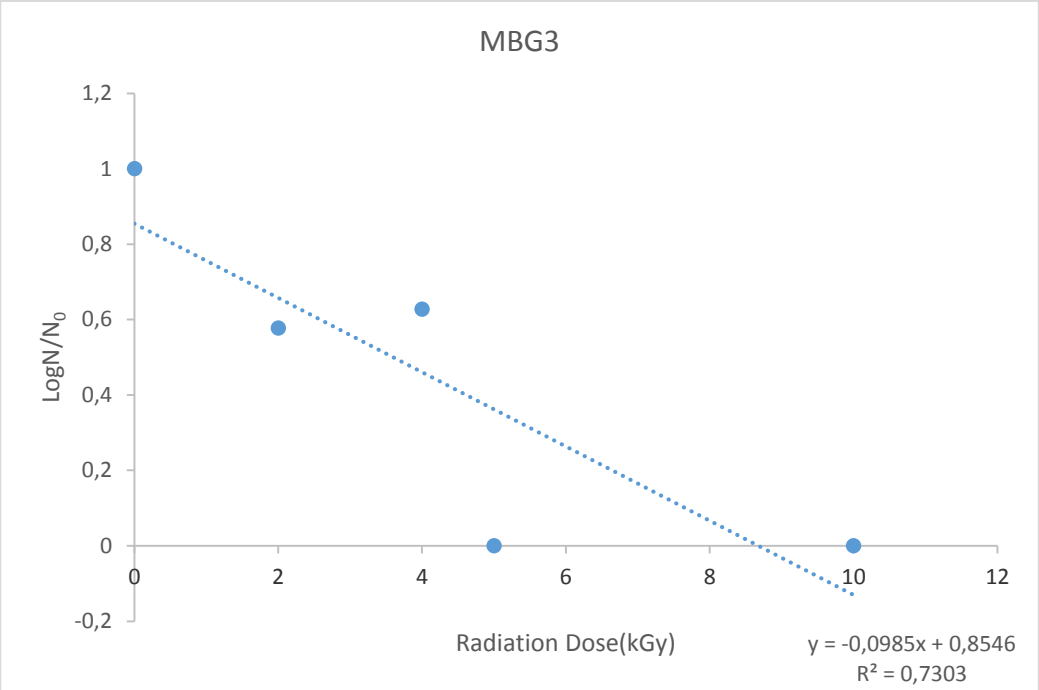

Figure 4.61. Microorganism dead graphic of MBG3 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

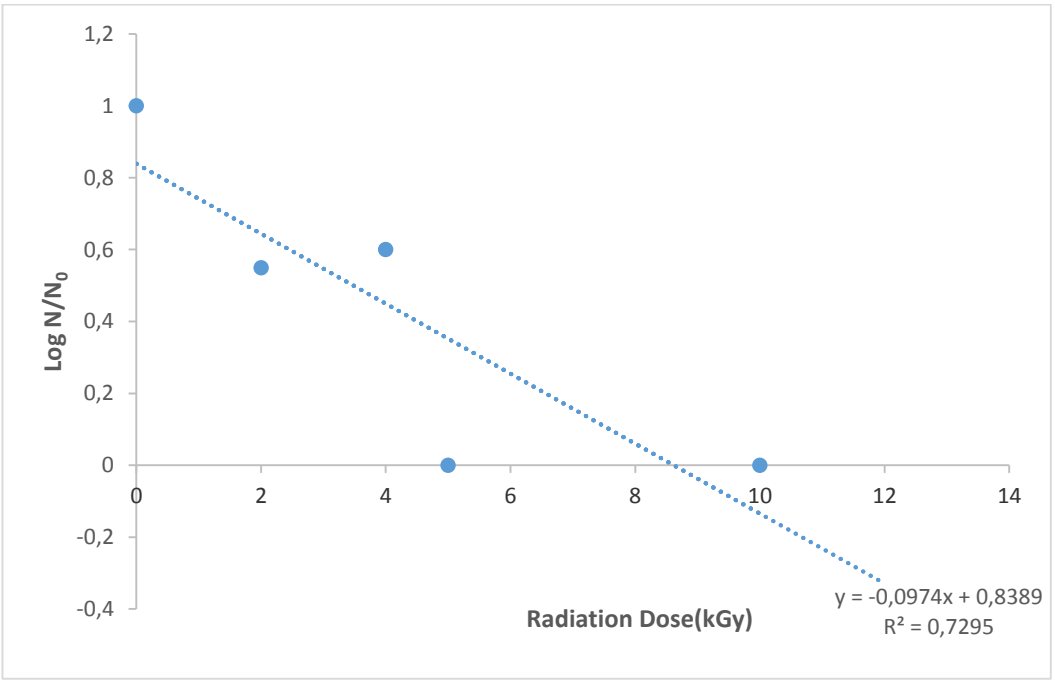

Figure 4.62. Microorganism dead graphic of MDG2 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

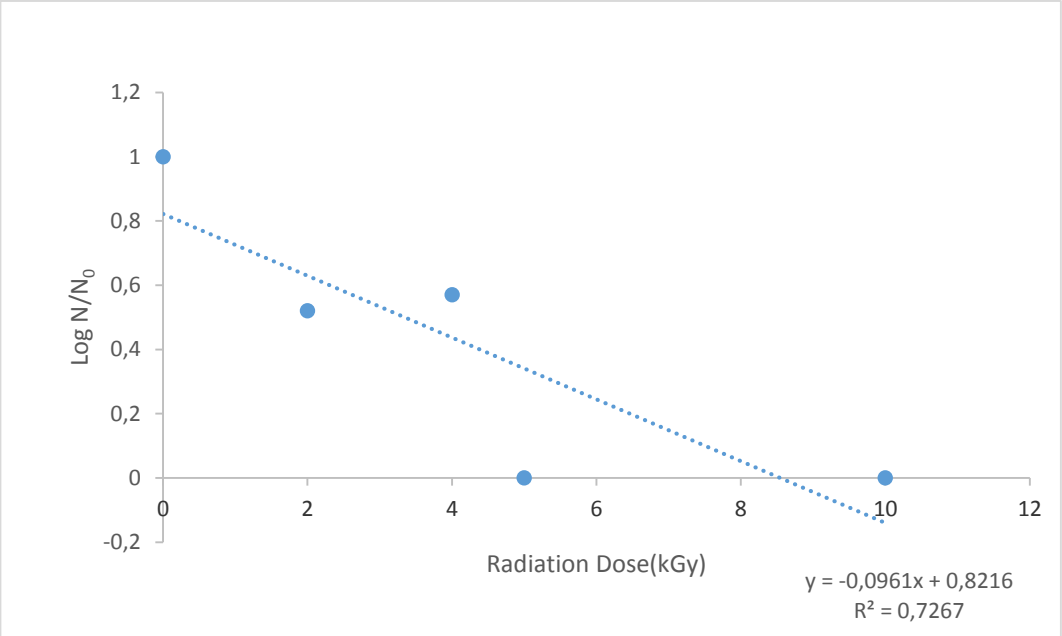

Figure 4.63. Microorganism dead graphic of PBG1 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N0= microorganism number before irradiation).

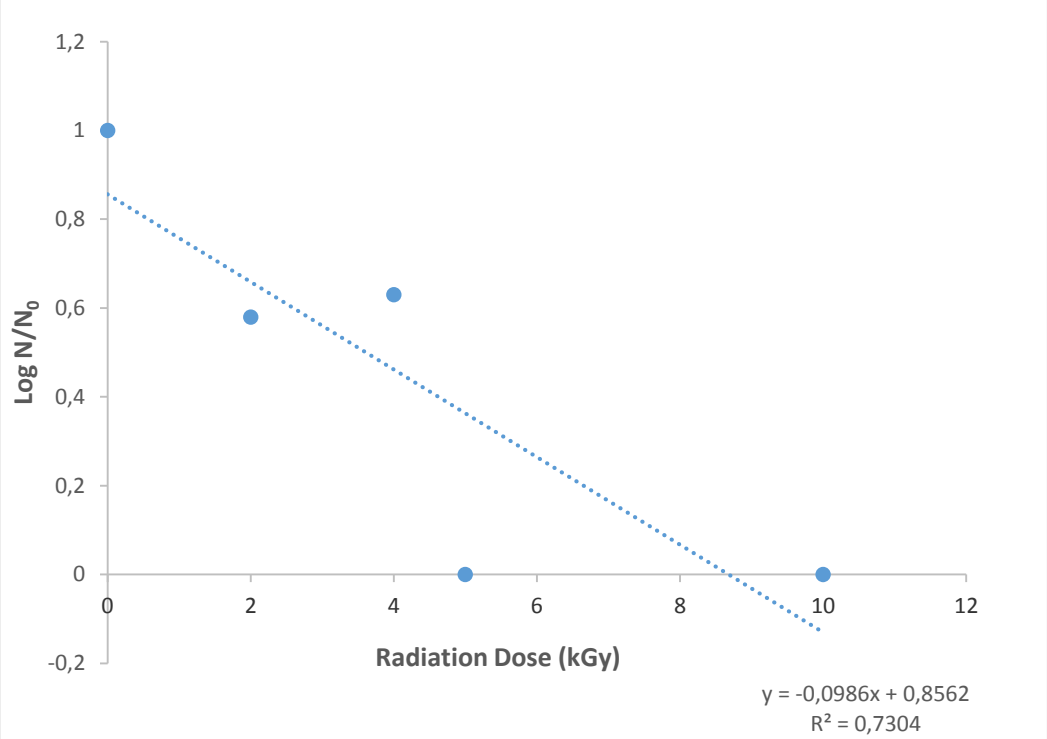

Figure 4.64. Microorganism dead graphic of PDG1 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N0= microorganism number before irradiation).

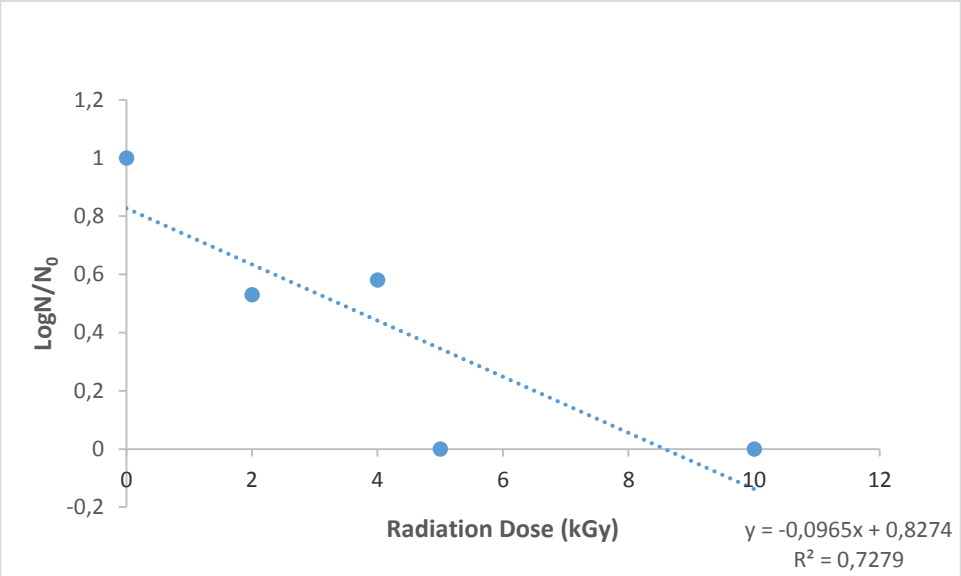

Figure 4.65. Microorganism dead graphic of PDG3 coded graft irradiated by gamma ray at 2, 4, 5, 10 kGy. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

Pyrogen Test Results

Endotoxin test was done by using a LAL Rapid Endotoxin Detection kit as explained in Section 3.5.2. For medical devices, using the extraction volume recommendations described below, the limit is 0.5 $EU.mL^{-1}$ or 20 EU/device for products that directly or indirectly contact with the cardiovascular system and lymphatic system (85). Based on the results from pyrogen test, endotoxin amount for only PDG1 coded graft was determined as more than 0.25 $EU.mL^{-1}$ .So the result is compatible to FDA.

4.3. Analyses of Grafts Pre-and Post-Sterilization with Microwave Radiation

All analyses were performed on the microwave irradiated grafts as were performed for gamma irradiated ones and explained in Section 3.5 and 3.6.

4.3.1. Physicochemical Properties

Organoleptic Analyses

Organoleptic characteristics of all grafts were detected before and after microwave sterilization (1 min, 2 min, 3 min, 4 min) as given in Section 3.5.1. The results of organoleptic evaluation of grafts before and after microwave sterilization were given in Table 4.24.

Table 4.24. Organoleptic results of grafts before and after microwave irradiation.

Grafts	Organoleptic Features				
	Before radiation	Radiation Time (Min)			
		1	2	3	4
HBG1	White Porous,Fragile	–	–	–	–
HL1	Cream Elastic	–	–	–	+
MBG3	Cream Porous, Strict	–	–	–	–
MDG2	Cream Elastic	–	–	–	+
PBG1	Cream Porous,Fragile	–	–	–	–
PDG1	White Elastic,Strict	–	–	–	+
PDG3	White Elastic	–	–	–	+

+ Slightly change - No change

FTIR Analysis Results

FTIR analysis was performed as explained in Section 3.5.1. All the spectra for each graft were given in Figure 4.66 to 4.72.

In the evaluation of HBG1 coded graft as designated in Figure 4.66. 3291 (O-H str., N-H str., amide A, hydrogen bonded), 3067, 2921 (C-H str., amide B), 1645 (C=O str., amide I), 1547 (N-H def. + C-N str. amide II), 1408 (C-H bend., C-O str., CO_3^{-2}), 1014, 871 (P-O str., PO_4^{-3}) peaks were observed. Based on these results, a significant change after and before irradiation by microwave was not observed as seen in Figure 4.66.

The FTIR peaks of HL1 coded graft were observed as; 3301 (N-H str., amide A, hydrogen bonded), 3076, 2929 (C-H str., amide B), 1639 (C=O str., amide I), 1542 (N-H def. + C-N str. amide II), 1448, 1335 (C-H bend., C-O str., CO_3^{-2}), 1234 (C-N str., N-H def., amide III), 1081, 1032, 648 (P-O str., PO_4^{-3}) in Figure 4.67. Based on these results, a significant change after and before irradiation by microwave was not detected as seen in Figure 4.67.

The FTIR peaks of MBG3 coded graft were observed as; 3285 (N-H str., amide A, hydrogen bonded), 3071, 2923, 2853 (C-H str., amide B), 1643 (C=O str., amide I), 1538 (N-H def. + C-N str. amide II), 1414 (C-H bend., C-O str., CO_3^{-2}), 1014, 871 (P-O str., PO_4^{-3}) in Figure 4.68. Based on these results, a significant change after and before irradiation by microwave was not detected as seen in Figure 4.68.

The FTIR peaks of MDG2 coded graft were observed as; 3291 (N-H str., amide A, hydrogen bonded), 3081, 2924, 2854 (C-H str., amide B), 1635 (C=O str., amide I), 1541 (N-H def. + C-N str. amide II), 1449, 1333 (C-H bend., C-O str., CO_3^{-2}), 1235 (C-N str., N-H def., amide III), 1077, 1021, 645 (P-O str., PO_4^{-3}) in Figure 4.69. Based on these results, a significant change after and before irradiation by microwave was not detected as seen in Figure 4.69.

The FTIR peaks of PBG1 coded grafts were observed as; 3294 (N-H str., amide A, hydrogen bonded), 3076, 2922, 2852 (C-H str., amide B), 1648 (C=O str., amide I), 1542 (N-H def. + C-N str. amide II), 1453, 1415 (C-H bend., C-O str., CO_3^{-2}), 1013, 872 (P-O str., PO_4^{-3}) in Figure 4.70. Based on these results, no significant change after and before irradiation by microwave was detected as seen in Figure 4.70 .

For PDG1 coded grafts as given in Figure 4.71, 3299 (N-H str., amide A, hydrogen bonded), 3072, 2926 (C-H str., amide B), 1631 (C=O str., amide I), 1539 (N-H def. + C-N str. amide II), 1447, 1328 (C-H, bend., C-O str., CO_3^{-2}), 1230 (C-N str., N-H def., amide III), 1083, 1030, 645 (P-O str., PO_4^{-3}) peaks were observed. Based on these results, a significant change after and before irradiation by microwave was not detected as seen in Figure 4.71.

In the evaluation of PDG3 coded graft as designated in Figure 4.72, 3299 (N-H str., amide A, hydrogen bonded), 3076, 2923, 2853 (C-H str.), 1637 (C=O str., amide I), 1542 (N-H def. + C-N str. amide II), 1456, 1337 (C-H bend., C-O str., CO_3^{-2}), 1236 (C-N str., amide III), 1082, 1029, 668 (P-O str., PO_4^{-3}) peaks were observed. Based on these results, no significant change after and before irradiation by microwave was detected as seen in Figure 4.72 .

All FTIR spectral data were observed in accordance with the assumed structures. Functional groups identified in the FTIR spectra were amide A, B, I, II and III corresponding to collagen. The samples of collagens had amide A (N-H stretching) bands at 3301-3285 cm^{-1} and C-H stretching peaks at 3081-3067 cm^{-1} for amide B. Aliphatic C-H stretching bands were seen at 2929-2921 and 2853-2852 cm^{-1} corresponding to groups CH_3 and CH_2. The strong bands were recognised at 1648-1631 cm^{-1} (C=O stretcing) corresponding to amide I and 1553-1538 cm^{-1} corresponding to Amide II N-H) and C-N. In the spectra, while CH bending and CO bands associated with CO_3^{-2} were observed at 1456-1447 and 1337-1328 cm^{-1}, amide III (NH) + (CN) bands were seen at 1236-1230 cm^{-1}. There were also PO_4^{-3} P-O stretching bands located at 1083-1077, 1035-1013, 872-871 and 668-645 cm^{-1}.

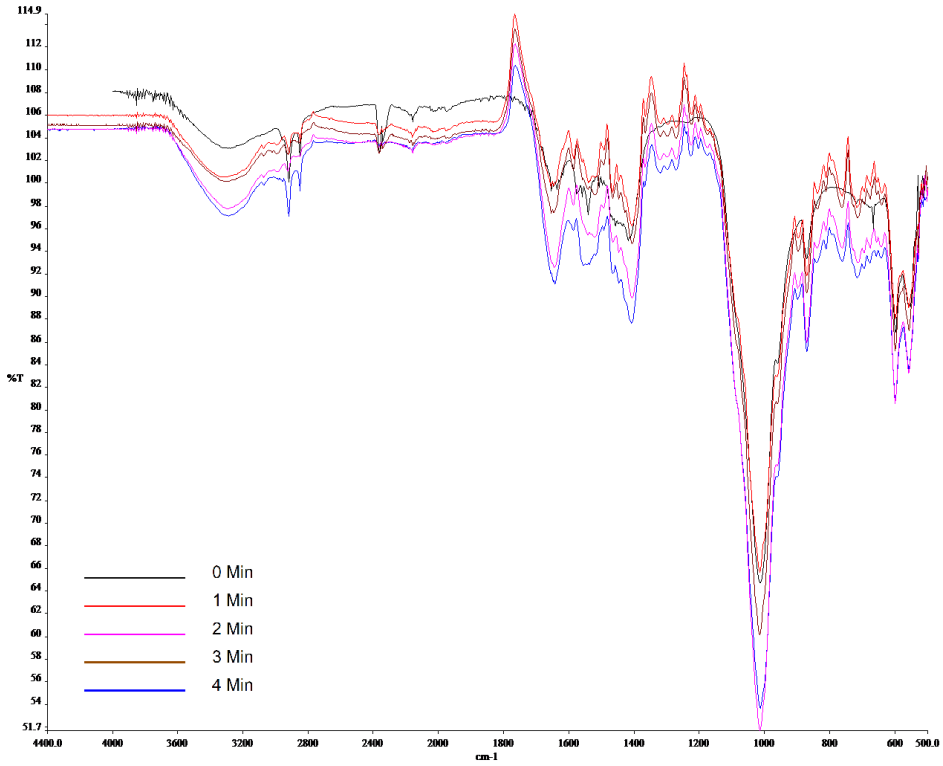

Figure 4.66. FTIR spectra of HBG1 coded grafts irradiated by microwave at 1, 2, 3, 4 min and unirradiated.

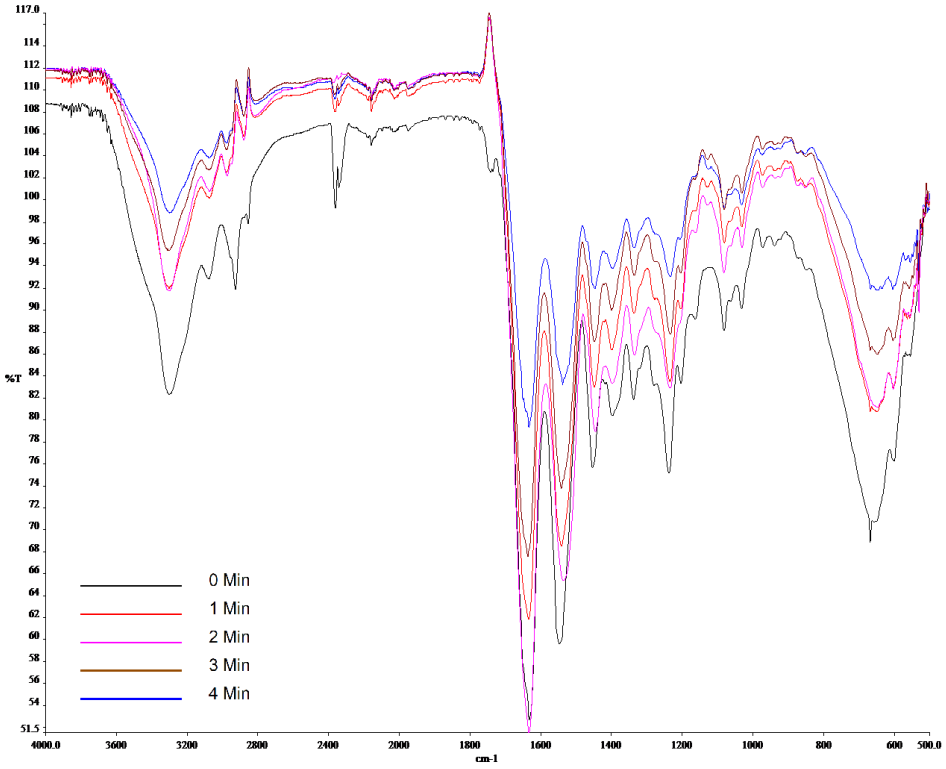

Figure 4.67. FTIR spectra of HL1 coded grafts irradiated by microwave at 1, 2, 3, 4 min and unirradiated.

