IN VITRO STUDIES OF DRUG ELUTING BIODEGRADABLE POLYMERIC STENT

by

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ABSTRACT

IN VITRO STUDIES OF DRUG ELUTING BIODEGRADABLE POLYMERIC STENT

Cardiovascular disease which is the prominent reason for morbidity and mortality, affects large number of world population. Coronary artery disease (CAD) constitutes large class of cardiovascular disease and mostly eventuate heart attack and death. Therefore, CAD treatment is extremely important. The studies proved that stent implantation is the most ideal and beneficial treatment way of CAD. There are different types of stents as bare metal stents, drug eluting coated metal stents, polymer stents and drug eluting polymeric stents. The objective of this research was to investigate the *in vitro* convenience of paclitaxel eluting biodegradable polymeric stent for coronary arteries using PLLA/PCL/PBS polymer blends. For this purpose, biocompatibility, cytotoxicity and thrombogenicity of the stent body material and the drug loaded stent coating were studied.

In order to examine the biocompatibility of the stent body, MTS and Calcein AM assays were performed in this study. In addition to biocompatibility, thrombogenicity properties of the stent body are substantially significant due to thrombosis and restenosis risks. Therefore, thrombogenicity assay was applied to the polymers that were used for stent body. According to the results, the most appropriate polymer compositions due the biocompatibility and thrombogenicity were determined and chosen as a potential polymer blend for stent construction.

In the mean time, cytotoxicity of the drug loaded stent coating is very important due to its apoptotic effect to the surrounding cells, smooth muscle cells and inflammatory cells. Therefore, the biocompatibility of the unloaded stent coating was searched first by MTS assay. Then, the apoptosis effect of the paclitaxel loaded stent coating was studied by Caspase-3 apoptosis assay. The results of the assays were considered and the potential stent coating blend was decided. For future prospects, the design and the morphology of the stent will be decided and *in vivo* experiments will be performed.

ÖZET

İLAÇ SALAN BİYOBUZUNUR POLİMERİK STENT İÇİN *İN VİTRO* ÇALIŞMALAR

Hastalıkların ve ölümlerin en belirgin sebeplerinden biri olan kardiyovasküler hastalıklar dünya nüfusunun büyük bir çoğunluğunu etkiler. Koroner arter hastalığı (KAH) kardiyovasküler hastalıkların en büyük sınıflarından birini oluşturur ve genellikle kalp krizi ve ölümle sonuçlanır. Bu nedenle KAH tedavisi oldukça önemlidir. Çalışmalar stent implantasyonunun en ideal ve en yararlı tedavi yöntemi olduğunu kanıtlamıştır. Stentlerin metal stentler, ilaç salan kaplı metal stentler, polimer stentler ve ilaç salan polimerik stentler olarak farklı çeşitleri bulunur. Bu çalışmanın amacı, PLLA/PCL/PBS polimer karışımları kullanılarak koroner arterler için paklitaksel salan biyobozunur polimerik stentin *in vitro* uyumluluğunun araştırılmasıdır. Bu amaçla, stent çatı materyalinin ve ilaç yüklü stent kaplamasının biyo-uyumluluğu, sitotoksisitesi ve pıhtılaşma özelliği çalışılmıştır.

Bu çalışmada stent çatısının biyo-uyumluluğunu incelemek için MTS ve Calcein AM testleri uygulanmıştır. Biyo-uyumluluğun yanısıra, tromboz ve restenoz riskleri açısından, stent çatısının pıhtılaşma özellikleri oldukça önemlidir. Bu nedenle stent çatısında kullanılan polimerlerin pıhtılaşma özellikleri test edilmiştir. Sonuçlar değerlendirilerek, biyo-uyumluluk ve pıhtılaşma özelliklerine göre en uygun polimer kompozisyonları belirlenmiş ve stent yapımı için potansiyel polimer karışımları seçilmiştir.