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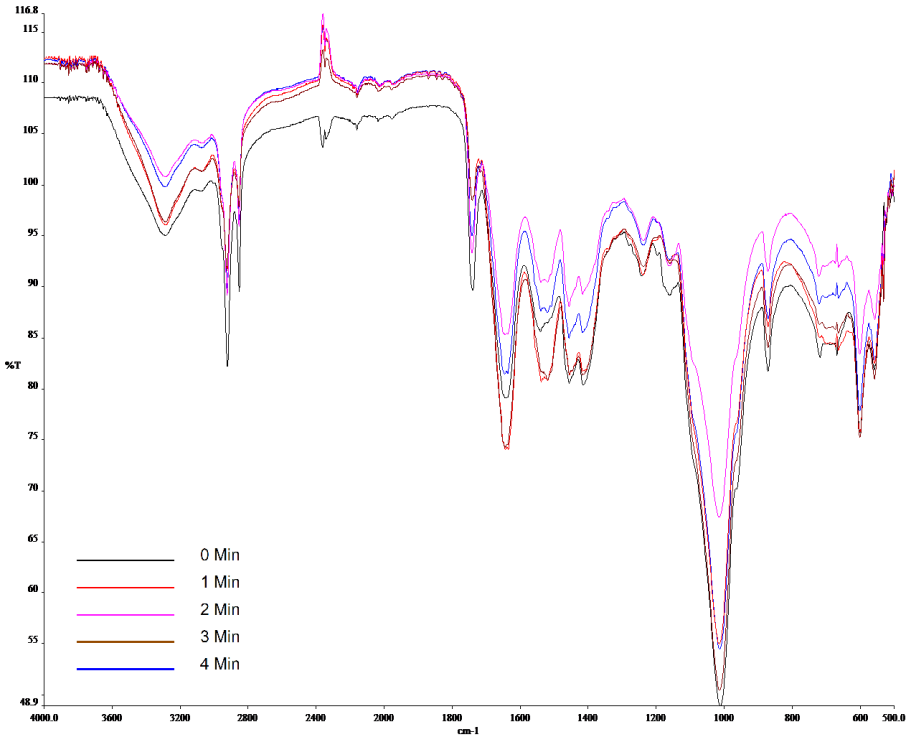


Figure 4.68. FTIR spectra of MBG3 coded grafts irradiated by microwave at 1, 2,3,4 min and unirradiated.

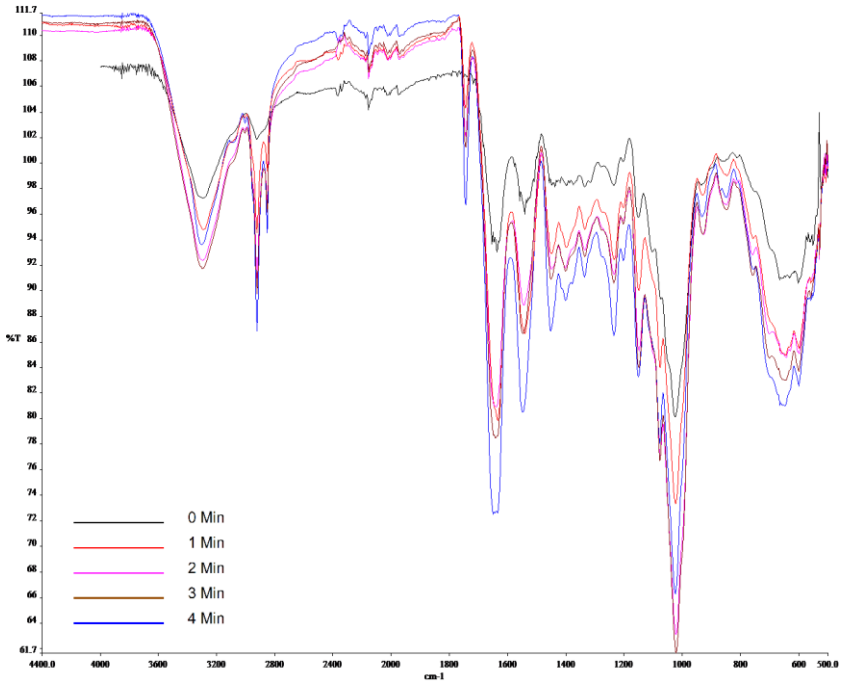


Figure 4.69. FTIR spectra of MDG2 coded grafts irradiated by microwave at 1, 2, 3, 4 min and unirradiated.

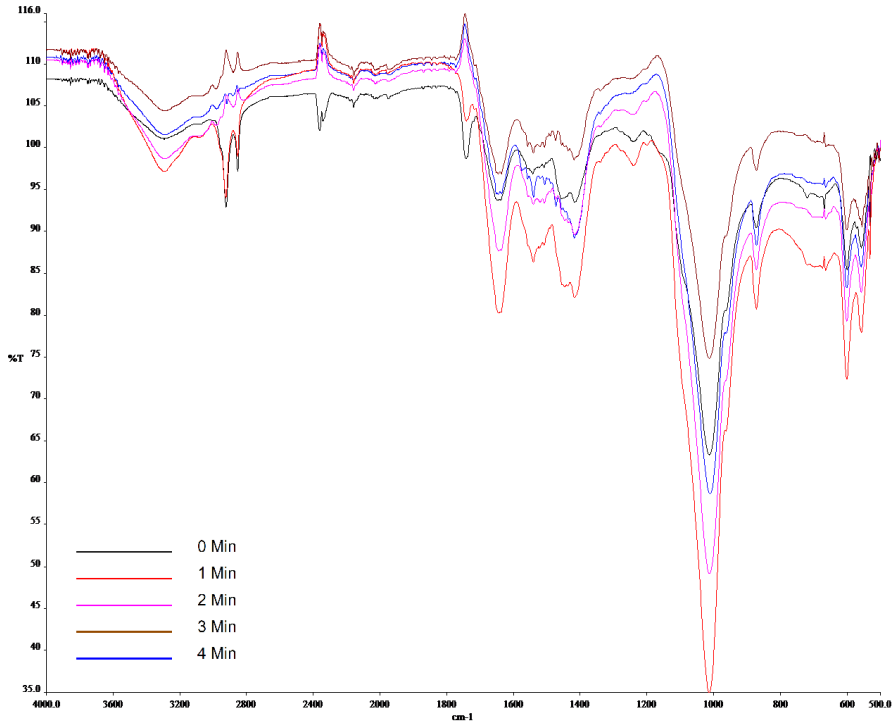


Figure 4.70. FTIR spectra of PBG1 coded grafts irradiated by microwave at 1, 2, 3, 4 min and unirradiated.

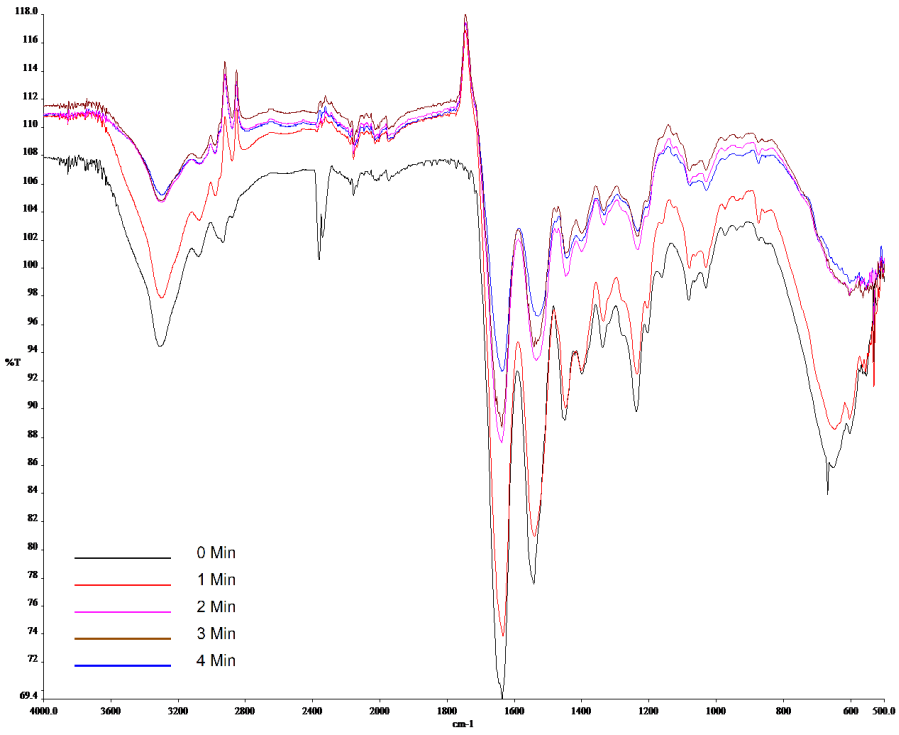


Figure 4.71. FTIR spectra of PDG1 coded grafts irradiated by microwave at 1, 2, 3, 4 min and unirradiated.

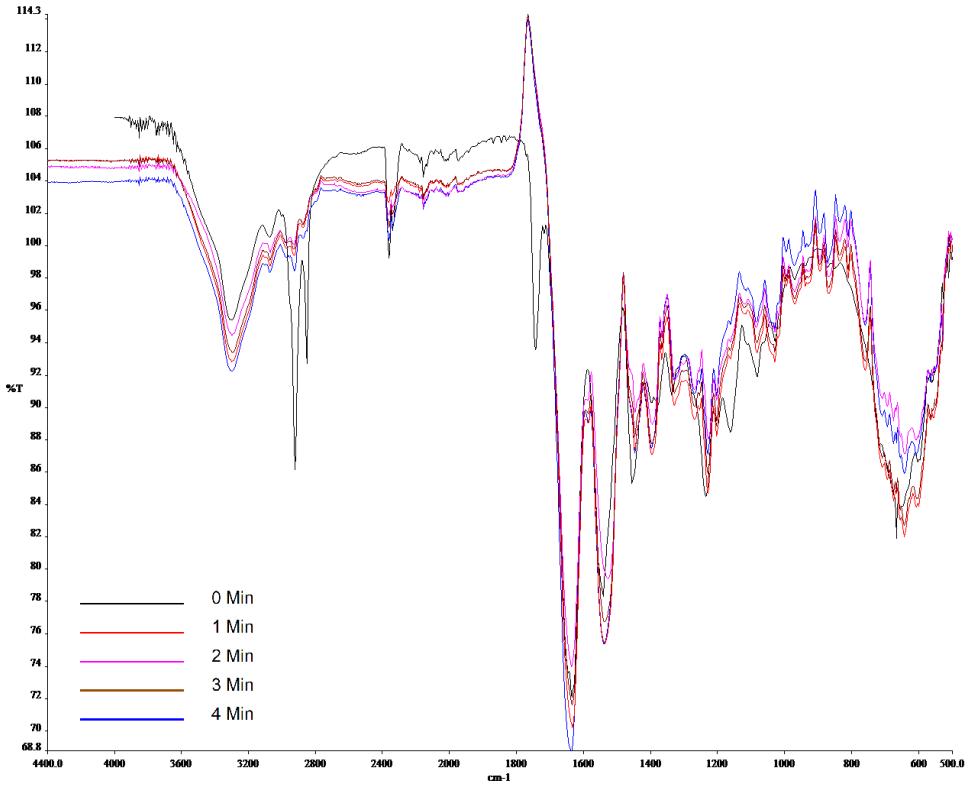
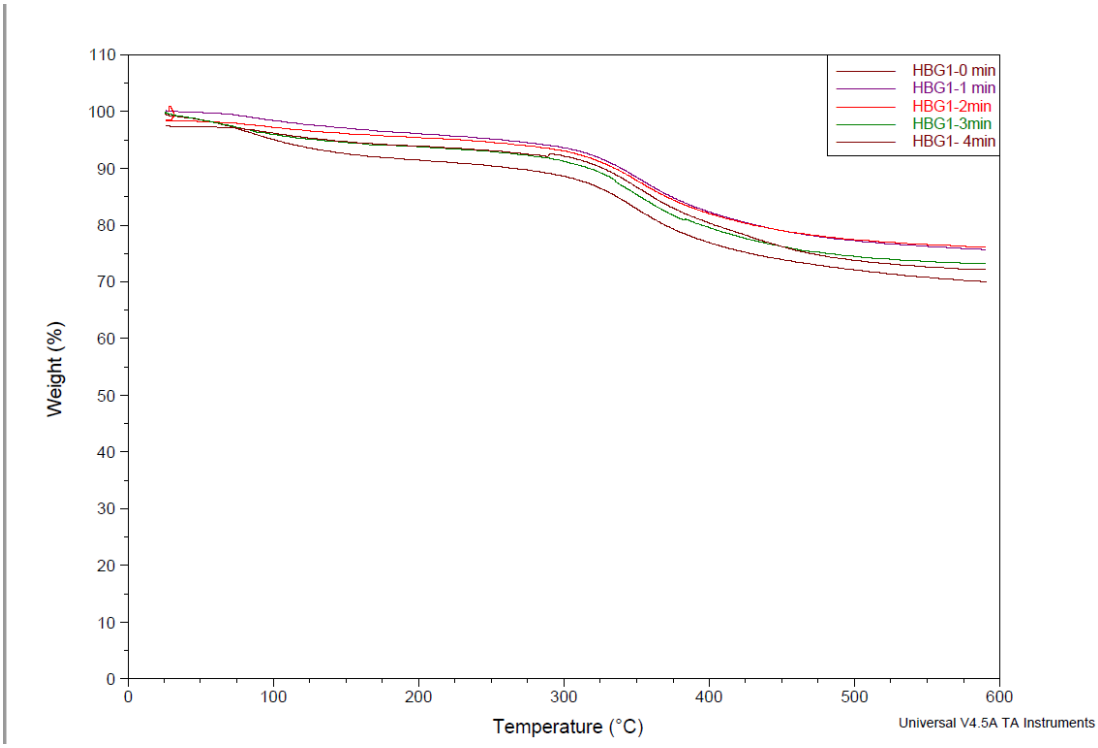


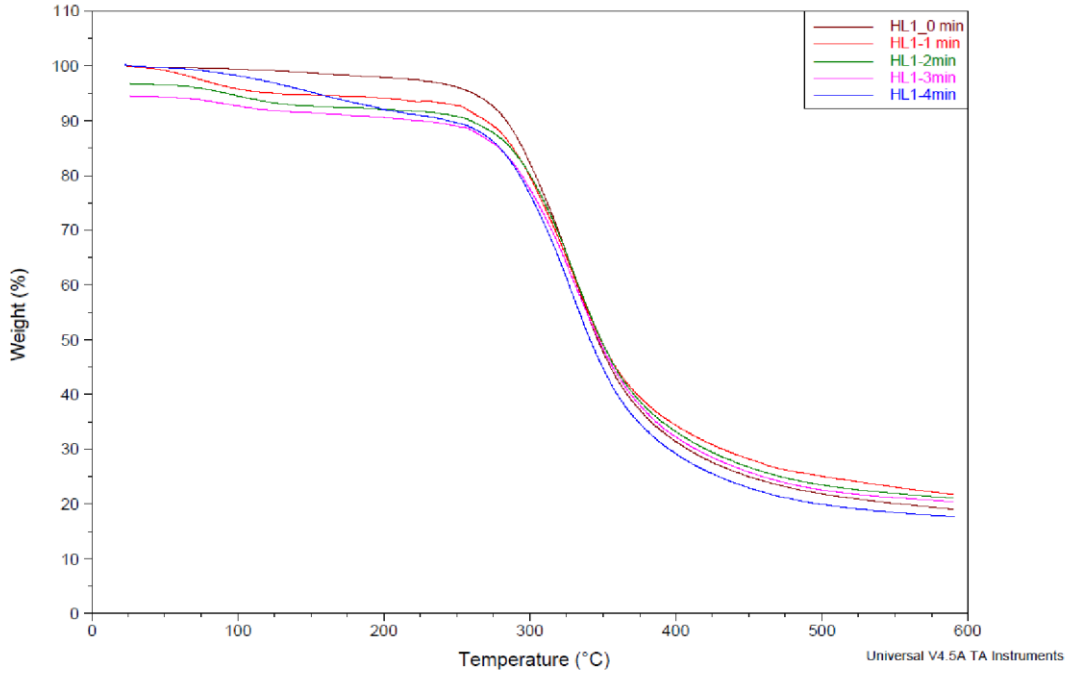
Figure 4.72. FTIR spectra of PDG3 coded grafts irradiated by microwave at 1, 2, 3, 4 min and unirradiated.

TGA Analysis Results

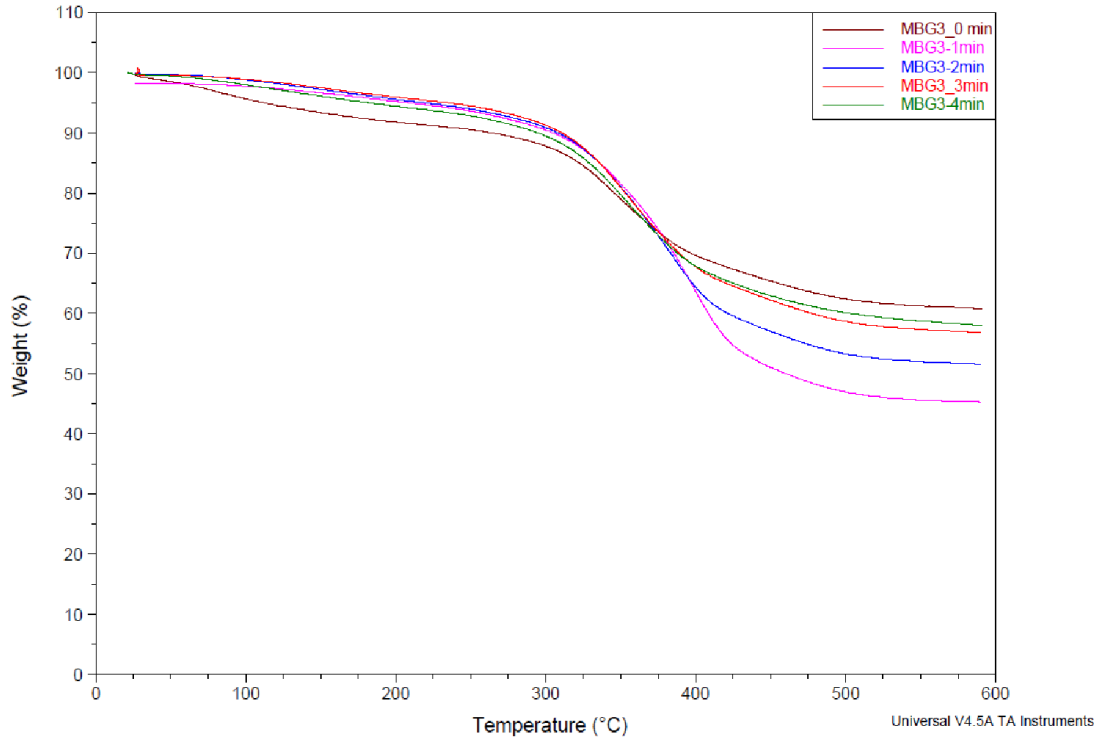
TGA results of grafts irradiated at different time intervals (1, 2, 3, 4 min) were performed as described in Section 3.5.1. The thermal analysis curves obtained were shown in Figures 4.73 to 4.79. To determine the thermal stability of unirradiated and irradiated samples, the temperature for half-life ($T_{1/2}$) were found directly from their dynamic thermograms were given in Table 4.25. Moreover, residual percentage of grafts at 550 ^{0}C for different radiation intervals was also determined from TGA thermograms (Table 4.26) and the temperature for maximum weight loss (T_{max}) was determined by first derivative curve of TGA thermograms (Figure 4.80 to 4.86 and Table 4.27)



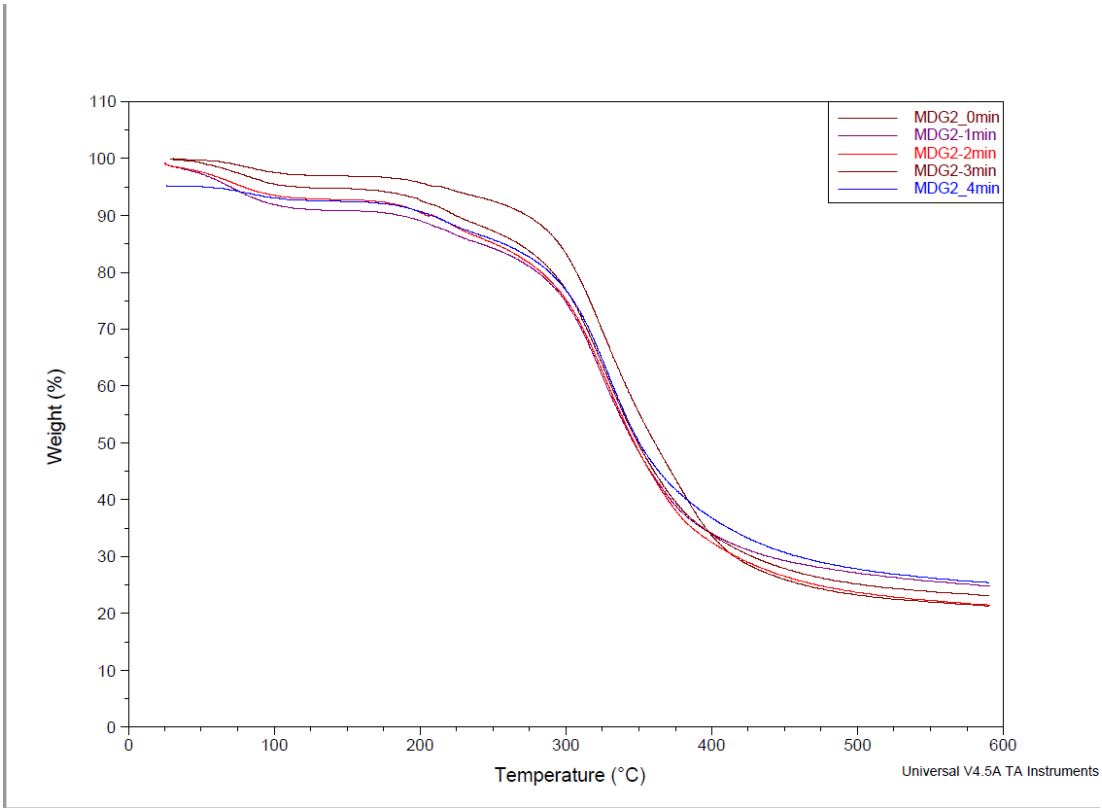
4.73. TGA thermograms of HBG1 irradiated at 1, 2, 3, 4 min and unirradiated.



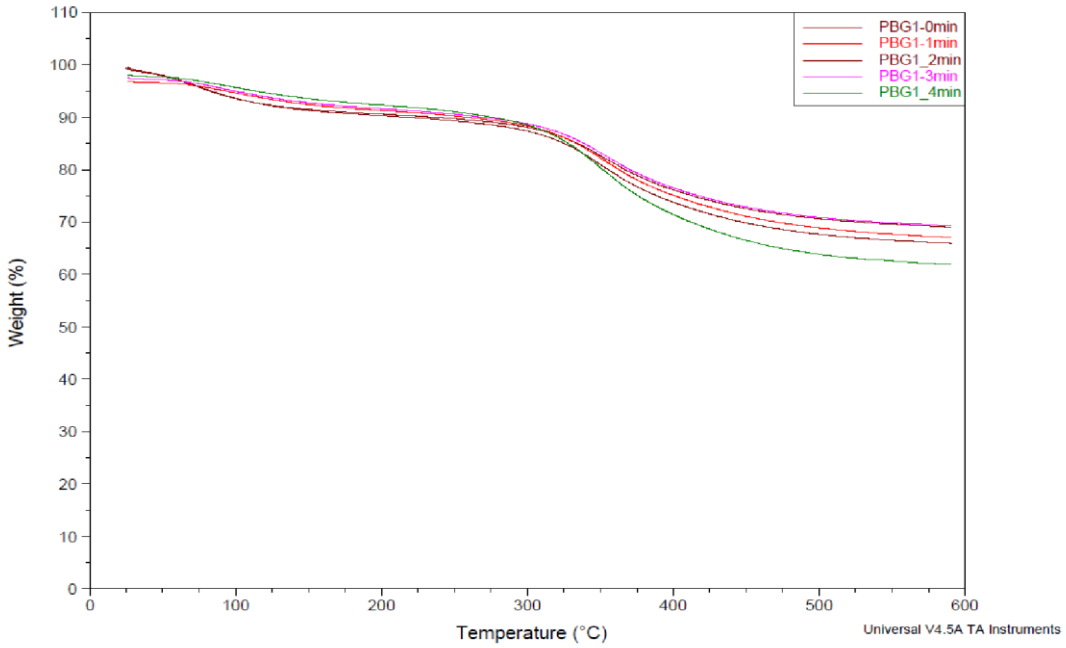
4.74. TGA thermograms of HL1 irradiated at 1, 2, 3, 4 min and unirradiated.



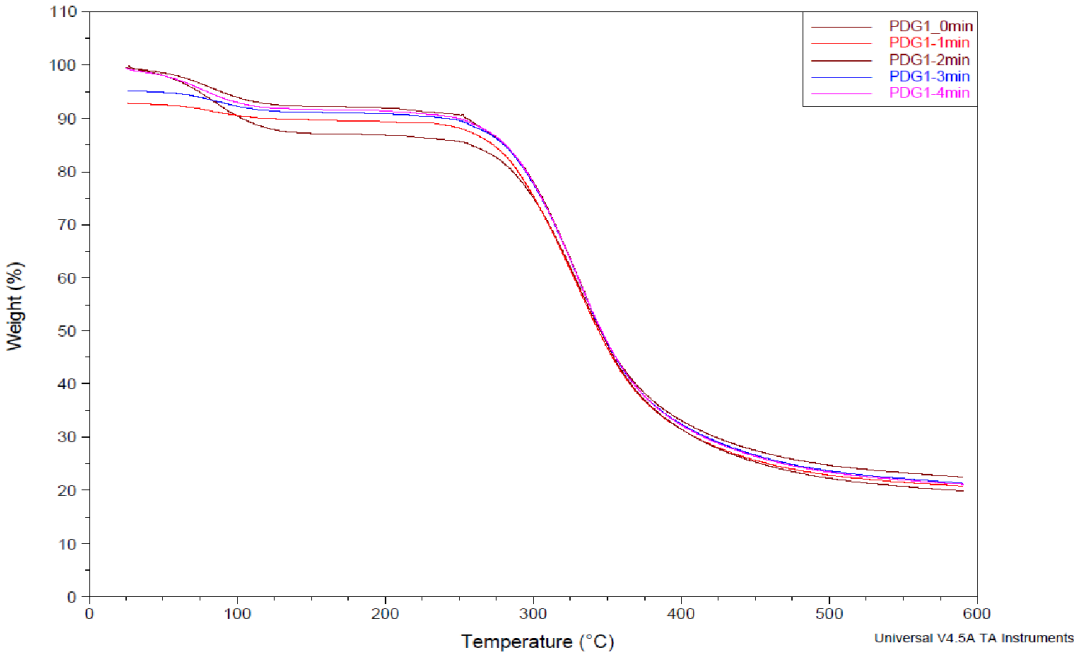
4.75. TGA thermograms of MBG3 irradiated at 1, 2, 3, 4 min and unirradiated.



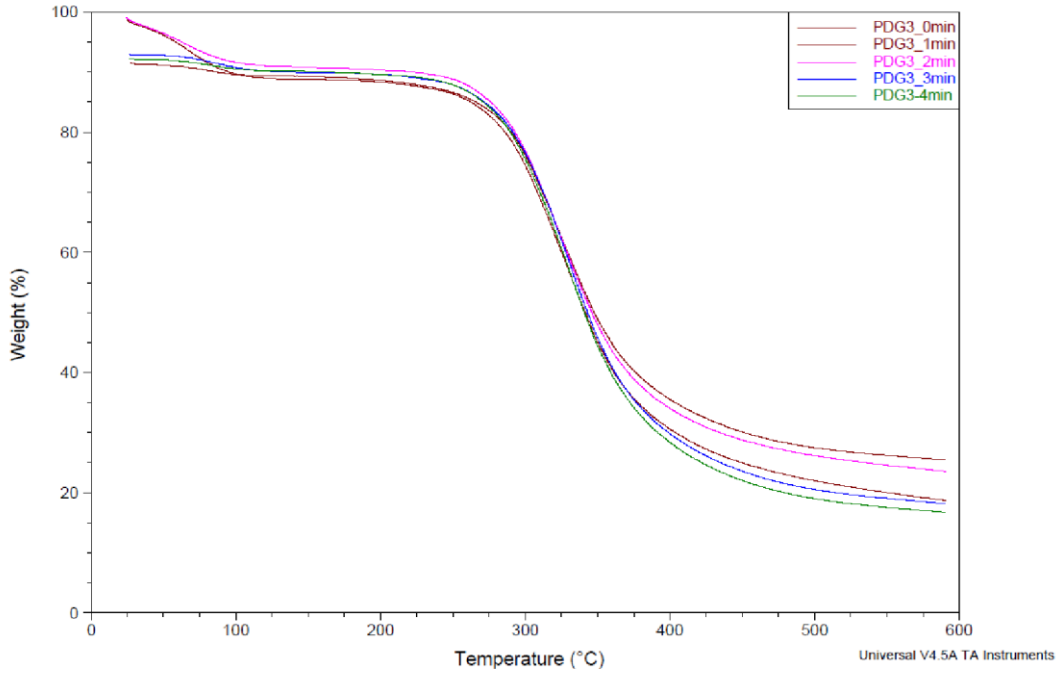
4.76. TGA thermograms of MDG2 irradiated at 1, 2, 3, 4 min and unirradiated.



4.77. TGA thermograms of PBG1 irradiated at 1, 2, 3, 4 min and unirradiated.



4.78. TGA thermograms of PDG1 irradiated at 1, 2, 3, 4 min and unirradiated.



4.79. TGA thermograms of PDG3 irradiated at 1, 2, 3, 4 min and unirradiated**.**

Table 4.25. The temperature for half-life ($T_{1/2}$) (^{0}C) for different radiation intervals

Sample	The temperature for half-life ($T_{1/2}$) (°C)				
	Irradiation Time (min)				
	0	1	2	3	4
HBG1	-	-	-	-	-
HL1	350	350	350	349	347
MBG3	-	462	-	-	-
MDG2	352	349	348	349	347
PBG1	-	-	-	-	-
PDG1	348	348	349	349	349
PDG3	335	333	336	334	335

Table 4.26. Residual percentage at 550 ^{0}C for different irradiation intervals

Sample	Residue at 550 °C (%)				
	Irradiaton Time (min)				
	0	1	2	3	4
HBG1	76	75	75	73	71
HL1	20	23	22	20	17
MBG3	63	44	52	55	58
MDG2	24	23	20	21	23
PBG1	69	68	66	65	60
PDG1	23	21	22	20	19
PDG3	25	18	22	17	16

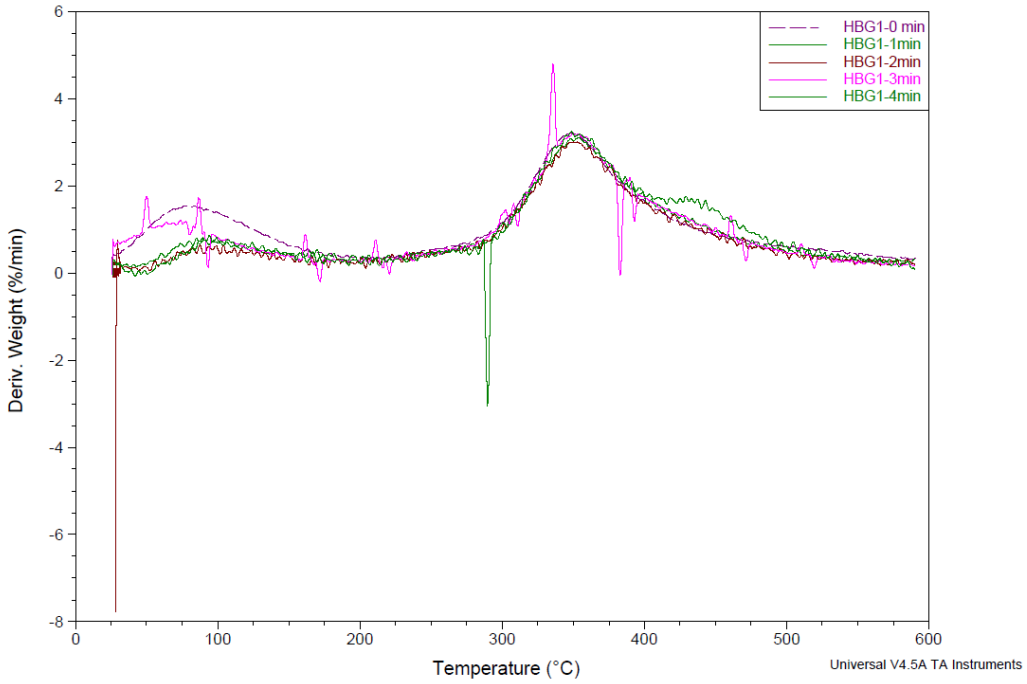


Figure 4.80. First derivative curves of TGA thermograms of HBG1 coded graft for different irradiation intervals by microwave.

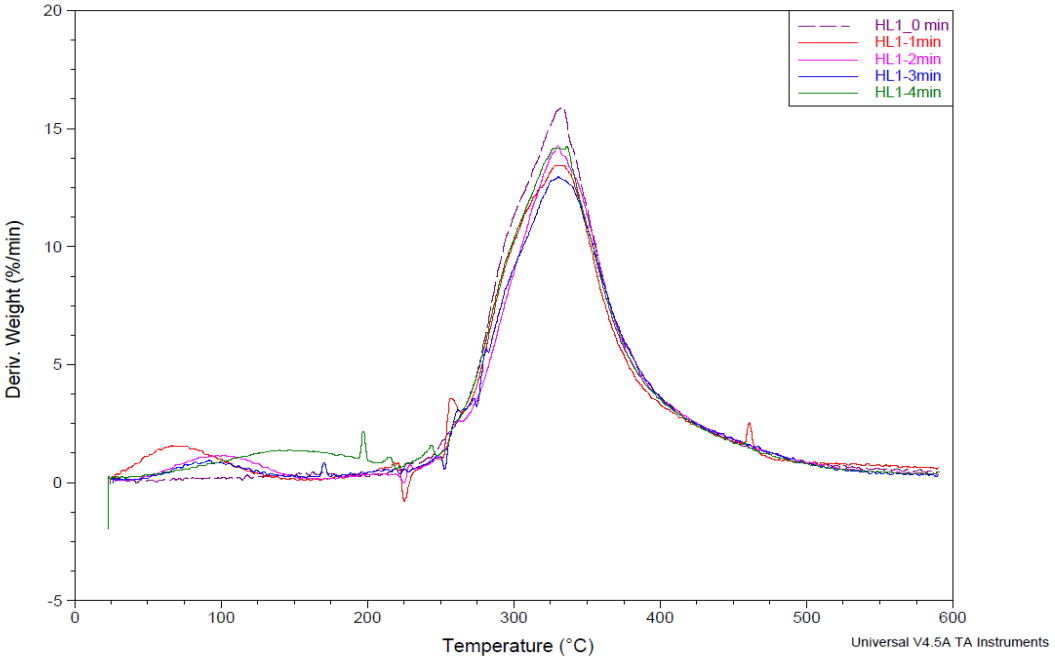


Figure 4.81. First derivative curves of TGA thermograms of HL1 coded graft for different irradiation intervals by microwave.

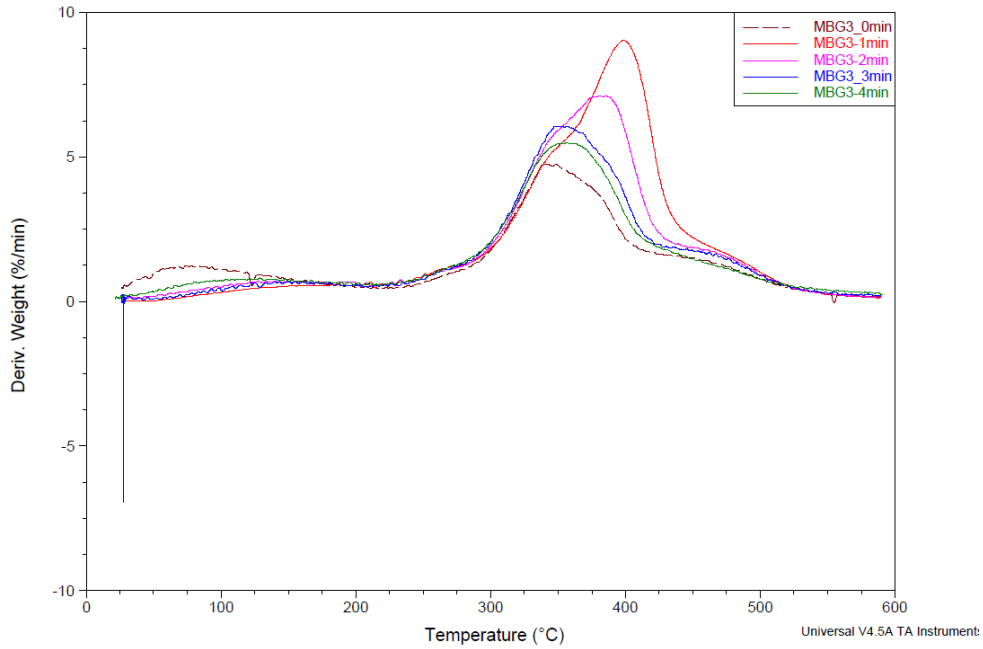


Figure 4.82. First derivative curves of TGA thermograms of MBG3 coded graft for different irradiation intervals by microwave.

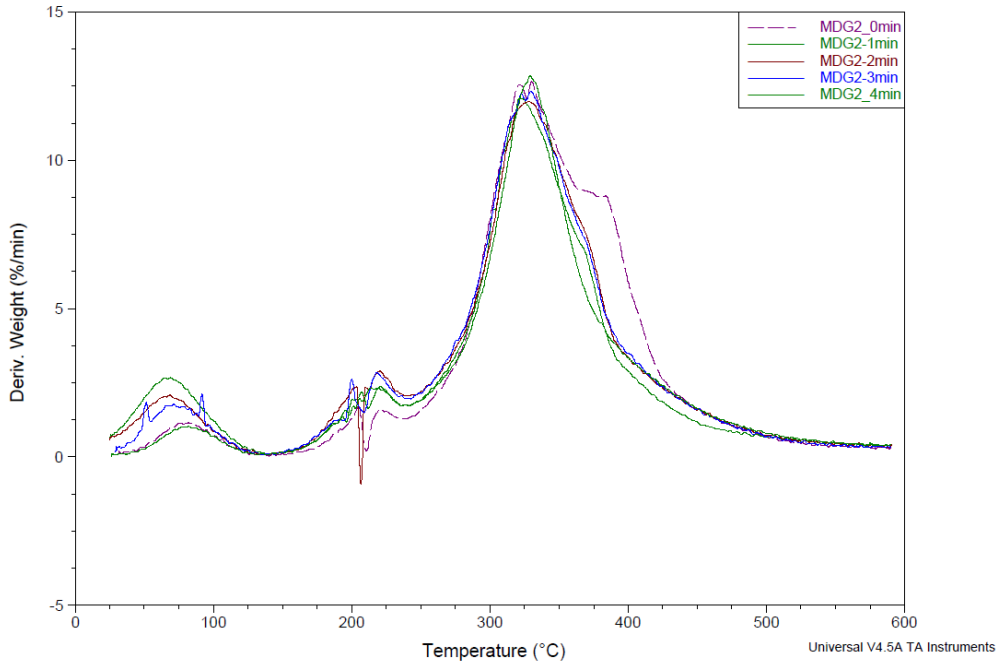


Figure 4.83. First derivative curves of TGA thermograms of MDG2 coded graft for different irradiation intervals by microwave.

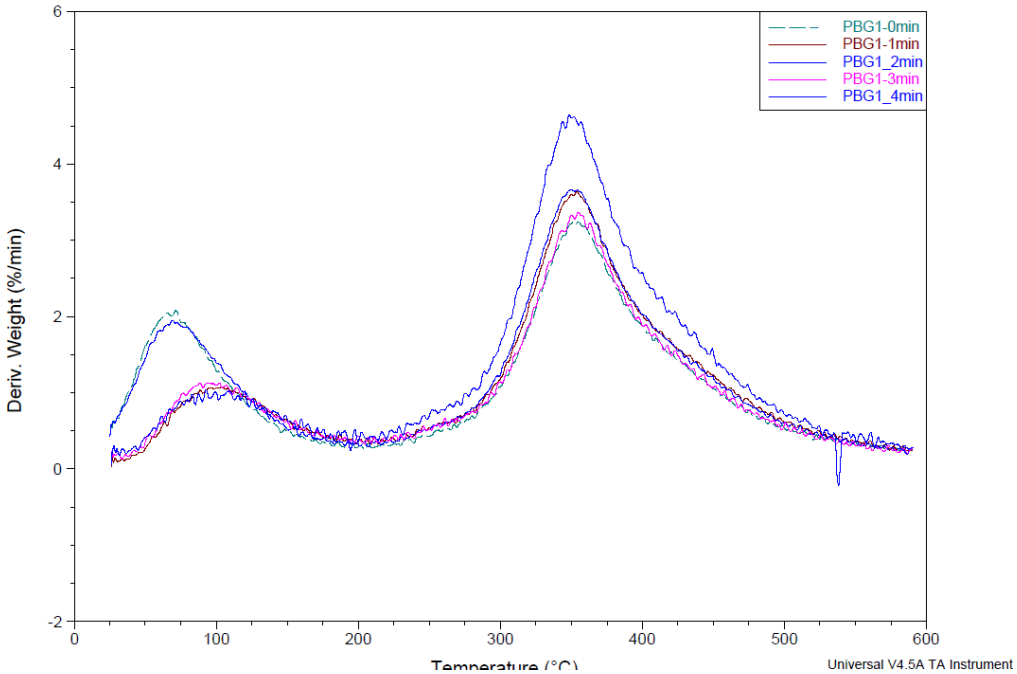


Figure 4.84. First derivative curves of TGA thermograms of PBG1 coded graft for different irradiation intervals by microwave.

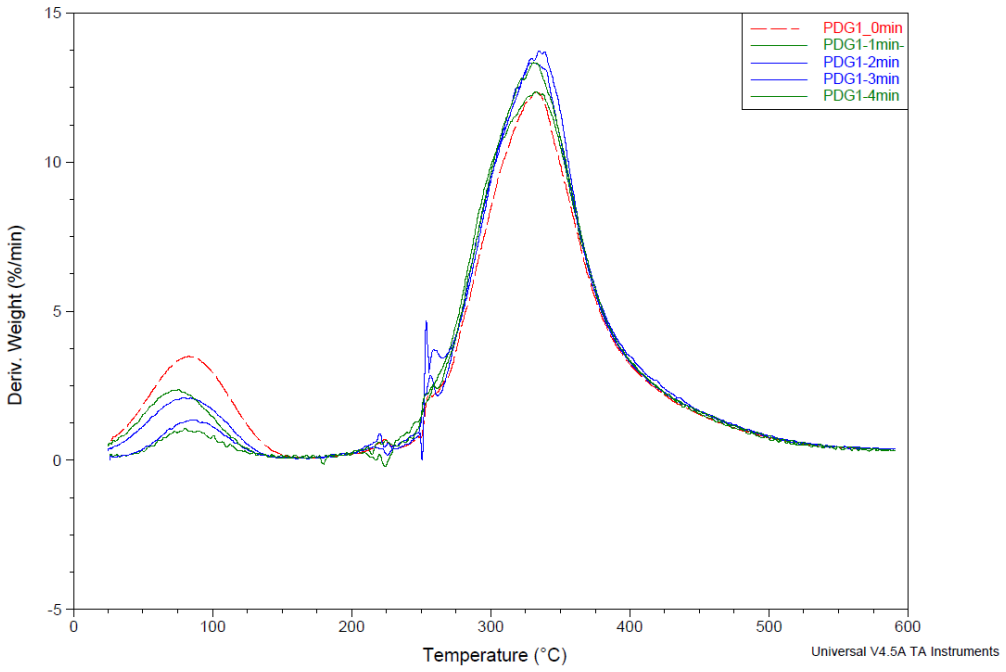


Figure 4.85. First derivative curves of TGA thermograms of PDG1 coded graft for different irradiation intervals by microwave.

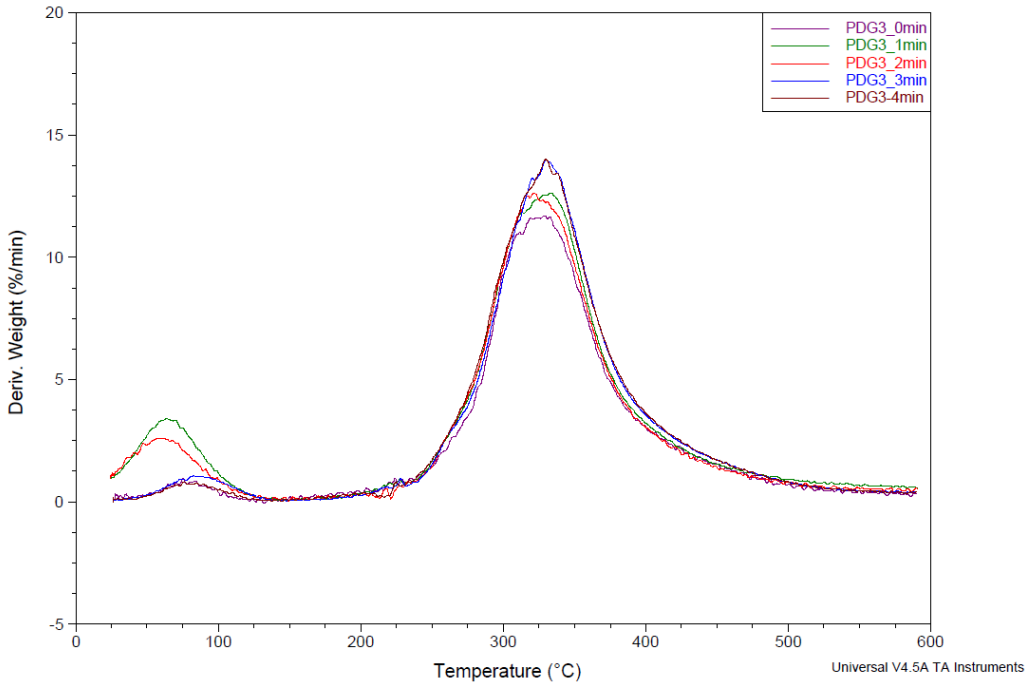


Figure 4.86. First derivative curves of TGA thermograms of PDG3 coded graft for different irradiation intervals by microwave.