Bunun yanı sıra, ilaç yüklü stent kaplamasının sitotoksisitesi, çevresindeki hücrelere, düz kas hücrelerine ve inflamatuar hücrelere olan apoptotik etkisinden dolayı çok önemlidir. Bu sebeple ilk olarak stent kaplamasının biyo-uyumluluğu MTS testi ile araştırılmıştır. Daha sonra Caspase-3 apoptoz testi ile paklitaksel yüklü stent kaplamasının apoptotik etkisi araştırılmıştır. Deney sonuçları değerlendirilmiş ve potansiyel stent kaplaması karışımına karar verilmiştir. İlerleyen aşamalarda, stentin dizaynına ve morfolojisine karar verilecek ve *in vivo* deneyleri yapılacaktır.

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ACD	Citrate-Dextrose Solution
BMS	Bare metal stents
CAD	Coronary Artery Disease
DES	Drug eluting coated metal stents
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HA	Hyaluronate
HUVEC	Human Umbilical Vein Endothelial Cells
MA	Methacrylate
MTS	CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay
OC	Only Cell
PBMA	Poly n-butyl methacrylate
PBS	Polybutylene succinate
PC	Phosphorylcholine
PCI	Percutaneous coronary intervention
PCL	Polycaprolactone
PET	Polyethylene terepthalate
PEVA	Polyethylene-co-vinyl acetate
PHBV	Polyhydroxybutrate-co-hydroxyvalerate
PLGA	Poly-L-glycolic acid
PLLA	Poly-L-lactic acid
PU	Polyurethane
PXT	Paclitaxel
P/S	Penicillin-Streptomycin solution
SEM	Scanning Electron Microscope
VSMC	Vascular Smooth Muscle Cells

1. INTRODUCTION

It has been reported that, since the first clinical application of bare metal stents in 1986, there are several attempts in order to construct new coronary stents for eliminating the disadvantages and side effects of stent implantation. The main approach of stent implantation is to hold the artery in an open state for preventing the occlusion. However, the bare metal stents, although providing the support to artery, they cause restenosis and thrombosis in the vessel. Therefore, the researchers have improved a new era, drug eluting stents. By incorporation of antiproliferative drugs to stent coating, the restenosis and thrombosis ratios after stent implantation have been reduced [1, 2]. However, there are still additional drawbacks such as permanency of the stent in the vessel and unknown long-term effects of metals in the vessel. The most creative idea has been developed to overcome this situation; construction of a coronary stent to provide drug elution in the artery and disappear after healing process. For this purpose, drug eluting biodegradable polymeric stents have been generated [3, 4]. But the survey of the most suitable polymeric blend and drug incorporation process is still in progress.

The ultimate aim of this study was to investigate the *in vitro* convenience of drug eluting biodegradable polymeric stent to the human body for the treatment of coronary artery disease.

For designing the ideal scaffold, determining the biocompatibility and cytotoxicity of the stent body material and the drug loaded stent coating are crucial. For this purpose, biocompatibility and cytotoxicity of the stent body polymer were investigated. Besides, the drug loaded stent coating polymer was examined due to its cell death effect.

Biocompatibility of the stent body material is extremely important. Since the stent is constructed from biodegradable polymer, full stent degradation is expected after 12 months. If it degrades before this time period, whole system may collapse and stent failure may occur. Because of that reason, the type and the degradation profile of the polymer are very important parameters. Polymer chosen for the stent body should be highly

biocompatible to prevent hypersensitivity reactions, late thrombosis and delayed endothelization. For that purpose, MTS and Calcein AM assay were chosen in this study to test the biocompatibility of the stent. In addition to biocompatibility, thrombogenicity property of the stent body is considerable due to thrombosis and restenosis risks. Therefore, thrombogenicity assay was applied to the polymers that were used for stent body.