Table 4.27. The temperature for maximum weight loss [T_{max} (^{0}C)]for different radiation intervals.

Sample	The temperature for maximum weight loss (T_{max}) (°C)				
	Irradiation Time (min)				
	0	1	2	3	4
HBG1	347.96	354.77	351.93	335.47	350.23
HL1	332.63	333.20	330.36	330.93	332.06
MBG3	339.44	373.02	382.02	357.61	358.18
MDG2	330.36	323.55	328.09	330.93	329.22
PBG1	353.07	354.77	350.80	355.91	353.07
PDG1	334.33	332.06	333.77	332.06	332.06
PDG3	329.79	335.47	322.98	330.93	329.79

SEM Analysis Results

SEM analyses were performed on all grafts as explained in Section 3.5.1. Results before (0 min) and after microwave irradiation with different radiation times (1, 2, 3, 4 min) were given in Figure 4.87 to 4.93.

HL1 sample has a kind of smooth, non-porous surface with a few numbers of not deep holes in the surface. No significant change was observed on the surface of HL1 after sterilization with microwave radiation.

A very stable elemental structure of the material and the porotic properties of HBG1 were observed after sterilization with microwave radiation. HBG1 did not cause any change in the surface and porosity of the sample after application of microwave radiation.

In MBG3 case, a strict and relief view of the surface was observed before and after sterilization at different time intervals. The same tough view was also observed. Moreover, when compared to the gamma irradiated MBG3 graft with microwave irradiation, the surface looked tougher due to the lost of water and has a completely dry view. This can be due to the thermal effect of microwave radiation which causes high temperature and evaporation of water but in general, it can be concluded that a significant change was not detected after irradiation.

In MDG2 case, as a dermal graft, had a fibrillary view from a close watch not porous; and after radiation, some little change in the fibrillar string order shapes was observed. However, it can not be approved that the irradiation made a significant change on it.

In PBG1, like other bone grafts it has a porous surface view and the size of pores was different and it had not a homogenous view and after sterilization also, the elemental structure of the material and the porotic properties have been observed stable. Therefore, sterilization with gamma radiation, PBG1 did not cause any change in the surface and porosity of the sample.

In PDG1, it had a combination of both globular and fibrillary view. Moreover, a dry fibrillary surface especially in high radiation, due to the loss of water caused by microwave thermal effect, was observed.

For PDG3 which was another dermal graft, a complex fibrillary matrix was observed. After sterilization, especially in high radiation doses, a slight change in the matrix shape was detected .An evaporation of water and dryer surface were observed in MBG3 and PDG1.

When grafts were compared with each other, HBG1 and PBG1 bone grafts were found stable showing that bones are more stable than dermal grafts.

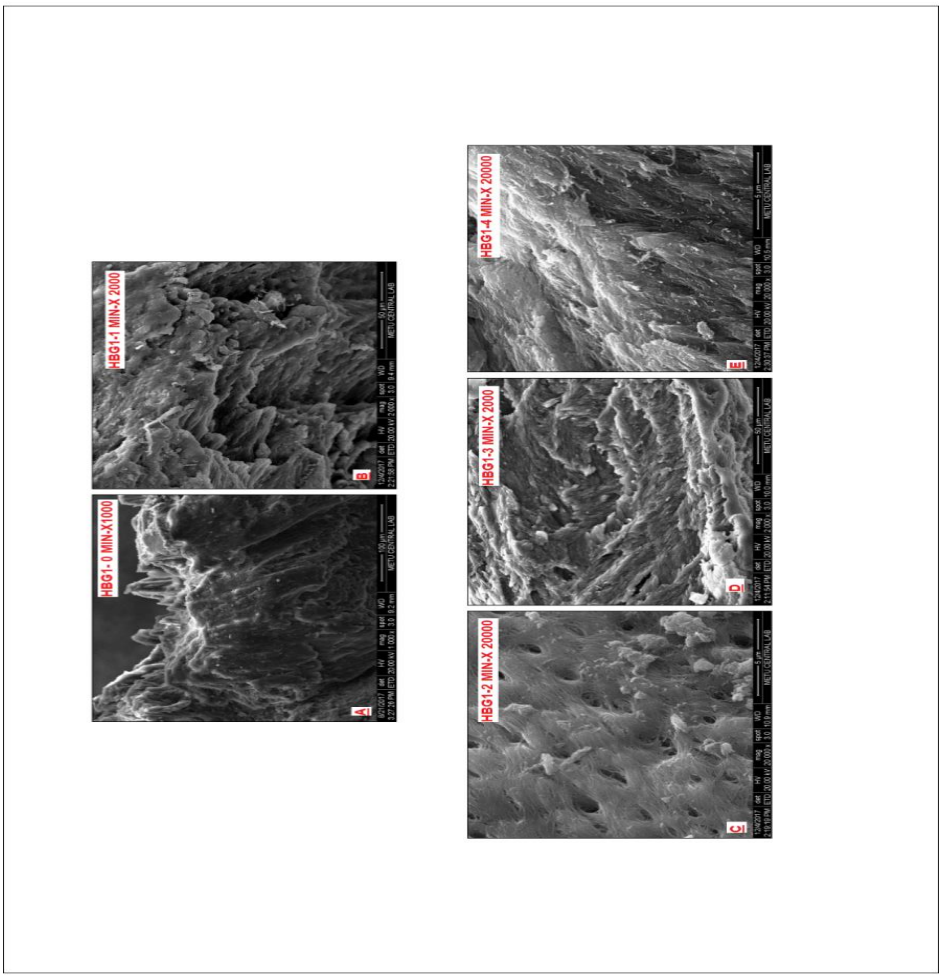


Figure 4.87. SEM images of HBG1 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one. A)HBG1-0 MIN-X1000, B)HBG1-1 MIN-X2000, C)HBG1-2 MIN-X2000, D)HBG1-3 MIN-X2000, E)HBG1-4MIN-X20000.

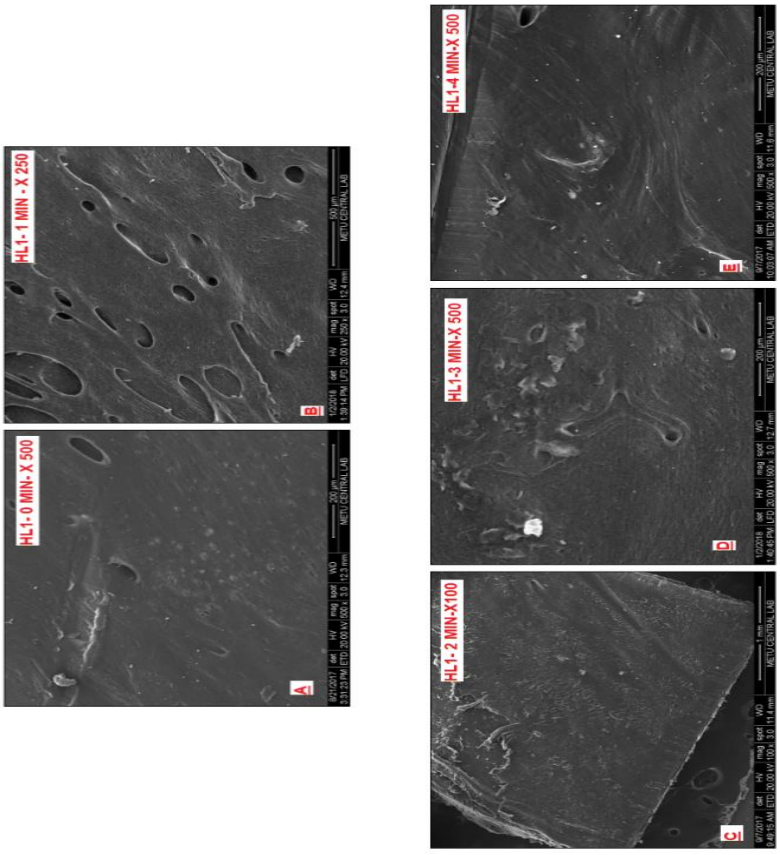


Figure 4.88. SEM images of HL1 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one A) HL1-0 MIN -X500, B) HL1-1 MIN-X250, C) HL1-2MIN-X100, D)HL1-3 MIN-X500, E)HL1-4 MIN-X500.

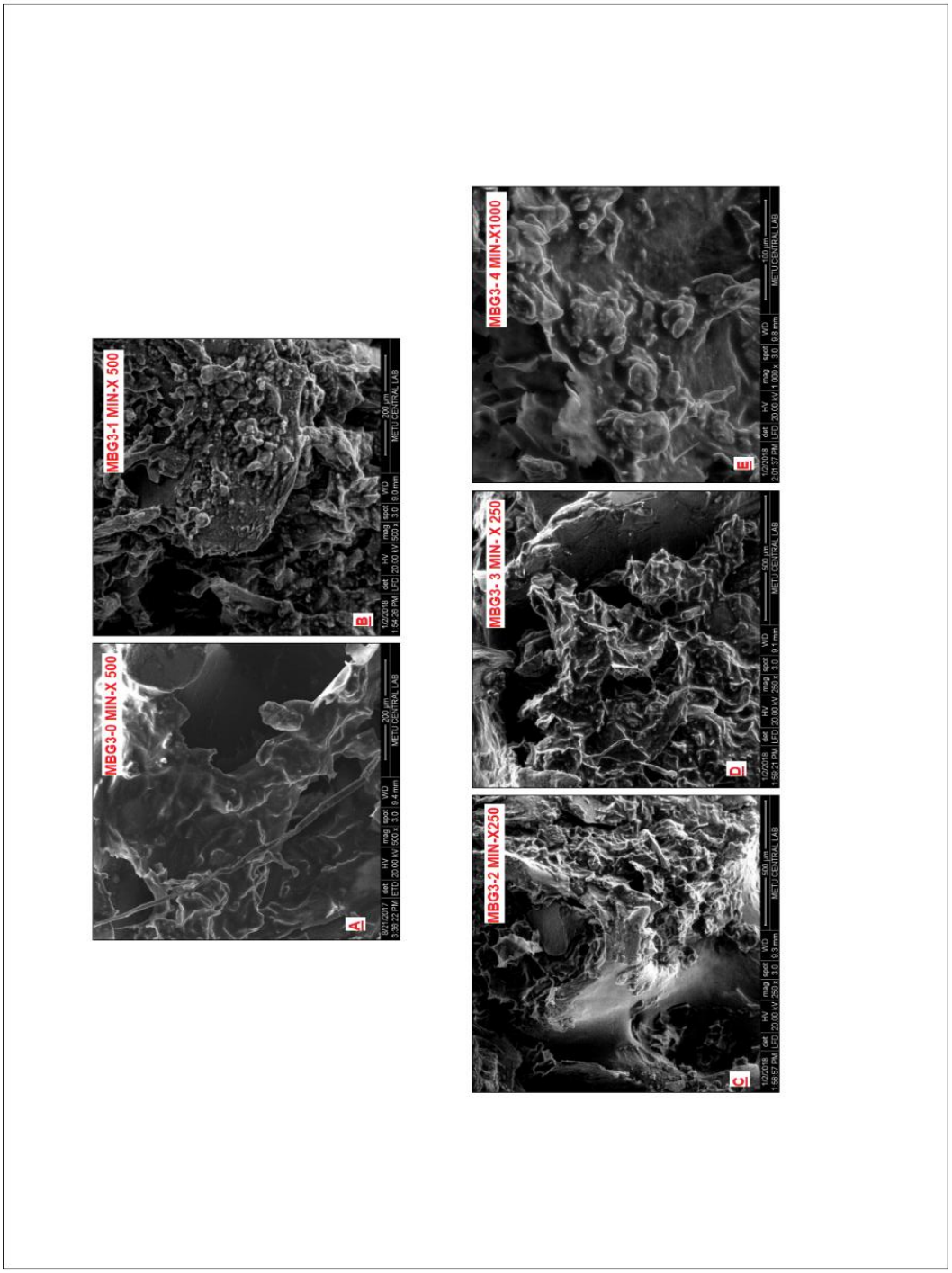


Figure 4.89. SEM images of MBG3 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one. A) MBG3-0 MIN-X500, B) MBG3-1 MIN-X500, C) MBG3-2MIN-X250, D) MBG3-3 MIN-X250, E) MBG3-4MIN-X1000.

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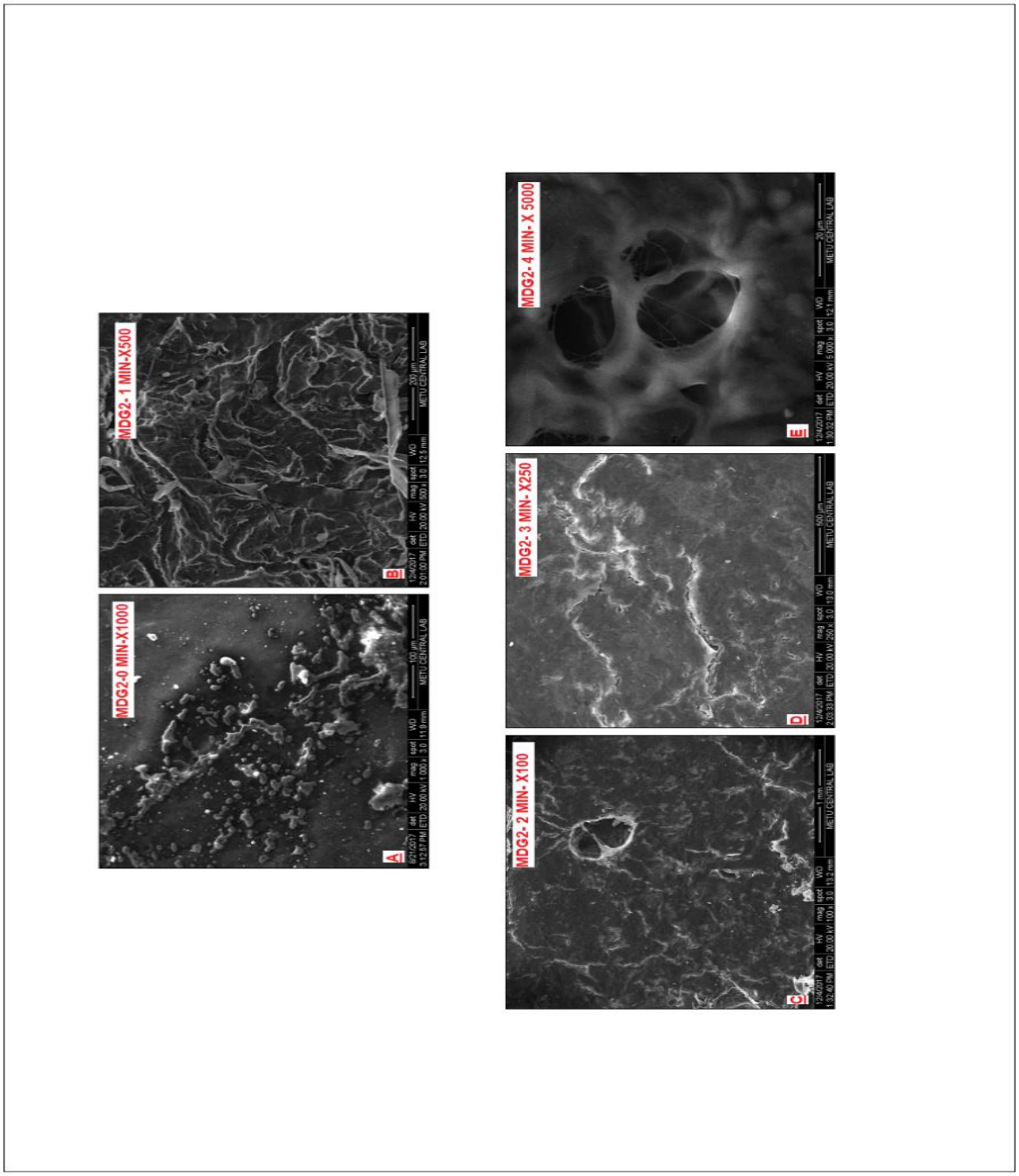

Figure 4.90. SEM images of MDG2 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one. A) MDG2-0 MIN-X1000, B) MDG2-1 MIN-X500, C) MDG2-2MIN-X100, D) MDG2-3 MIN-X250, E) MDG2-4MIN-X5000.

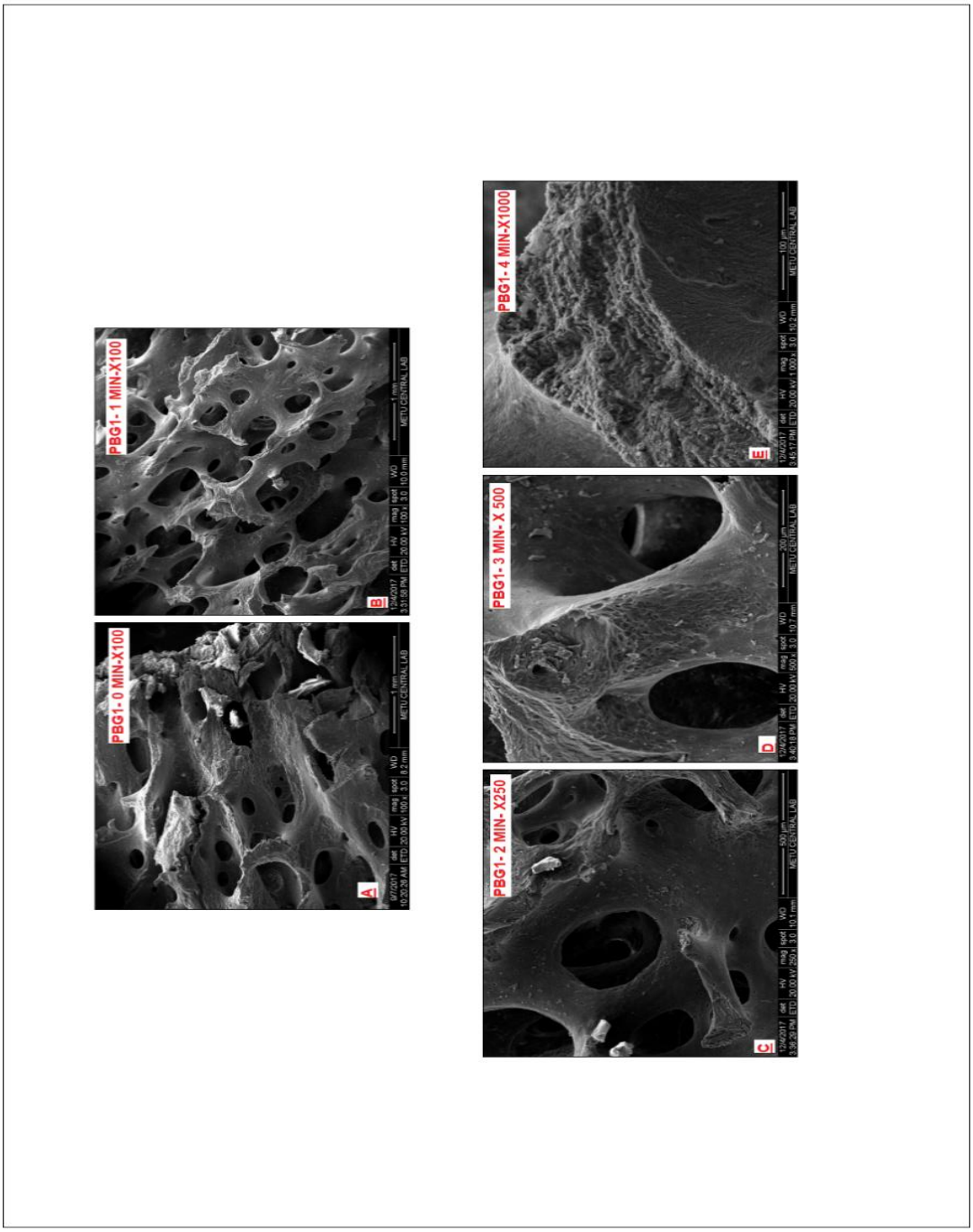

Figure 4.91. SEM images of PBG1 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one. A) PBG1-0 MIN-X100, B) PBG1-1 MIN-X100, C) PBG1-2MIN-X250, D) PBG1-3 MIN-X500, E) PBG1-4MIN-X1000.

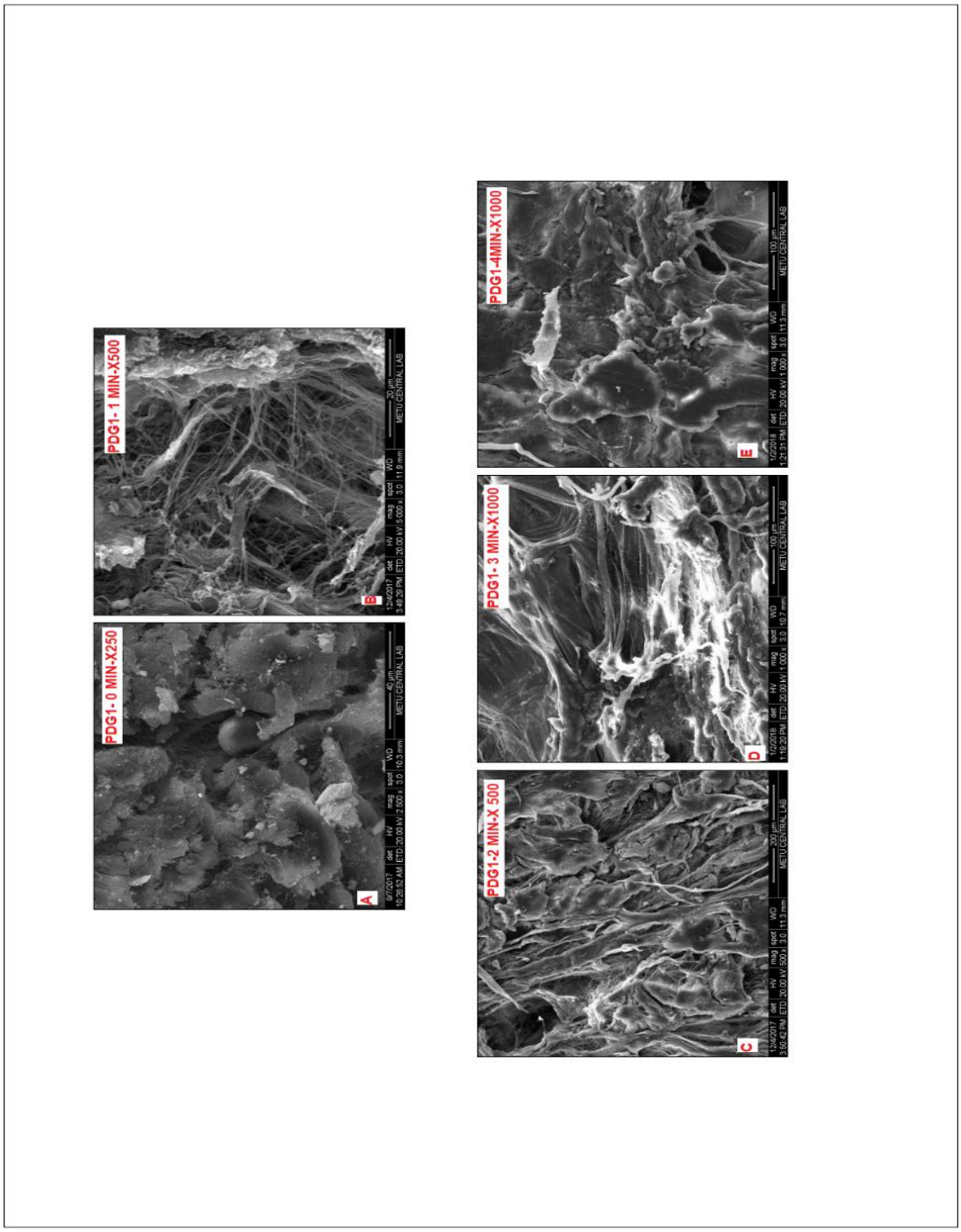

Figure 4.92. SEM images of PDG1 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one. A) PDG1-0 MIN-X250, B) PDG1-1 MIN-X500, C) PDG1-2MIN-X500, D) PDG1-3 MIN-X1000, E) PDG1-4MIN-X20000.

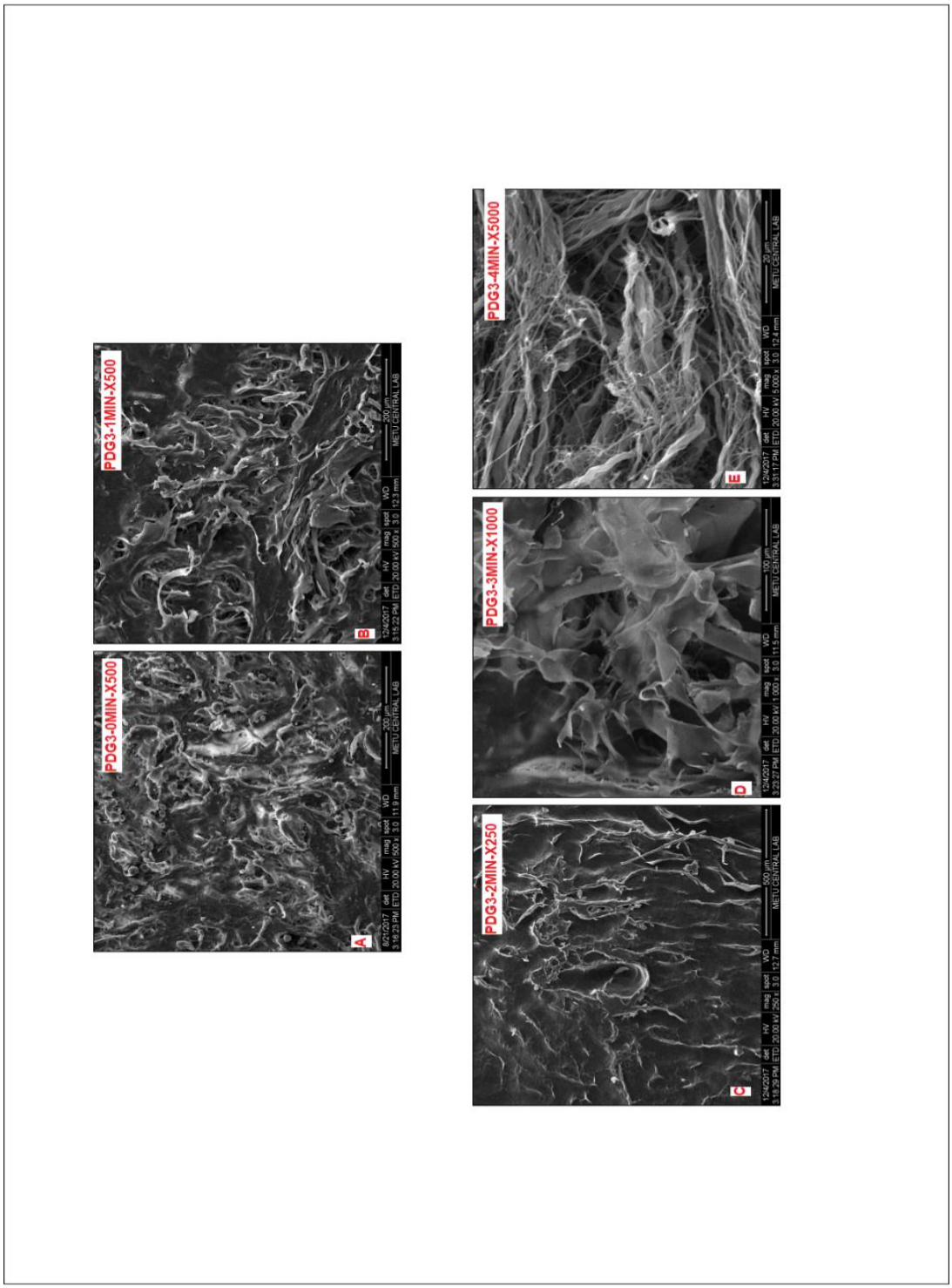

Figure 4.93. SEM images of PDG3 coded grafts irradiated at different time intervals (1, 2, 3, 4 min) of microwave radiation and unirradiated one. A) PDG3-0 MIN-X500, B) PDG3-1 MIN-X500, C) PDG3-2MIN-X250, D) PDG3-3 MIN-X1000, E) PDG3-4MIN-X5000.

SAXS Analysis Results

SAXS analysis was performed as explained in Section 3.5.1. SAXS analysis were performed for optimum irradiation time of microwave in which no growth of microorganism was detected and sterilization process was found successfull at 3 min for all grafts. On the other hand, other irradiation times were not analyzed due to the necessity of long time for SAXS analysis. Double distribution function graphics was given in Figure 4.94. Globular 3D nano structure images of the irradiated grafts at 3 min were given in Figure 4.95 .

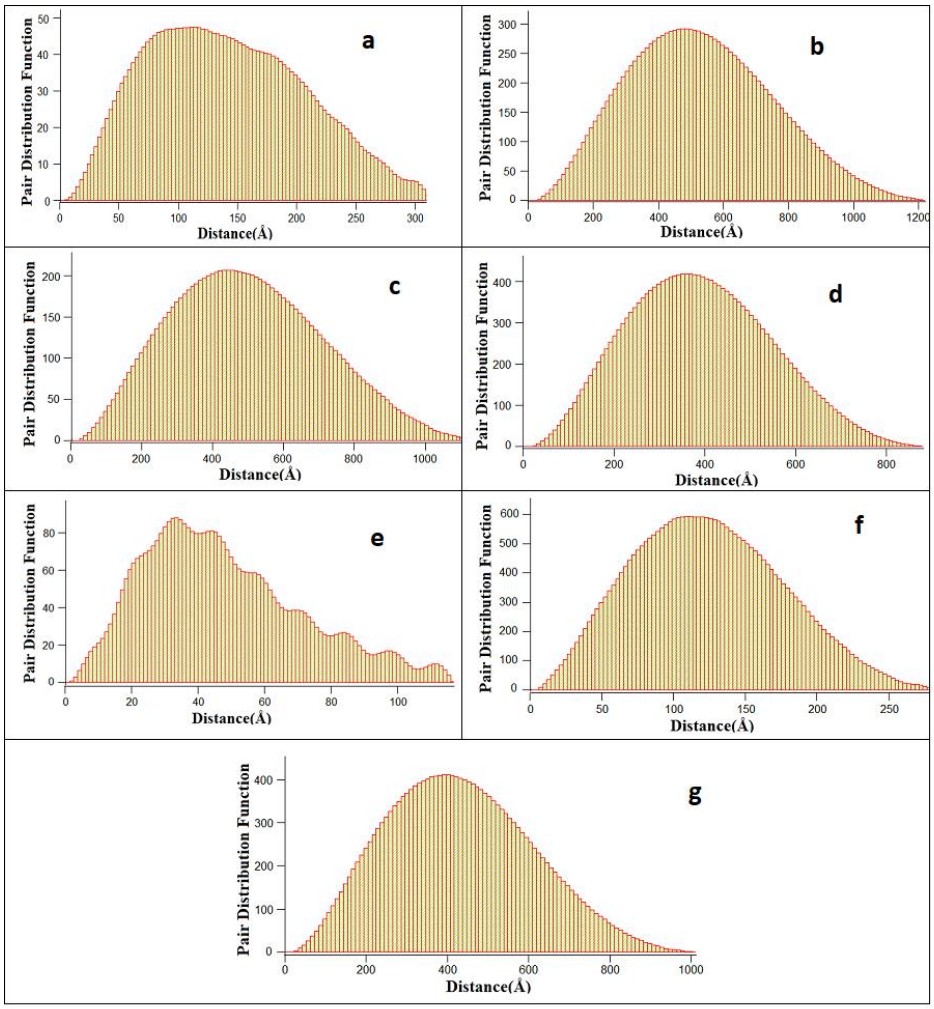

Figure 4.94. Double distant distribution function with SAXS analysis for a) HBG1, b) HL1, c) MBG3, d) MDG2, e) PBG1, f) PDG1, g) PDG3 coded grafts irradiated at 3 min.

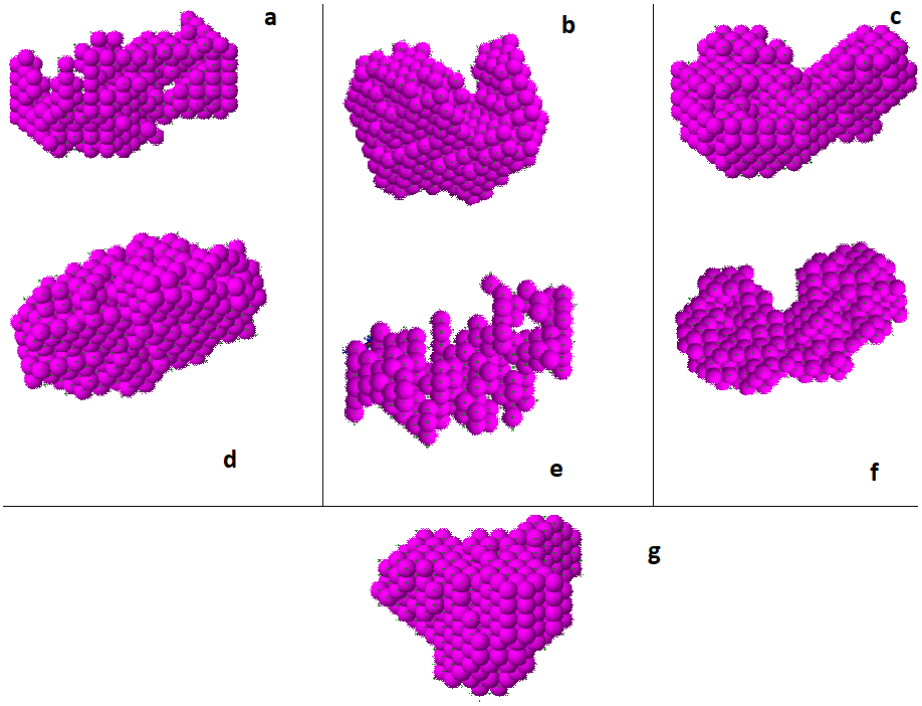

Figure 4.95. Nano globular struacture of a) HBG1, b) HL1, c) MBG3, d) MDG2, e) PBG1, f) PDG1, g) PDG3 coded grafts irradiated 3 min with microwave radiation.

ESR Analysis

ESR analysis was performed as explained in Section 3.5.1.

ESR Spectra of Irradiated Sampels of Unexposed and Different Doses

Microwave irradiation (even up to 8 minutes) did not caused significant difference in the ESR spectra of the samples ; So, ESR spectra of microwave irradiated samples have not given.

Dose-Response Findings

ESR Dose-response curves of all grafts after irradiation with microwave and for unirradiated as given in Section 3.5.1.

Microwave irradiation did not change the pattern of ESR spectra of HBG1. Only slight decreases were observed in the intensities of the resonance peaks of the samples and/or the area (second integral of the spectrum) of the sample (Figure 4.96).

Microwave irradiation did not change the pattern of ESR spectra of HL1. Besides there was no significant change in the intensities of the resonance peaks of microwave irradiated HL1 samples. As the intensity of the resonance peaks of the samples was relatively low, the change of the area (second integral) of the spectra of microwave irradiated samples were taken into consideration (Figure 4.97).

Microwave irradiation did not change the pattern of the ESR spectra of MBG3, only slight decrease was observed in the intensities of the resonance peaks of MBG3 and/or the area (second integral of the spectrum) of the sample (Figure 4.98).

Microwave irradiation did not change the pattern of the ESR spectra of MDG2 but continuous decreases in the resonance lines (also area) of the samples was observed upon high microwave irradiation doses (Figure 4.99).

Microwave irradiation did not change the pattern of ESR spectra of PBG1 but, slight continous decreases were observed in the intensities of the resonance peaks of PBG1 and/or in the area (second integral of the spectrum) of the sample (Figure 4.100).

Microwave irradiation did not change the pattern of ESR spectra of PDG1, a significant increase in the intensities of the resonance peaks of PDG1 was observed for 8 min microwave irradiated PDG1 sample (Figure 4.101).

Microwave irradiation did not change the pattern of ESR spectra of PDG3, but continous increases were observed in the intensities of the resonance peaks of PDG3 and/or the area (second integral of the spectrum) of the samples (Figure 4.102).

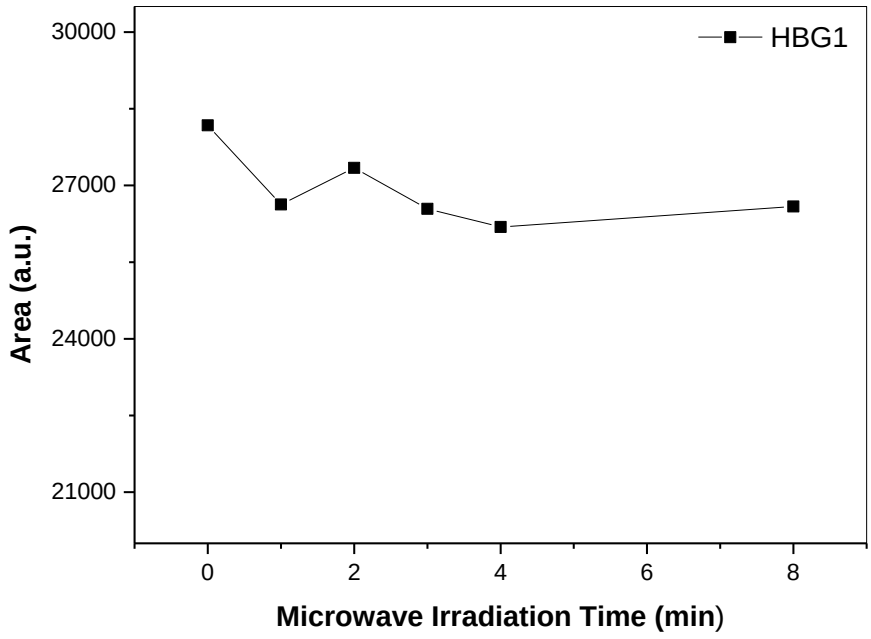

Figure 4.96. Variations of area of the spectra of HBG1 with absorbed microwave irradiation.

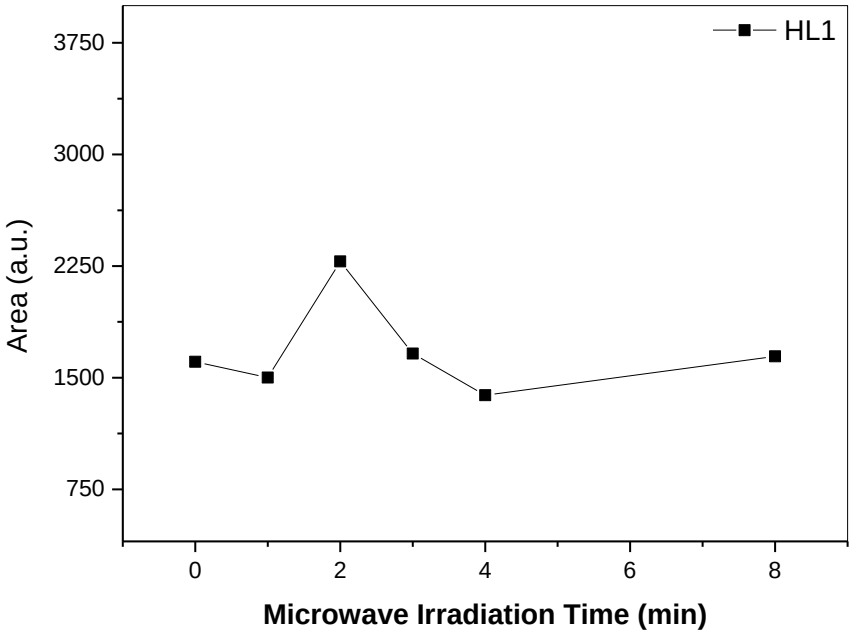

Figure 4.97. Variations of area of the spectra of HL1 with absorbed microwave irradiation.

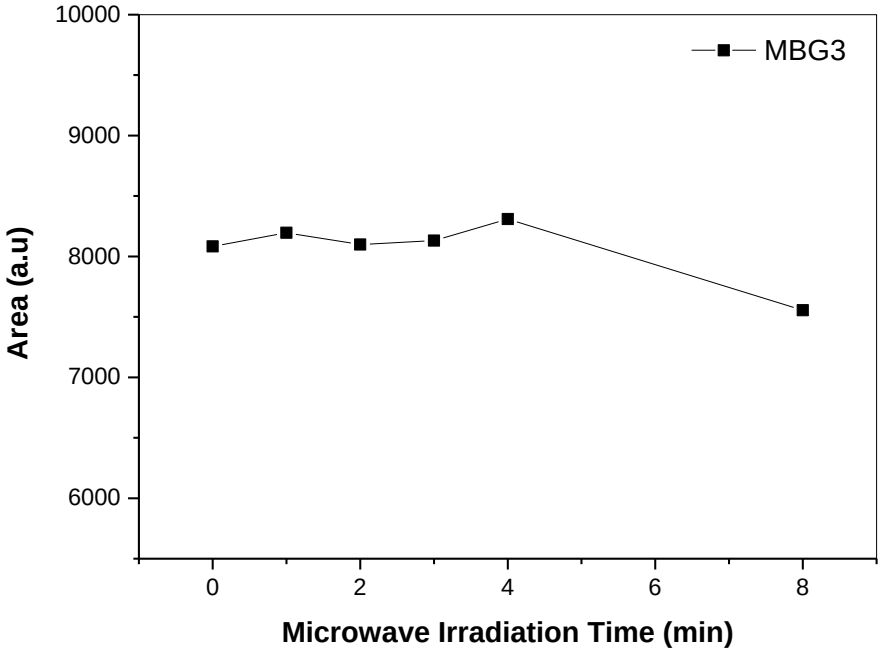

Figure 4.98. Variations of area of the spectra of MBG3 with absorbed microwave irradiation.

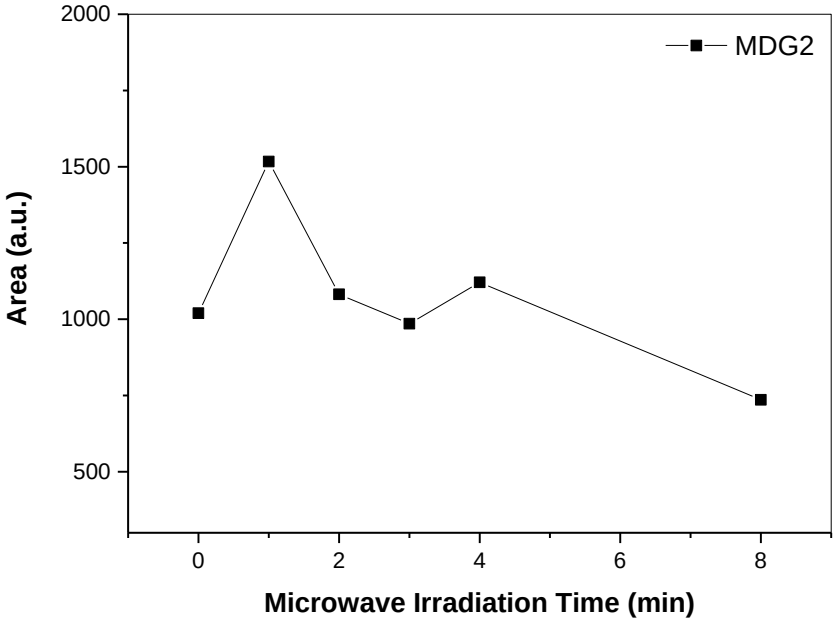

Figure 4.99. Variations of area of the spectra of MDG2 with absorbed microwave irradiation.

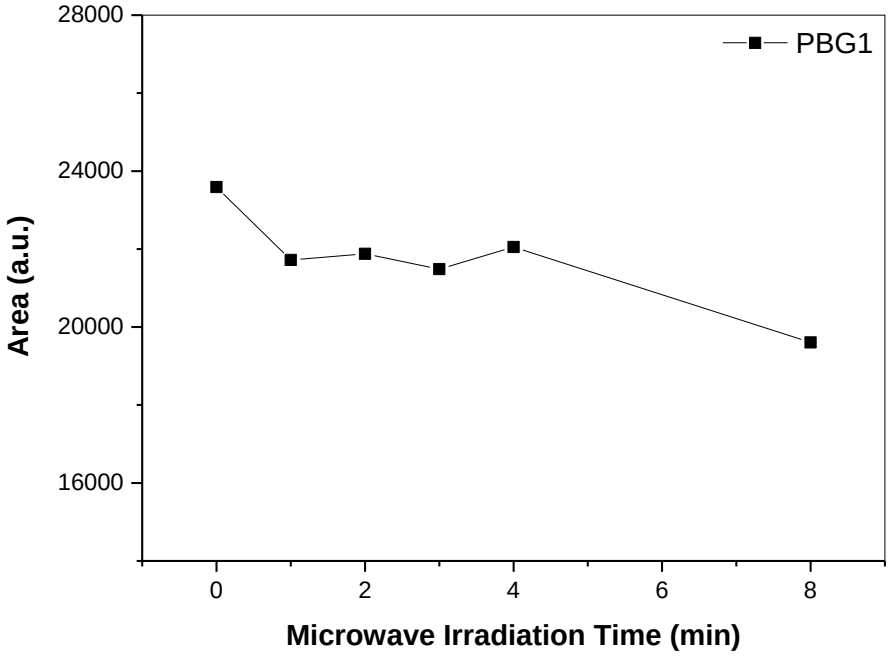

Figure 4.100. Variations of area of the spectra of PBG1 with absorbed microwave irradiation.