In the mean time, cytotoxicity of the stent coating is very important. Anti-proliferative drugs are incorporated into the stent coatings in drug eluting stents and thus, the coating should be fully degraded after 1 month without inhibiting healing process and endothelization. However, the drug incorporated into the stent coating should inhibit the cell growth and accumulation in the vein during the first month. Therefore, stenosis and plaque formation possibilities after stent implantation can be eliminated. In the second part of the study, the biocompatibility of the unloaded stent coating was tested with MTS assay. Then, the apoptosis effect of the paclitaxel loaded stent coating was studied by Caspase-3 apoptosis assay.

2. THEORETICAL BACKGROUND

2.1. CARDIOVASCULAR SYSTEM

The cardiovascular system consists of the heart and the blood vessels. These two components constitute the pulmonary circulation and the systemic circulation. In pulmonary circulation, the blood is pumped throughout the lungs and oxygenated. In systemic circulation, the oxygenated blood is delivered to the rest of the body and provides nutrients [1].

The main blood vessels; arteries are thick-walled tubes and they are covered with circular elastic fibers, which contain a filling of muscle that absorbs the great pressure wave of a heartbeat and slows the blood down. The arteries divide into smaller arterioles and then into smaller capillaries, the smallest of all blood vessels in order to supply oxygen and nutrients to all over the body, tissues and cells. On the other hand, the capillaries form the small veins which connect to the larger veins. The veins are complementary to the arteries. They collect the deoxygenated blood from the body and deliver it back to heart. Conversely to the arteries, the veins have thin, slack walls because the deoxygenated blood has low pressure which forced it out of the heart. In the heart, the veins join into another vessel; pulmonary arteries and the blood is delivered to the lungs for collecting oxygen again by pulmonary arteries. After reoxygenation, the circulation starts again from the beginning [5, 6].

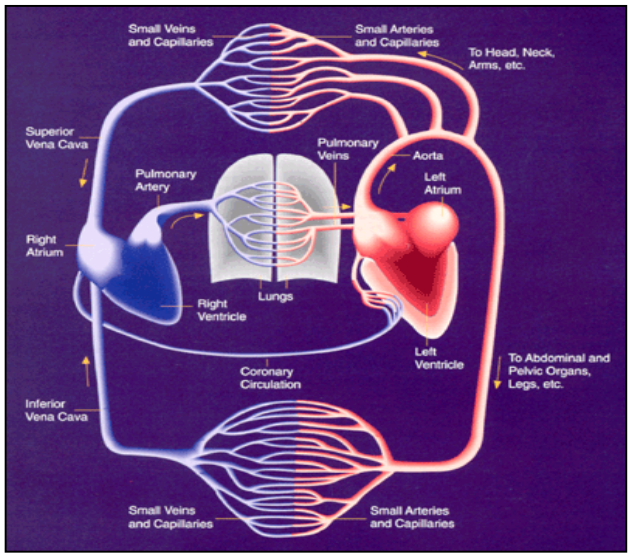

Figure 2.1. Diagram of cardiovascular system [6]

2.2. CARDIOVASCULAR DISEASE

Cardiovascular disease is affecting large number of world population and it is the prominent reason for morbidity and mortality [1, 2]. Coronary artery disease (CAD) constitutes large class of cardiovascular disease and mostly eventuate heart attack and death. It affects both blood vessels and heart. CAD occurs with narrowing and blockage of blood vessels by buildup of cholesterol-rich plaque. This condition is referred as *Atherosclerosis.* The plaque formed in the arteries includes lipids (intracellular and extracellular cholesterol and phospholipids), inflammatory cells (macrophages, T cells), smooth muscle cells, connective tissue (collagen, glycosaminoglycans, elastic fibers), thrombi and Ca^{+2} deposits. Therefore, the blood flow in the vessel is obstructed by this formation, which can lead to decrease in O_2 supply and heart attack. The risk factors that cause *atherosclerosis* comprise diabetes, cigarette smoking, obesity, family history, sedentary lifestyle, dyslipidemia and hypertension [2].