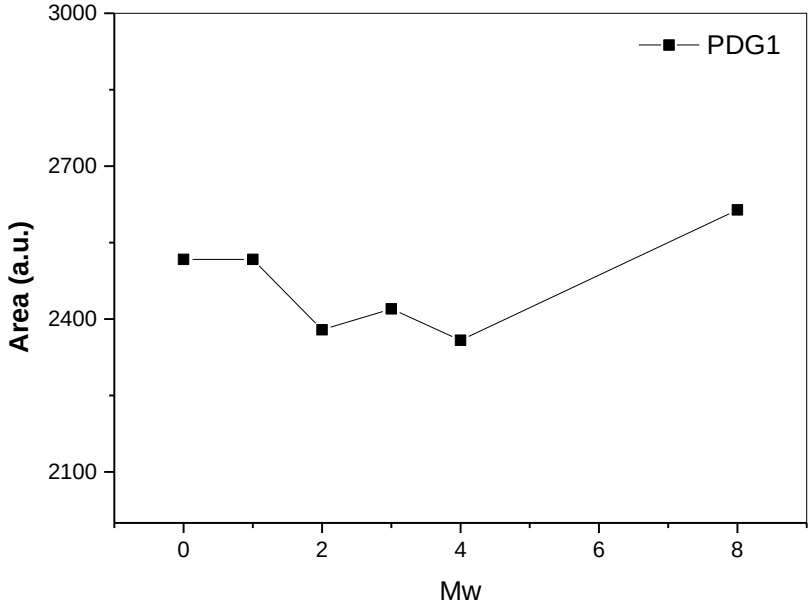

Figure 4.101. Variations of area of the spectra of PDG1 with absorbed microwave irradiation.

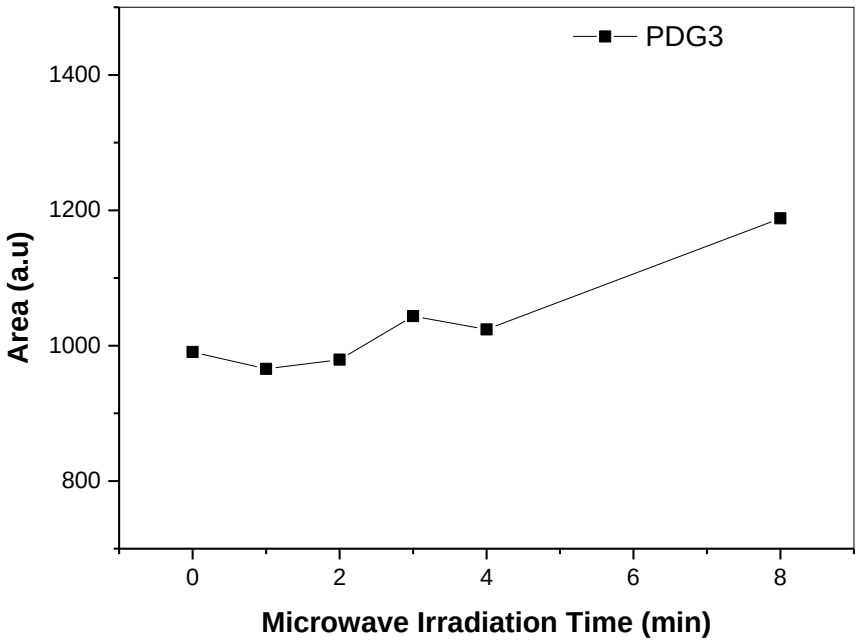

Figure 4.102. Variations of area of the spectra of PDG3 with absorbed microwave irradiation.

Long Term Studies

Due to limited time , a long term study was not performed for microwave irradiated grafts.

4.3.2. Results of Microbiological Tests

Microbiological tests were performed on all grafts as explained in Section 3.5.2 and 3.6.2.

Sterility Test Results

As microbiological tests, the sterility test, the determination of SAL doses and pyrogen test were evaluated. Sterility test for microwave irradiated HBG1, HL1, MBG3, MDG2, PDG1, PDG3, PBG1 coded grafts at different irradiation times as 1, 2, 3, 4 min was done as explained in Section 3.5.2.

The results of the sterility test on grafts after irradiation after 14 days were given in Table 4.28. No growth of microorganism was detected for 3 and 4 min.

Table 4.28. Sterility test results of grafts after sterilization with microwave irradiation for 3 and 4 min exposure time .

Grafts	Medium	
	FTM (35 ^{0}C)	SCDM (25 ^{0}C)
HBG1	-	-
HL1	-	-
MBG3	-	-
MDG2	-	-
PBG1	-	-
PDG1	-	-
PDG3	-	-

(-) No Microbial Growth.

SAL Test Results

SAL test was performed for all grafts as explained in Section 3.5.2. Microbial death rates for grafts were plotted and SAL values were determined (Log N/N_0 – Radiation Dose) (Figure 4.103 to 4.109).

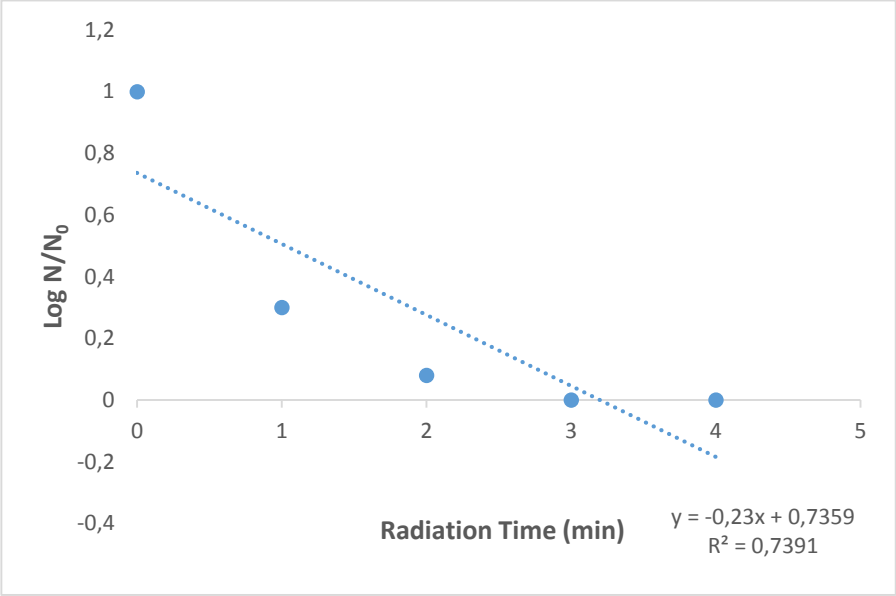

Figure 4.103. Microorganism dead graphic of HBG1 coded graft irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

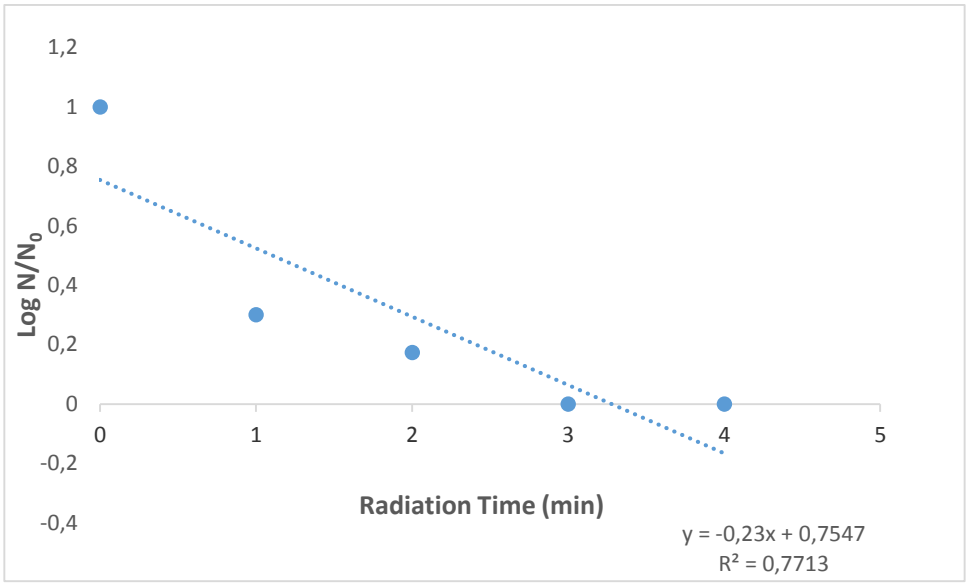

Figure 4.104. Microorganism dead graphic of HL1 coded grafts irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

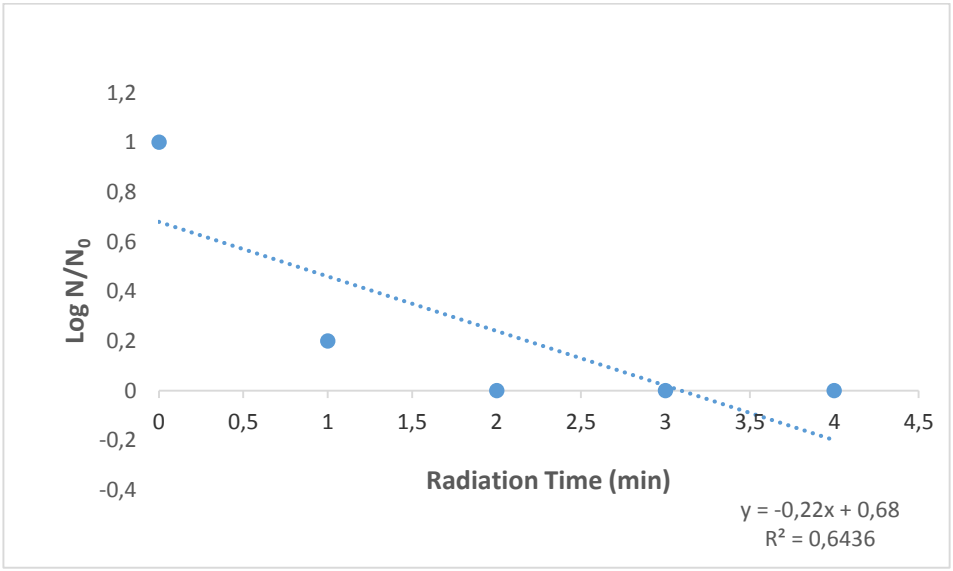

Figure 4.105. Microorganism of dead graphic MBG3 coded grafts irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

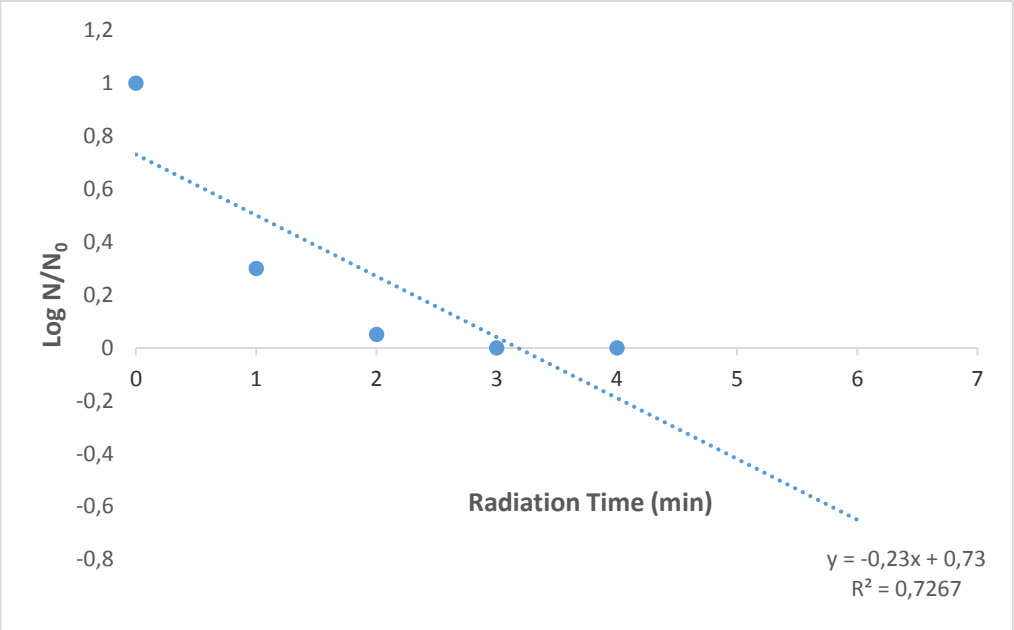

Figure 4.106. Microorganism dead graphic MDG2 coded grafts irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

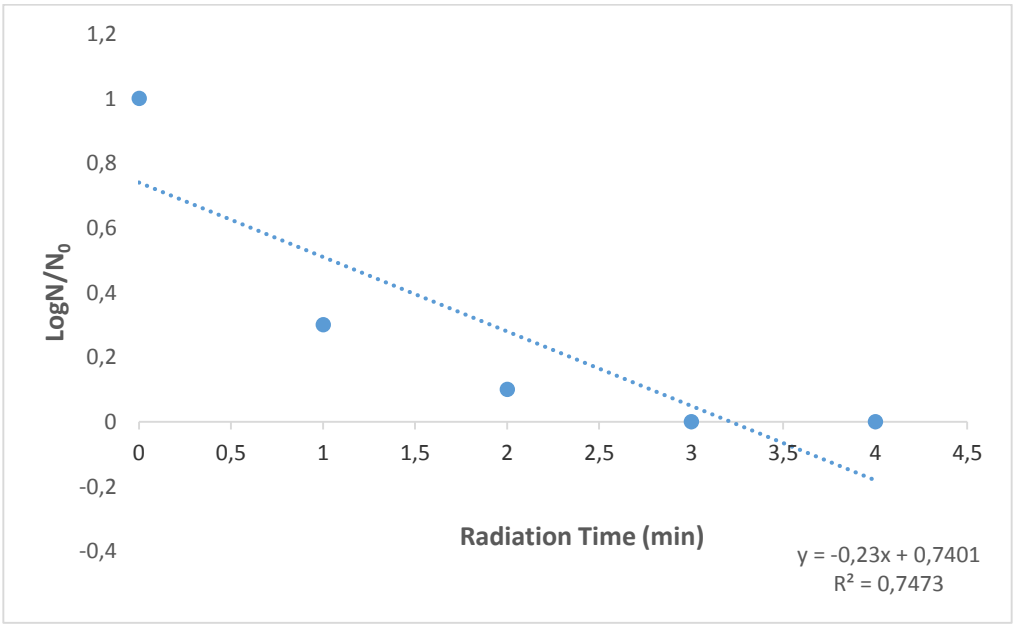

Figure 4.107. Microorganism dead graphic PBG1 coded grafts irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

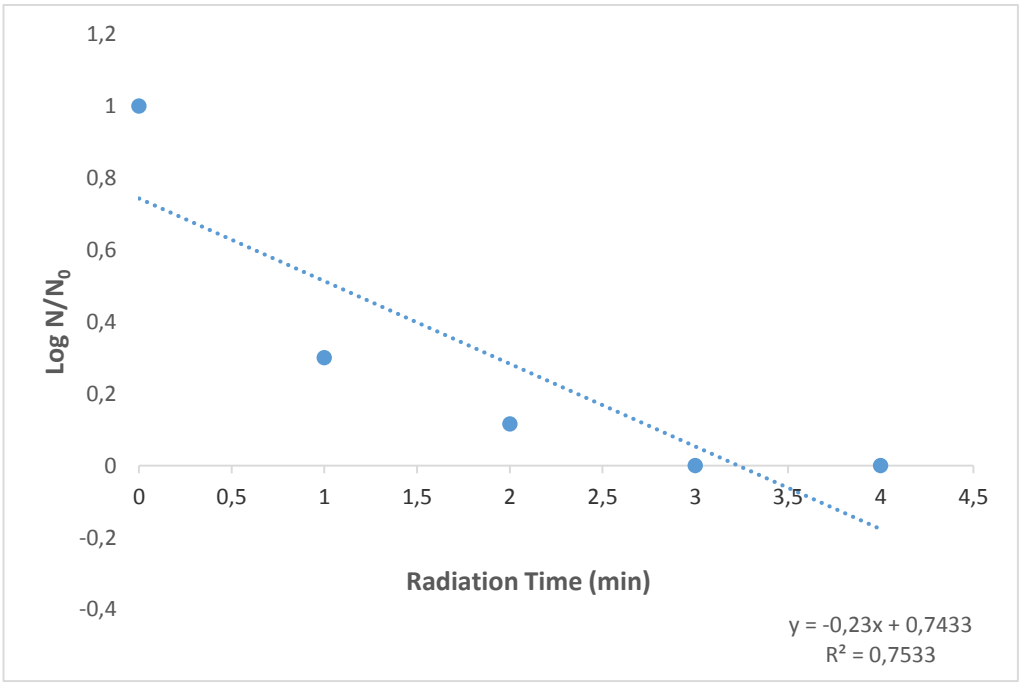

Figure 4.108. Microorganism dead graphic PDG1 coded grafts irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

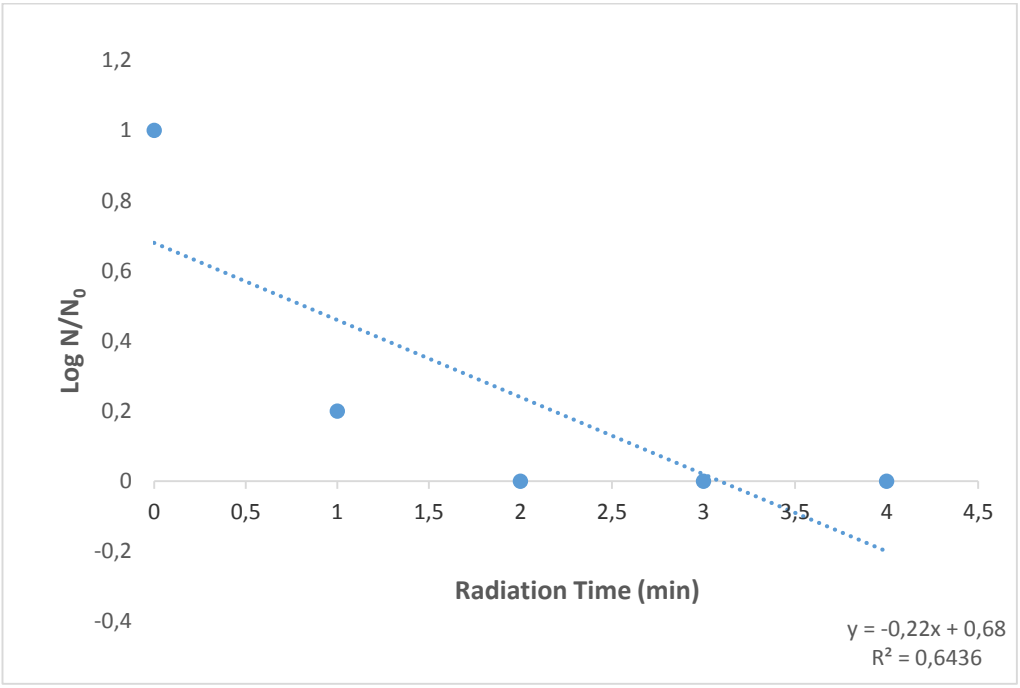

Figure 4.109. Microorganism dead graphic PDG3 coded grafts irradiated by microwave at 1, 2, 3, 4 min. (N= microorganism number after irradiation, N_0= microorganism number before irradiation).

Pyrogen Test Results

Endotoxin test was done by using a LAL Rapid Endotoxin Detection kit as explained in Section 3.5.2. for PDG1 coded graft irradiated at 900 Watt and 2450 MHz microwave radiation for 3 min. For medical devices, using the extraction volume recommendations described below, the limit is 0.5 EU.mL $^{-1}$ or 20 EU/device for products that directly or indirectly contact with the cardiovascular system and lymphatic system (85).Based on the results from pyrogen test , endotoxin amount for PDG1 coded graft was determined less than 0.25 EU.mL $^{-1}$.

4.4. Optimum Grafts for Two Sterilization Methods (Comparison)

At last when all the results of all alyses before and after sterilization process by both gamma and microwave irradiation have been evaluated in order to understand having the optimum features for two different sterilization methods and different analyses performed on the grafts . Moreover, optimum ones of dermal , bone and human , animal sourced also has been evaluated and given in Table 4.29 to 4.31.

Table 4.29. Optimum grafts chosen based on the sterilizatiom methods

Physicochemical Analyses	**Optimum Graft for Sterilization**	
	MICROWAVE STERILIZATION	**GAMMA STERILIZATION**
ORGANOLEPTIC	HBG1, MBG3, PBG1	HBG1, MBG3, PBG1
FTIR	HBG1, MBG3, PBG1	PBG1, HBG1
SEM	PBG1, HBG1, HL1	HBG1, MBG3, PBG1
TGA	HBG1, MBG3, PBG1	HBG1, MBG3, PBG1
ESR	-	PDG3
SAXS	MDG2, PDG3	HL1, PDG3

- Not determined.

Table 4.30. Optimum grafts chosen based on their type and sterilization method**.**

Physicochemical Analyses	Optimum Graft for Sterilization			
	DERMAL GRAFTS		BONE GRAFTS	
	MICROWAVE	GAMMA	MICROWAVE	GAMMA
ORGANOLEPTIC	PDG1, PDG3, MDG2	PDG1	MBG3, PBG1, HBG1	MBG3, PBG1
FTIR	PDG3	PDG3	MBG3	PBG1
SEM	PDG3	MDG2	PBG1	HBG1
TGA	PDG1	PDG3	HBG1	HBG1
ESR	-	PDG3	-	MBG3
SAXS	PDG3	PDG3	MBG3	HL1

- No optimum graft .

Table 4.31. Optimum grafts chosen based on their source and sterilization method.

Physicochemical Analyses	Optimum Graft for Sterilization					
	HUMAN SOURCED		ANIMAL SOURCED			
	Gamma	Microwave	PORCINE		HORSE	
			Gamma	Microwae	Gamma	Microwae
ORGANOLEPTIC	MBG3	MBG3	PBG1	PBG1	HBG1	HBG1
FTIR	MBG3	MBG3	PBG1	PBG1	HBG1	HBG1
SEM	MBG3	MBG3	PBG1	PBG1	HBG1	HBG1
TGA	MBG3	MBG3	PBG1	PBG1	HBG1	HBG1
ESR	MBG3	-	PDG3	-	HL1	-
SAXS	MDG2	MDG2	PDG3	PDG3	HL1	HL1

- No optimim graft.

5. DISCUSSION

5.1. Materials Used for Sterilization

Dentistry is an occupation that helps tooth to have straight and decent function and esthetics (5). Dentistry treatments can expand from a simple teeth whitening operation to different complex bone or dermal (soft tissue as gingival) orofacial surgeries (86) like GTR. Grafts that are evaluated in this thesis are periodontal materials that are used in periodontology (branch of dentistry) for the purpose of tissue regeneration as directed tissue regeneration. These materials have the ability to regenerate lost tissues. Grafts as physical barriers are placed in the dental soft tissue, bone or surface of the teeth during the surgery and act as a barrier in these places. Placed grafts as barriers make a gap between the gingiva and tooth root surface and make an environment around the lost tissue area and in the remaining periodontal bond tissue. It helps selective fibroblast grow to the tooth root surface. These fibroblasts inhibit epithelium cells migration and allow the relatively slow-growing periodontal ligament fibroblasts to be repopulated over the root surface (87).

Due to the many cross contamination and infection reports in dental care and especially in GTR method, disinfection and asepsis gained a significant importance during last years. Different companies apply various sterilization methods for sterilizing their products based on the materials' composition and the required sterility level. Generally gamma irradiation is used by these companies as a safe and reliable method of sterilization. Additionally, microwave radiation can be a successful radiation sterilization to replace with gamma radiation. Because, it is easy for the application of sterilization process and has a lower cost in comparison to gamma radiation. In this study, microbiological aspect and different physicochemical effects caused by both gamma and microwave radiation methods were evaluated.

In this thesis, seven different grafts from three different companies were evaluated. These grafts are categorized into dermal and bone grafts from human, porcine and horse sources.

Composition of the materials used in this thesis were given in Table 3.1 and Table 3.2. Beta calcium phosphate, bioglass and collagen are commonly used biodegradable polymers. Polylactic acid (PLA) and poly (lactic acid-co-glycolic acid) (PLGA) are materials mostly used in dental repair and GTR. Materials selected for this study are the materials used in clinical dentistry and periodontology very frequently. Sterilization of these materials used in periodontology is important .. Apart from microbiological changes, physicochemical characteristics of materials are also vital subject. In the scope of this thesis, microwave and gamma radiation sterilization were applied to all grafts in different doses initially. Afterwards, physicochemical and microbiological characteristics of the material were compared for all grafts before and after sterilization. Based on these results, the optimum sterilization conditions were determined (88).

5.2. Sterilization Methods and Dose/Time Selection

Gamma radiation is approved as an appropriate sterilization method for both pharmaceuticals and various kinds of medical devices accepted by European Pharmacopiea (43, 82). The most important advantageous of gamma radiation are its high penetration ability and the capability of sterilization of materials that are sensitive to heat than the other sterilization methods. Lethal effect of ionizing radiation on the microorganisms is a big discovery in the first year of the past century. Sterilization by ^{60}Co radiation is a fast, reliable, effective and easy method. This sterilization method gives the producer companies the possibility of sterilization their products in their final package. Sterilization dose that is suggested by pharmacopeias (14,82) and frequently used is 25 kGy (43).

Although there are various studies (21,22,89) about introducing gamma radiation as an appropriate sterilization method for various medical devices, there are still some discussions about disadvantageous and adverse effects of gamma radiation on the materials after sterilization. As an example, 25 kGy which is the sterilization dose for the most of the pharmaceuticals, 1-5% degradation has been detected (44). Critical point in sterilization with gamma radiation can be the radiation dose because, the effect of lower doses can be lesser when compared with higher doses in general. Sterilization dose for pharmaceuticals depends on the microbial burden, the

radioresistancy of the microorganisms and the chemical structure of the products (44, 90). Therefore, in the scope of this thesis, other radiation doses (lower and higher doses than standard 25 kGy) as 2, 4, 5, 10, 50 kGy were investigated. The reason for choosing 50 kGy as a very high radiation dose was based on some recent studies that the inactivation dose for HIV viruses is more than 50kGy (12) . Inactivation of HIV as a big threat for human being, should be considered as an important subject in sterilization. Additionally, 50 kGy provided accelarated condition for the sample has a potentional for degradation.

In general, 25 kGy is accepted as a dose that can provide 10^{-6} SAL based on the GMP conditions (36). 10 kGy is chosen as the dose for decontamination (for foods,cosmetics, herbal drugs and etc.). Moreover, other lower doses were investigated in this study for the determination of precise irradiation dose for each sample in parallel to the SAL level. The effect of gamma radiation on different molecules is a subject that needs a deep investigation because of its effect on different molecules might be different. So, it is not acceptable to make a generalization about the exact effect of gamma radiation on the materials.

On the other hand, microwave radiation sterilization as another option has gained attention of researchers, recently (91,61). Microwave radiation as a sterilization method can be a good alternative for gamma radiation due to its easily applicable condition and low costs in comparison to gamma ray. However, it is still a novel method and its reliability is not approved completely and its different possible effects on different materials are still not confirmed. Electromagnetic energy in the microwave region (225 MHz to 100 GHz, typically 2450 MHz) is extensively studied as one of the alternative energy sources for sterilization. The electromagnetic field strength and the exposure time are two factors that affect the efficiency of microwave sterilization. The electromagnetic energy is expressed largely in two groups:

(i) **Thermal effect** : The factors that depend on the dielectric properties of the dipole molecules of the irradiated materials in the form of heat ,

(ii) **Nonthermal effect** :The factors that do not depend on the dipole molecules in the form of a direct effect of the radiofrequency.

However, the differentiation of thermal and non-thermal effects of microwave sterilization is still controversial (46). The changing factor in microwave sterilization (in 900 Watt and 2450 MHz) is exposure time.

Singh and Singh have evaluated microwave and gamma radiation sterilization for bone allografts (62). Bone allografts were irradiated in microwave oven (2450 MHz) at different radiation times as 1, 2, 3, 4, 5 and 6 min after the determination of microbial load. According to this study, the sterilization of allografts was found successful at 2 min of irradiation.

Based on another study performed by Katsufumi and co-workers (63), microwave radiation sterilization as a developed heating method, was applied to bovine femoral head and the thermal effect of microwave radiation was detected. The average temperature of the bovine femoral head became 80°C in 15 min of microwave irradiation during sterilization process. The use of microwave irradiation enables quick heating for disinfection of large allograft bones when a hot-air supply was used as well. An effective sterilization was achieved.

In this thesis, grafts were contaminated with Bacilus pumulis in the same way that it was done for gamma radiation sterilization. Then the materials were irradiated by microwave radiation in microwave oven with 900 Watt and 2450 MHz at four different irradiation times as 1, 2 , 3 and 4 min.

The main goal of this research is to compare the effects of two different sterilization methods on the grafts and to evaluate physicochemical characteristics and microbiological load before and after sterilization process inorder to determine the most effective and appropriate sterilization method. During this study, dermal and bone grafts from human and animal sources coded as HBG1, HL1, MBG3, MDG2, PDG1, PDG3 and PBG1 were irradiated by gamma radiation in 2, 4, 5, 10, 25, 50 kGy and another group as the same materials were irradiated by microwave radiation at 1, 2, 3, 4 min. Physicochemical and microbiological tests were done on all the irradiated materials and the results were compared. So, the effects of two different sterilization methods were evaluated on the grafts and the feasibility of both method were investigated.

5.3. Validation Method Before Sterilization

Validation processes before and after sterilization for both gamma and microwave irradiated grafts as biological and dosimetric validations were done as explained in Section 3.3.3. According to the dosimetric validation, the dose of gamma radiation that was absorbed by materials were found the same to the dose that was calculated for all doses except 1 and 4 kGy (Table 4.1). The sterilization doses of 5, 10, 25, 50 kGy were found as 5.09, 10.15, 25.08 and 50.05 kGy as actual absorbed doses, respectively. According to the biological validation results, there was no growth of B. pumulis and B. atrophaeous spores used in the validation process in which both of these spores are radioresistant. These results were both valid for both gamma and microwave irradiation.

5.4. Analyses of Pre- and Post-Sterilization with GAMMA Radiation of Grafts

All results about analyses performed before and after gamma radiation sterilization were given at Section 4.2. In the following section, results of physicochemical and microbiological analyses were evaluated.

5.4.1. Physicochemical Analyses

The results covering organoleptic tests, SEM, SAXS, TGA, FTIR, ESR analyses and microbiological tests (sterility, pyrogen, SAL dose determination) were discussed in this section.

Organoleptic Analysis

Organoleptic features of gamma irradiated and unirradiated grafts were compared. According to the results, there was no significant change in both dermal and bone grafts after irradiation and a slight change in color and in HL1 at 25 and 50 kGy irradiation, MDG2 at 25 and 50 kGy and PDG3 at 50 kGy were observed. In a study performed by Rodrigues and co-workers reported the effects of a 25 kGy dose of gamma radiation on the mechanical properties of enamel and its resistance to demineralization were evaluated and it showed color changes in bone tissues after

gamma irradiation .It was mentioned that these changes in color could be the effect of denaturation of organic components (92).

In another study performed by Ruben et al showed a change in color at 25 kGy of gamma irradiation of dental materials (93). A study done by Turker et al showed color change in grafts even at 5 kGy irradiation gamma ray . In this study two types of grafts were irradiated by gamma radiation at 5, 10, 25 and 50 kGy and change in color at all radiation doses were observed.

As a result in general, it can be concluded that grafts used in this study have shown a great stability after irradiation even at high exposure doses of gamma ray like 50 kGy . HBG1, MBG3, PBG1 and at last PDG1 showed a great stability and no changes were observed even at high doses. HL1, MDG2 and PDG3 showed lower stability and radioresistance dose and it can be said that, this change can be due to the weaker structure of dermal matrix in comparison to bone for MDG2 and PDG3 as two different dermal grafts.

FTIR Analysis

FTIR is a method which determines the structure of molecules with the molecular characteristics of absorption of infrared radiation. Infrared spectrum is a molecular vibrational spectrum. When exposed to infrared radiation, sample molecules selectively absorb radiation of specific wavelengths which cause the change of dipole moment of sample molecules. Consequently, the vibrational energy levels of sample molecules transfer energy from ground state to excited state. The frequency of the absorption peak is determined by the vibrational energy gap. The number of absorption peaks is related to the number of vibrational freedom of the molecule. The intensity of absorption peaks is related to the change of dipole moment and the possibility of the transition of energy levels. Therefore, by analyzing the infrared spectrum, one can readily obtain abundant structure information of a molecule. So, the bonds of molecules can be detected based on the different absorption characteristics of every bond (C=C, C-O, N-H, O-H). FTIR application for molecules should be in the range of 650-4000 cm^{-1}. FTIR can be used for the diagnosis and the evaluation of the structure of functional groups.

Based on the FTIR results given in Section 4.2.1. for all grafts either dermal or bone sourced and coded as HBG1, HL1, MBG3, MDG2, PBG1, PDG1 and PDG3, no change was observed even up to 50 kGy in FTIR spectra. For instance, in animal sourced dental grafts coded as HBG1 and PBG1, mineral part characteristic peaks (PO_4^{-3} and CO^{-2}_3 vibration) did not show any difference after gamma irradiation even at 50 kGy dose (Figure 4.1 and 4.5) and this showed the high stability of these materials even in high doses .

According to a study performed by Kubisz and his group, there was not any change detected at FTIR spectra of the mineral part of bone grafts from human source even up to 100 kGy of gamma radiation and showed a high stability-radioresistancy. However; some changes in spectra related to collagen as wavelength shift and some expansion in the peaks, were detected (76, 94,95).

In another study done by Turker et al, dental grafts were irradiated by gamma radiation at 5, 10, 25, 50 kGy and they also didn't detect a change in two types of dental grafts coded G1 consisting of B-TCP and G4 from animal bone source showed high stability even after a high dose of 50 kGy. On the other hand in another type of dental graft coded G10 consisting of collagen , has shown a change in 1647 cm^{-1} peak which is related to primary amine groups after irradiation with gamma; It was interpreted as the sign of breaking of protein bonds after irradiation and presenting free radical formation (80).

As a result of this study, it can be said that, a significant change in characteristic functional groups of grafts after irradiation by gamma ray even in high doses as 50 kGy was not detected. However, HBG1 and PBG1 dental grafts from human and porcine source FTIR spectra seemed more uniform at different irradiation doses when compared with the other grafts and might be more stable.

TGA Analysis

TGA is one of the most helpful method in gaining both physical and chemical information. Perhaps, the most used technique of thermal analysis is TGA. TGA can provide information about bonding of components within the sample and it can nearly

be used in all types of materials. TGA, as a good technique to find out about the physicochemical changes of the materials before and after sterilization, measures the absolute amount and rate of change in weight of a sample in a controlled environment. Also, TGA determines if and how different components within a material are bonded differently (96). TGA analysis was done for all grafts as it was explained in Section 3.5.1. The results were given at Section 4.2.1. To investigate the thermal properties of unirradiated and irradiated HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 samples by gamma, their thermograms were recorded. To determine the thermal stability of unirradiated and irradiated samples, the temperature for half-life ($T_{1/2}$) were found directly from the dynamic thermograms (Table 4.4). The grafts' residues at 550°C for every sample were also observed and given at Table 4.5. The temperature for maximum weight loss (T_{max}) were observed and to determine this value the first derivative curves of thermograms were used (Figure 4.80 to 4.86 and Table 4.6).

HBG1 coded samples unirradiated and irradiated at 2, 4, 5, 10, 25 and 50 kGy irradiation doses by gamma, gave residues at 550°C in between (73-63) %. The temperature for maximum weight loss was in between T_{max}=348-354 °C depending on the irradiation dose by gamma. The temperature for half-life couldn't be gotten because the residue was higher than 50% for bone containing gamma irradiated samples.

HL1 coded samples unirradiated and irradiated at 2, 4, 5, 10, 25, 50 kGy irradiation doses by gamma gave residues at 550°C between (20-19) %. The temperature for maximum weight loss of HL1 coded samples was between T_{max}=332-328 depending on the irradiation dose by gamma. The temperature for half-life was $T_{1/2}$=352-353°C.

Thermograms of MBG3, MDG2, PBG1, PDG1, PDG3 samples were given in Figure 4.10 to 4.14 and their $T_{1/2}$, residues at 550°C and T_{max} values were given in Tables 4.4, 4.5 and 4.6. The thermal stabilities of the samples were observed as type of sample dependant (such as based on bone and dermal). The thermal stabilities of samples containing bone were higher than dermal ones. T_{max}, $T_{1/2}$, residues at 550°C values of the bones were also higher than the dermal grafts.

In a study performed by Turker and co-workers, material loss percentage of gamma irradiated dental grafts were observed after irradiation by gamma at 5, 10, 25 and 50 kGy. Based on this study G1, G4 and G10 coded dental grafts showed material loss after gamma irradiation. G10 coded dental bone graft as an animal sourced graft showed the most loss in material and degradation after irradiation with gamma. G4 coded graft also showed a 3.2 % loss of material after irradiation (80).

In another study performed by Descamps and colleagues, a kind of dental graft showed a high stability up to 1120 ^{0}C and a degradation and material loss observed after it (97).

There is another research that showed material loss for animal sourced bone graft as in between 100 and 500 ^{0}C (98).

Martel-Estrada and co-workers evaluated the effects of radiation on the thermal properties of chitosan/mimosa tenuiflora and chitosan/mimosa tenuiflora/multiwalled carbon nanotube composites for bone tissue engineering. Based on this study, two stages of degradation in the TGA curves of all samples were observed. The initial weight loss of the composites (100–150 ◦ C) was due to evaporation of the absorbed moisture (99).

This study was somehow a new study about evaluating gamma irradiated bone and dermal dental grafts. The results were found in this study were parallel to literature. It can be said that bone dental grafts from porcine and human source coded as PBG1 and HBG1 showed a higher stability .

SEM Analysis

SEM images of the collagen sheets from bone grafts exhibited a porous and fibrous surface network in PBG1, MBG3 and HBG1 coded grafts (human , porcine and horse bone grafts) and a fibrillary view for dermal grafts for PDG1, PDG3, MDG2 as it was shown in Section 4.2.1. Interdependant porosity appearance is desirable in any implantable material. Based on SEM results, radiation dependant significant change in porous and elementary structures were not detected. SEM pictures of materials were observed very similar before and after sterilization.

In a study performed by Turker and co-workers, different kinds of grafts were irradiated by gamma radiation for sterilization at different irradiation doses as 5, 10, 25, 50 kGy and a significant change in pore sizes or surface shapes were not detected for G1 and G4 coded grafts. Additionally, in the same study for another graft coded as G10, a reduction in surface pore sizes depending on the radiation dose has been detected (80).

In another study performed by Szarska and co-workers showed that the dental grafts they used has shown a good stability up to 300 kGy of radiation, however; after 300 kGy of irradiation with gamma, changes in the microstructure and the surface of the material used were observed after irradiation (100).

A study done on gamma irradiated animal sourced bone dental grafts showed no change even up to high doses of 50 kGy (101). There are other studies showing simillar results confirming no significant change in SEM images of irradiated grafts (102, 103 ,94).

In dermal grafts, fibrillar arrangement of the collagen was not affected from the irradiation. Some deformation have been detected due to the cutting process of the materials in their preparation process for sterilization. There is no study about evaluating gamma irradiated dermal dental grafts by SEM analysis. Our study is novel in this issue.

In general basing on the results, it can be said that, bone grafts coded as PBG1, MBG3 and HBG1 have shown more radiostability when compared to dermal grafts. In bone grafts, porcine sourced one (PBG1) was observed more radiostable than human sourced one (MBG3).

SAXS Analysis

Small-angle scattering (SAS) of X-rays (SAXS) and neutrons (SANS) are powerful methods for the analysis of biological macromolecules in solution. Great progress has been made over the years in applying this technique to extract structural

information from non-crystalline samples in the fields of physics, materials science and biology (94). SAXS and SWAXS in the natural environments in which biological materials have been used in the last 20 years and studies are being actively carried out to investigate the properties of such materials.

As it is known, X-rays have been used effectively in structural analyses since 1895 when they were discovered. These structural analyses have spread to a wider scale with nano-dimensional structural analyses as well as developing X-ray sources and detector technology. The most important development is to gain experimental possibilities to use X-ray scattering methods that we can examine information simultaneously in electron density of liquid materials, dynamic structure changes on both molecular and nanoscale simultaneously. Conventional X-ray tubes or synchrotron sources are commonly used in current X-ray scattering studies. The great advantage of closed X-ray tubes is their ability to be used in small environments.

However, the intensity of the X-ray emitted from the conventional closed X-ray source is much smaller than the synchrotron rays (SR) which is known as a disadvantage for the studies. Also, because of the limited working wavelength range, synchrotron sources are generally preferred when compared to the X-ray tubes. In summary, structural information such as shape, size, gyration/ effective radius (Rg), maximum 3D size (Dmax) and distances in between macromolecular structures such as proteins and nano formations can be reached using the data generated by the SAXS method (104). 3D orientation maps using 3D scanning SAXS help to quantify and understand structure–function relationships in bone (105). The arrangement of atoms in an ordered manner at an atomic level is called crystallinity. However, the hardness of the polymer chains in a polymer suppresse the tendency of atoms to form crystalline structure. But, polymers are normally semi-crystalline. Determination of the structure of the crystal with SAXS is possible and 3D pictures of the unit cell can be obtained according to the positions and intensities of the peaks in the X-ray diffraction profiles of the samples (106).

In this study, SAXS analysis was also used for evaluating crystallinity and nonstructural and 3D structures of the dermal and bone grafts irradiated by gamma rays at different doses. A comparative evaluation of the structural shapes before and

after gamma irradiation was performed. Based on the results given at Section 4.2.1. for SAXS analysis, increase in radiation dose caused an increase in the density of nonglobular structure of the grafts. This result showed a desirable effect on the material whereas, it caused unwanted effect on the others. While, HL1 and PDG3 grafts were observed as the best; PDG1 and HBG1 grafts on the contrary were determined as the worst nonglobular and nonstructural shapes among the grafts after gamma irradiation. Moreover, based on the double distribution function graphics it can be concluded how homogenous or heterogenic are the materials in nano structure view. As it is shown in Figure 4.31, HBG1 graft nano structure's homogeneity increased by radiation dose increase especially after 25 and 50 kGy. Figure 4.32 showed that HL1 has a heterogenous structure, however, after radiation even at low doses like 5 kGy it showed a smoother double distribution function and homogenity. Figure 4.33 and 4.34 also showed the same results i.e. showed a positive effect of radiation on homogeneity.

PBG1 graft showed a different pattern from the others. It exhibited a completely heterogenous pattern in unirradiated and irradiated at 5, 10, 25 kGy and it considerably changed the homogenity after 50 kGy of irradiation (Figure 4.35). PDG1 and PDG3 also showed homogenity after irradiation (Figure 4.36. and Figure 4.37). Moreover, the average electron density weighted squared distance of the scatters from the centre of the object explained as Radius of gyration has been calculated for every radiation dose and the smallest one considered as the most desirable has been given .For HBG1, HL1, MBG3 at 50 kGy MDG2, PBG1 at 10 kGy, PDG1 at 5 kGy and PDG3 at 25 kGy showed the bests results. The smaller measurment the more desirable nanostructure (Table 4.7).

Evaluation of gamma irradiated dental grafts by SAXS analysis is a new study there is not many researches on this subject in literature .However, there are some other studies evaluating gamma irradiated polymers by SAXS analysis, performed by Feng and co-workers observed an increase in the crystallization by chain scission during irradiation up to 100 kGy (107).

In another study, carbon fiber crystallization has been evaluated by SAXS analysis after gamma irradiation up to 100 kGy and according to the results, crystallization has been enhanced by radiation (107).

As a result, in general it can be said that HL1 as a horse source dermal graft and PDG3 as a porcine dermal graft showed the most desirable and optimum reaction to gamma radiation and this showed that animal sourced grafts were affected by gamma radiation in a good way more than human source grafts. On the other hand, PDG1 and HBG1 grafts were observed as the worst reacting grafts to gamma radiation.

ESR Analysis

Due to the thermal, mechanic, ionizing radiation and photochemical applications on the materials, some changes happened in the materials and depending on the type of the bonds in molecules some free radicals were produced. These free radicals are detectable by Electron Spin Resonance Spectroscopy (ESR) or Electron Paramagnetic Resonance (EPR) which is a technique for studying chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals or inorganic complexes possessing a transition metal ion. The radicals typically are produced an unpaired spin on the molecule from which an electron is removed. Study of the radicals produced by radiation gives information about the location and mechanism of radiation damage.

Magnetic resonance techniques are normally based on the interaction of a nuclear or electron spin with a magnetic field in the presence of either radio- or microwave. Putting the sample with free radicals in this field causes an arousal in the spins of the radicals and when these spins go back to their normal level, they give of an energy as an ESR signal. The signal intensity gives information about the amount of free radicals in the samples and also we can get information about the type of free radicals based on the lines location and number in spectra.

Splitting factor multiplier called as g factor, shows characteristics of radicals and it is like a finger print for the special radical. It can be calculated from the location of the lines of spectra. Lines width of spectra and their changes during time intervals give some information about free radicals settlement and their interaction with other molecules. In ESR spectra, lines of figures and location stayed unchanged and the reduction against intensity is called amortization. The h_r (magnetic field) peak to peak distance called ΔH_{pp} showed the distance between the two peaks in ESR spectrum in

a magnetic field and the smaller this distance is ,the relaxation time for exited spin and time for going back to normal level is bigger and the frequency is smaller (20). Depending on the ESR analysis results, ESR spectra of irradiated samples and dose-response curves were discussed in the following section.

ESR Spectra of Pre- and Post-Sterilization with Gamma Radiation of Grafts

ESR were done on all samples coded as HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 irradiated at different doses (2, 4, 5, 10, 25 and 50 kGy) by gamma radiation as it was explained in Section 3.5.1. and all results were given in Section 4.2.1. The radiation sterilization feasibility of these samples was examined in detail. As it was seen in results, unirradiated samples indicated ESR signals with relatively low intensities while gamma irradiation caused an increase in the intensities of ESR signals with the increasing absorbed dose in all irradiated grafts.

Gamma irradiated HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 samples exhibited complex ESR spectra consisting of 7, 5, 5, 4, 7, 5, and two resonance lines, respectively, where g values for the central resonance lines of the samples were found to be g_{HBG1} = 2.0022, g_{HL1} = 2.0049, g_{MBG3} = 2.0020, g_{MDG2} = 2.0059, g_{PBG1} = 2.0021, g_{PDG1} = 2.0043, g_{PDG3} = 2.0054 . As it was mentioned before, g factor for a radical is like the radical's finger print and the more the amount of g factor of the special radical which was calculated is close to the g (free e^- =2,0013) the more it is dependent to the nucleus . Moreover, peak-to-peak widths of the central resonance lines of the investigated samples have been found to be $\Delta H_{pp,HBG1}$ = 3.4 G, $\Delta H_{pp,HL1}$ = 11.6 G, $\Delta H_{pp,MBG3}$ = 3.6 G, $\Delta H_{pp,MDG2}$ = 12.3 G, $\Delta H_{pp,PBG1}$ = 3.6 G, $\Delta H_{pp,PDG1}$ = 12.0 G and $\Delta H_{pp,PDG3}$ = 9.8 G and as it was mentioned before , bigger ΔH_{pp} showed a smaller relaxation time for the special free radical. An increase in absorption dose caused an increase in the intensity of basic resonance line without changing the spectrum shape for almost all grafts as it was shown in Figure 4.38 to 4.44 in and Section 4. So, the amount of irradiation dose did not affect the shape of the spectrum. In the evaluation of ESR spectra increasing in absorbed dose and not change in the spectral shapes and just cause an increase in the basic resonance lines intensity .So it can be said that the radiation dose of gamma ray did not cause any change in the spectral shape.

In a study performed by Turker and co-workers ,different dental grafts were irradiated by gamma radiation in different doses and based on this study radiation doses even up to 50 kGy did not change the shape of ESR spectra of gamma irradiated grafts coded as G1, G4, G10 and it just caused an increase in the basic resonance lines intensity (80).

Dose-Response Curves

Dose-Response curves are significant in gaining information about dosimetric characteristics of samples. Dosimetric features of the materials based on the dose that they have been irradiated by, showed changes in signal intensities. A fine dosimetric material normally shows linear dose-intensity curves. It is possible to calculate the irradiation dose for a sample in which its irradiation dose is not known, If it is a material, the dose-intensity curve is known. As it was mentioned before, the dose-intensity curves slope shows how much the material is sensitive to radiation and as the slope of the curves increases it shows that this material is a good dosimetric material. However, being a good dosimetric material is not something that we seeking for in radiation sterilization. Because a good dosimetric material is the one which responds to radiation easier than the others and produce more free radicals. Based on the dose-intensity curves of all grafts given in Section 4.2.1., it can be said that HBG1, HL1, MDG2 and PBG1 grafts were not found having good dosimetric materials, on the other hand ,PDG3 ,MBG3 ,PDG1 were good dosimetric materials due to their dose-intensity curves.

In the study performed by Turker and co-workers, the graft samples were irradiated by gamma radiation. G4 coded graft‘s dose response curve showed different free radicals and also affirmed that it can be a good dosimetric material, however, for another graft used in the same work coded as G10 graft could not be considered as a good dosimetric material after 20 kGy of irradiation (80).

The same results have been found in a study about a group of graft including bioglass performed by Sharaf and collegues (108).

Long Term Stability Studies

Long term stability tests performed at 25 kGy on gamma irradiated samples have indicated that 8% of HBG1, 68% of HL1, 8% of MBG3, 38% of MDG2, 12% of PBG1, 75% of PDG1 and 33% of PDG3 decayed approximately during 3 month storage period held at room temperature (25℃) and atmospheric pressure (101325 Pa (1.01325 bar)). Depending on ESR results, it was concluded that HL1 (horse sourced) could be considered to be a good radiosensitive and PDG3 (porcine sourced) could be considered to be a good radioresistive material among the investigated samples. Moreover, it was understood that the radiation affected both dermal and bone grafts in different irradiation doses and caused formation of free radicals by the time of period and formation of a decay in the appeared radicals.

In a study performed by Duliu and co-workers, the long term stability study was done on collagen irradiated by gamma rays (1-15 kGy) and after three weeks of storage at room temperature, the concentration of some centers diminished by about 50% (109) like it has been seen in our study.

This study is novel in evaluating gamma irradiated dental grafts by ESR.

Briefly, it can be concluded that among all dental grafts irradiated by gamma radiation at different doses , PDG3 as a porcine sourced dermal graft can be mentioned as the best one showing a high resistance to radiation and has a high stability.

5.4.2. Microbiological Tests

Under microbiological tests; sterility and pyrogen tests and SAL dose determination were discussed.

Sterility Test

Based on the sterility test results of the grafts irradiated by gamma rays and cultured in two different media for aerobic and anaerobic microorganisms, no growth was observed in 5, 10, 25, 50 kGy irradiated grafts. However, microorganism growth was observed below 5 kGy nearly 2 and 4 kGy. So, it was evaluated that gamma radiation is an effective sterilization method even at lower doses (2 kGy and 5 kGy)

when compared with standard 25 kGy dose. These results of this research were found parallel to literature.

Turker and co-workers reported that irradiation dose of gamma ray even less than standard dose (25 kGy) for sterilization was successful in sterilization process of dental grafts irradiated by gamma rays .They proved that for G1, G4, G10 coded grafts gamma radiation sterilization can be achieved at 13.2, 11.5, 11.5 kGy, respectively (80).

Determination of SAL Dose

For SAL dose determination, grafts were irradiated by gamma radiation at 2, 4, 5, 10, 25, 50 kGy doses. No growth of microorganism was observed at 5, 10, 25, 50 kGy. So, the doses were reduced down to 2 and 4 kGy below 5 kGy. As a result, it can be concluded that sterilization of grafts at lower doses (which means lower exposure time) and cheaper costs is possible by gamma irradiation. In addition, time and cost importance, minimizing physical and mechanical effects on the materials should be considered when choosing the best method of sterilization for the grafts (110).

SAL dose results are compatible with the sterility test results. Singh and collagues declared that a 15 kGy of gamma irradiation can be enough for sterilization of bone allografts with gram negative bacteria and 20 kGy of irradiation or more for inactivation of gram positive bacteria (62).

As final result of this study it can be said that complete sterilization and a SAL level of 10^{-6} for dental grafts could be achieved at 5 kGy of gamma irradiation.

Pyrogen Test

As an in vitro end-product endotoxin test, LAL is a rapid endotoxin test for human and animal parenteral drugs, biological products and medical devices. This test is not intended for the detection of endotoxin in clinical samples or as an aid in the diagnosis of human disease. The LAL test is a qualitative test for bacterial endotoxin.

Endotoxin is also known as lipopolysaccharides and lipoglycans are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria. The presence of endotoxin can cause fever and septic shock in humans. So, it is essential that medical devices or grafts which will be implanted within the body should not consist endotoxin.

Supplied LAL should be reconstituted with LAL reagent water and then mixed in equal parts with the solution being tested. After incubation and in the presence of endotoxin, gelation forms in the absence of endotoxin. As opposite, gelation does not form in the absence of endotoxin. The use of LAL for the detection of endotoxin evolved from the observation by Bang, that Gram-negative infection of Limulus Polyphemus resulted in fatal intravascular coagulation. Levin and Bang later demonstrated that this clotting was a result of the action between endotoxin and a clotting protein in the circulating amoebocytes of Limulus blood. Following the development of a suitable anticoagulant for Limulus blood, they prepared a lysate from washed amoebocytes which was an extremely sensitive indicator of the presence of endotoxin (111).

This kit is available in sensitivity level down to 0.03 $EU.mL^{-1}$. Lower concentrations of endotoxin cannot be detected with Pyrosate kit. A limit test with Pyrosate gives a binary (positive or negative) result. The result depends on whether or not the sample contains endotoxin at a concentration of at least the stated sensitivity of the reagent. To perform a quantitative test for endotoxin concentration equal to or greater than the stated sensitivity, a series of dilutions of the sample are tested and an endpoint (the greatest dilution to clot) is determined. The error of the test is plus or minus a two fold of dilution (i.e. +/- a factor of two). The gel that forms as a result of

a LAL reaction is delicate and may be irreversibly broken if the vials are disturbed during incubation. Certain substances interfere with the test and may reduce (or increase) the sensitivity of Pyrosate or prevent the detection of endotoxins. Endotoxin limit for every materials and drugs in the body is different. The endotoxin limit for a medical device depends on the intended use of the device and what the device contacts (e.g., blood, the cardiovascular system, cerebrospinal fluid, intrathecal routes of administration, permanently implanted devices, and devices implanted subcutaneously) (112).

For medical devices, recommendations using the extraction volume is as follows, the limit is 0.5 $EU.mL^{-1}$ or 20 EU/device for products that directly or indirectly contact with the cardiovascular system and lymphatic system. For devices in contact with cerebrospinal fluid, the limit is 0.06 $EU.mL^{-1}$ or 2.15 $EU.mL^{-1}$. For devices that are in direct or indirect contact with the intraocular environment, a lower endotoxin limit may be applied (85).

In this study, as it was mentioned before due to the limited budget and time, the pyrogen test just on the grafts obtained as the optimum dose for gamma radiation sterilization (5 kGy) (explained in Section 3.6.2.). Endotoxin limit for gamma irradiated sample coded as PDG1 was determined as more than 0.25 EU. So it was concluded that the endotoxin amount for this sample is a little high as it was given at 4.2.1.

5.5. Analyses of Pre- and Post-Sterilization with MICROWAVE of Grafts

Implanted biomaterials require complete sterility before implanting them to the body. Generally, sterilization process for these materials can be done by gamma radiation and gas sterilization like EtO (113). However, in recent years EtO is not acceptable and reliable anymore due to its toxic effects like in-vivo hemodyalyses effects and need for quarantine after sterilization process (114). An effective sterilization method, should not cause any significant unwanted effects on chemical, physical and biological characteristics of the materials. Other sterilization methods like steam sterilization causes dehydration in some materials which contain inorganic compounds like calcium phosphate and calcium oxide (115). Moreover, using of autoclave for sterilization of some dental samples can cause softening (93). A

significant denaturation in chemical characteristics of heat and moisture sensitive polymers has been approved for using autoclave and dry heat oven for the sterilization process (95, 116).

As a result, after gamma irradiation as the optimal and first option for sterilization, microwave sterilization as an another radiation type can be a good and promising choice for sterilization. Microwave radiation, as a power which can have a lethal effect on microorganisms and has less unwanted effects on the materials with lower costs and easy applying. It was chosen to be the alternative sterilization method, to investigate in this study and the results were compared to gamma radiation sterilization. Although, this radiation sterilization method is a novel method and is not used as much as gamma radiation sterilization or other heat and gas methods, it was observed a very promising method for the future and there are new investigations on it to approve how much is it reliable. All grafts were sterilized with microwave radiation sterilization at 1, 2, 3, 4 min. This study is a novel for evaluating microwave sterilization.

5.5.1. Physicochemical Analyses

Physicochemical analyses were carried on all grafts coded as HBG1, HL1, MBG3, MDG2, PDG1, PBG1, PDG3 including organoleptic analysis FTIR, TGA SEM, SAXS and ESR were evaluated.

Organoleptic Analysis

Grafts irradiated by microwave radiation did not show any significant changes in organoleptic features (color, shape and odor) after irradiation (Table 4.23). Just a slight change in fragility and color for PDG1, MDG2, PDG3 in dermal graft group at higher radiation time of 4 min of microwave irradiation has been detected . It was observed that grafts coded HBG1, MBG3 and PBG1 showed more radioresistancy than the others and this showed that bone grafts are more stable than dermal grafts .From the aspect of organoleptic features in comparison to gamma irradiated grafts, it can be seen nearly the same radioresistancy in bone grafts when compared to microwave irradiated ones. On the other hand, it can be said that the unfavourable effects of gamma irradiation on grafts is lesser than microwave irradiation. Because,

grafts showed radioresistancy in high radiation doses like 25 and 50 kGy, however, they showed change in the color in a short radiation of 4 min of microwave.

Briefly it can be said that HBG1, MBG3, PBG1 coded grafts are more radioresistant than the others.

FTIR Analysis

Evaluation of FTIR analysis results showed that there are no change in functional characteristic groups . All FTIR spectral data are in accordance with the assumed structures. Functional groups identified in the FTIR spectra were amide A, B, I, II and III corresponding to collagen. The sample of collagens have amide A (N-H stretching) bands at 3301-3285 cm^{-1} and C-H stretching peaks at 3081-3067 cm^{-1} for amide B. Aliphatic C-H stretching bands can be seen at 2929-2921 and 2853-2852 cm^{-1} corresponding to the groups of CH_3 and CH_2. The strong bands at 1648-1631 cm^{-1} (C=O stretcing) corresponding to amide I and 1553-1538 cm^{-1} corresponding to Amide II N-H) and C-N. In the spectra, while the CH bending and CO bands associated with CO_3^{-2} were observed at 1456-1447 and 1337-1328 cm^{-1}. amide III (NH) + (CN) bands were seen at 1236-1230 cm^{-1}. There are also PO_4^{-3} P-O stretching bands located at 1083-1077, 1035-1013, 872-871 and 668-645 cm^{-1}.

In a study performed by Acharya and co-workers , microwave irradiated polymers were evaluated by FTIR analysis and these composite polymer films were irradiated by various doses of microwaves at 100 to 750 W at 10 min in commercial microwave oven . Based on this studies' results, nanocomposite did not exhibit any formation/deformation of chemical bonds at microwave irradiation power ranging from 180-750 W(117).

In another study performed by Muharram and co-workers, FTIR analysis were used in the study to evaluate the effect of microwave on the structural properties of cotton fibers and on the mercerization mechanism of these fibers. According to this study, it was found that microwave irrdiation caused no observable changes in their spectral features apart from slight changes in the intensities of the absorption bands (118). As a result, it can be said that like gamma irradiation which did not cause a significant change in FTIR spectra of irradiated grafts and HBG1 ,MBG3 and PBG1

were more radiostable than the others . On the other hand, when gamma rays were compared with microwave it can be said that the radiostability of grafts after irradiation with microwave was higher than the gamma irradiated ones .

TGA Analysis

TGA analysis was performed for all grafts as it was explained in Section 3.5.1. Results, were given at Section 4.3.1. To investigate the thermal properties of unirradiated and irradiated HBG1, HL1, MBG3, MDG2, PBG1, PDG1, PDG3 samples by microwave. Their thermograms were recorded and they were given in Figure 4.73 to 4.79. To determine the thermal stability of unirradiated and irradiated samples, the temperature for half-life ($T_{1/2}$) was found directly from the dynamic thermograms (Table 4.25). The residues at 550°C for every sample were also observed and given at Table 4.26. The temperature for maximum weight loss (T_{max}) was also observed and to determine this value the first derivative curves of thermograms were used (Figure 4.80 to 4.86 and Table 4.27).

Unirradiated and irradiated HBG1 coded samples at 1, 2, 3, 4 min of microwave gave residues at 550°C in between (76-71) %. The temperature for maximum weight loss was in between (T_{max}) 348-350 °C. The temperature for half-life couldn't be obtained because of the residue was higher than 50% for bone containing samples for both gamma-irradiated and microwave irradiated samples.

Unirradiated and irradiated HL1 coded samples at 1, 2, 3, 4 min of microwave showed residues at 550°C in between 20-17%. The temperature for half-life are $T_{1/2}$=350-347°C. The temperature for maximum weight loss was in between (T_{max}) 332-330 °C.

In comparison of HL1 and HBG1 samples irradiated by gamma and microwave depending on the irradiation dose and time, there was no important differences and their thermal stabilities were approximately the same and their thermal degradation mechanisms were similar to each other. The thermograms showed only one stage of weight loss.

Thermograms of MBG3, MDG2, PBG1, PDG1, PDG3 samples were also given in Figure 4.73 to 4.79 and their $T_{1/2}$ residues at 550°C and T_{max} values were given in Tables 4.25 , 4.26 , 4.27. The thermal stabilities of the samples were depending on the source of samples (bone and dermal). It was higher for samples containing bone than dermal ones. T_{max} and $T_{1/2}$ residues at 550°C values of the bone grafts were also higher than the dermal sourced samples. In comparison of the thermal stabilities of all type of samples according to gamma and microwave irradiation, thermal degradation mechanism was similar to each other. For the irradiated samples by gamma (especially at 50 kGy irradiated samples), the degradation occurred very easily and thermal stabilities of this type of samples was lower than the other irradiated samples. When the bone sourced-gamma irradiated grafts were compared with the microwave irradiated samples, it was interpreted that there was a more or less similar results from thermal degradation and mechanism point of view.The thermograms showed only one stage of weight loss.

This study was a new study about microwave irradiated dental grafts.

As a result, in general it can be concluded that for both gamma and microwave irradiation of HBG1, MBG3, PBG1 as bone grafts showed more thermal stability than dermal grafts. Additionally, a significant difference between gamma and microwave irradiated samples was not detected.

SEM Analysis

All grafts coded as HBG1, HL1, MBG3, MDG2, PBG1, PDG3 and PDG1 were evaluated from nano scale point of view to evaluate the difference after irradiation by microwave. When SEM analysis of all images were taken into consideration at different magnifications and nano-scale structures (shown in Section 4.3.1.), HL1, HBG1, PBG1 did not exhibit a significant difference before and after irradiation at different time intervals there were no observation in the pore sizes and surface view. On the other hand, there were some signs of water lost at longer irradiation times due to the high temperature produced in the materials in all of them especially in PDG1, MDG2, PDG3 coded grafts. So heat produced by microwave, caused water loss in

microwave irradiated materilas.This can be a different aspect caused by microwave which can not be seen in gamma irradiated samples.

In a study performed by Popescu and co-workers , denture base acrylic were irradiated by microwave at 500, 650, and 750 W for 2, 3, and 5 min to evaluate morphological changes and based on the results superficial adaptation was discovered after 5 min of microwave irradiation at 500 W, 650 W, and 750 W and a significant roughness for 750 W(119).

In a study performed by Ewerton and co-workers the effect of microwave irradiation on morphological changes of a kind of polymer has been evaluated at 650 W for 1, 2, 3, 4, or 5 min irradiation and based on the results of SEM images showed alteration in cell morphology of sterilized samples and the effectiveness of microwave irradiation was improved as the exposure time increased (120).

So, the results of this study were found parallel to literature. As a result it can be said that bone grafts from animal sources as HBG1, HL1 and PBG1 were found more radioresistant than the others.

SAXS Analysis

SAXS analysis were applied for all grafts which were irradiated with microwave radiation at 3 min. Due to the limited time, SAXS analysis was applied as it was explained in Section 3.5.1. In order to determine the optimum radiation time in which sterilization process was completed successfully, SAXS results were given in Section 4.3.1. The best nonglobular structure after irradiation at 3 min of microwave exposure was observed for MDG2 and PDG3 coded grafts as denser and indicated cylindrical/ellipsoid nano aggregations. Also based on the double distribution function PBG1 has a heterogenous nanostructure in comparison with the others.

As a result, PDG3 and MDG2 coded grafts were cosidered as the optimum ones.

There is no study about grafts irradiated by microwave and this study is novel about evaluating microwave irradiated for dental grafts with SAXS analyses.

ESR Analysis

ESR Spectra of Pre- and Post-Sterilization with Microwave Radiation of Grafts

As it was mentioned before, microwave irradiation (even up to 8 minutes) did not cause significant difference in the ESR spectra of the samples. So, spectra of microwave irradiated samples have not been given and evaluated.

Dose-Answer Curves

Based on ESR results of microwave irradiated grafts, microwave irradiation at 1, 2, 3, 4 min did not change the pattern of ESR spectra of all grafts and only a slight decrease was observed in the intensities of the resonance peaks of the samples and/or the area (second integral of the spectrum) of the sample. Moreover, microwave irradiation even up to 8 min did not cause any significant difference in ESR spectra of the samples and no spesific peak could be differentiated from noise.

ESR evaluation of all dental grafts sterilized by microwave irradiation is a new study and there is no such study in the literature.

Long Term Study

As it was mentioned before due to the limited time, long term studies for microwave irradiated samples were not performed.

5.5.2. Microbiological Tests

Sterility Test

Based on the sterility test results of the grafts irradiated by microwave rays and cultured in two different media for aerobic and anaerobic microorganisms, no growth was observed in grafts irradiated at 3 and 4 min and even below 3 min (as 2 and 1 min). So, it can be concluded that microwave radiation has the ability to sterilize of dental grafts (either human or animal; bone or dermal; horse or porcine or human sourced) at even lower exposure times like 3 min.

SAL Dose Determination

SAL level for microwave irradiated grafts were determined and it was concluded that SAL for all grafts can be provided at 3 min of microwave irradiation at 2450 MHz and 900 W as conditions.

In a study performed by Singh and collegue, after the inoculation of bone grafts with gram-positive and negative bacteria, bone allografts were irradiated by microwave at different exposure times as 1 min, 2 min ,3 min ,4 min ,5 min and 6 min (2450 MHz and 900 W). Based on this study results sterilization was achieved successfully , after 2 min of exposure (62).

In another study performed by Shamis and collegues , microwave sterilization as a novel sterilization method for biomaterials was evaluated . Two common pathogenic species of bacteria, Escherichia coli and Staphylococcus aureus were used. Based on these results, inactivation of the contaminant bacteria was successful (61).

Ashure and co-workers also investigated that dry sterilization by microwave in the inactivation of pathogenic bacteria in blood wastes and depending on the results they found it successful (91).

Moreover, Everton and collegues also suggested that 3 min of microwave irradiation can be used for acrylic resin sterilization and preventing cross-contamination. For this porpuse, they irradiated samples at 650 W for 1, 2, 3, 4, or 5 min for sterilization (120).

Pyrogen Test

LAL is a rapid endotoxin test applied for microwave irradiated PDG1 sample like it has done for gamma irradiated ones. The endotoxin limit for this sample was determined as less than 0.25 $EU.mL^{-1}$ and as it was mentioned before, the endotoxin limit for medical devices is 0.5 $EU. mL^{-1}$. So it can be concluded that endotoxin limit for microwave irradiated sample was acceptable at 3 min.

This is a new research in this area.

6. CONCLUSION

When this research entitled “Sterilization of Grafts Used in Periodontology by Gamma and Microwave Radiation” was evaluated, the following conclusions have been obtained ;

Grafts as materials used in periodontology for regenerating lost tissue have an important role in dentistry and health science. So, in this study sterilization with gamma and microwave radiation for these materials as a subcategory of medical devices, has been investigated. Physicochemical analyses as organoleptic, FTIR, TGA, SEM, SAXS, ESR and microbiological tests (sterility, pyrogen and SAL determination) were performed on the grafts coded as HB1, HL1, MBG3, MDG2, PBG1, PDG1 and PDG3 before and after sterilization. Based on the results gamma radiation can provide a 10^{-6} SAL level at a minimum dose of 5 kGy. On the other hand, microwave radiation sterilization as a novel method also has been investigated and based on the results it can also provide a 10^{-6} SAL level at minimum irradiation time as 3 min (at 2450 MHz and 900 W).

Moreover, based on the physicochemical analyses performed before and after sterilization with gamma and microwave, a significant change after sterilization process has not been observed. Besides, it can be said that in general PBG1, HBG1, MBG3 coded bone grafts showed the most stability. So, it can be said that, in comparison of dermal and bone grafts, bone grafts showed a higher radiostability than dermal ones. When it comes to comparing human and animal sourced grafts, it was observed that HBG1 as an animal (horse) sourced graft exhibited more resistancy to irradiation for both gamma and microwave irradiation.

To sum up, it can be said that gamma radiation sterilization is an appropriate sterilization method for dental bone and dermal grafts and microwave sterilization can be a promising and acceptable alternative sterilization method for dental grafts. Additionally, HBG1, MBG3, PBG1 coded grafts were found more advisable as the most compatible materials to gamma and microwave radiation sterilization.

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