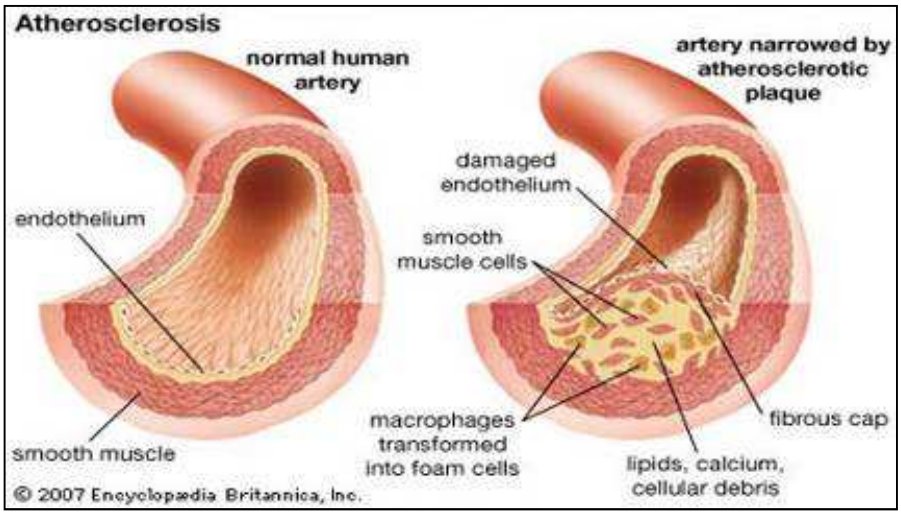

Figure 2.2. Cardiovascular Disease [7]

2.3. TREATMENTS FOR CAD

Current treatment ways for atherosclerosis include diet, usage of drugs, percutaneous coronary interventions and bypass graft operation [8, 9]. The cholesterol-poor nutrition is advised to CAD patients and there are several beneficial choices in these types of diets. As medicament, the drugs that are lowering the cholesterol level in blood are preferred. Bile acid resins, statins, niacin and fibric acid derivatives such as gemfibrozil are generally used to lower LDL-cholesterol. In addition, to prevent plaque fissure or rupture with platelet activation and thrombosis, antiplatelet drugs such as aspirin and clopidogrel are recommended. Percutaneous coronary intervention (PCI) is simply known as angioplasty and it is the therapeutic technique applied to the stenotic coronary arteries. The technique was firstly developed at 1977 and it was defined as percutaneous transluminal coronary angioplasty [9]. However, by the improvement of the method, as a general term, the technique is referred as percutaneous coronary intervention to specify the various methods. The branches of PCI can be classified as rotational or laser atherectomy, balloon angioplasty, implantation of stents and brachytherapy.

Atherectomy is the procedure applied to the obstructed region in the vessel to remove the plaque by using a laser catheter or a rotating shaver. The laser catheter vaporizes the plaque and the rotating shaver shaves off the plaque. However, generally after the

atherectomy operation, balloon angioplasty or stent implantation may be applied to the patient [10].

In balloon angioplasty, a tightly folded balloon is inserted into the artery and then inflated in the artery to crush the plaque into the vessel walls. By this procedure, widening of the vessel is aimed and normal blood flow in the arteries is tried to be supplied [11].

Brachytherapy is the operation performed to inhibit regrowth of the tissue. In atherosclerosis it is applied to prevent restenosis after balloon angioplasty or other treatments. Brachytherapy is a short-term operation and a radioactive source is used to deactivate tissue growth [12, 13]. As discussed above, all these techniques are complementary to each other. In addition to these applications, bypass graft operation is performed to patients who are at high risk.

Bypass surgery is generally applied for heart attack when the other treatment ways are inoperable due to the position and extent of the blockage [11]. This procedure is advantageous for the patients who have diabetes, disease of left main coronary artery, weakened heart muscle and valve disease. However, excluding these group of patients, the results of the researches prove that PCI methods applied together with stent implantation are superior to the bypass graft operation by eliminating drawbacks of bypass surgery such as death, heart attack, bleeding, chest infections and lung complications.

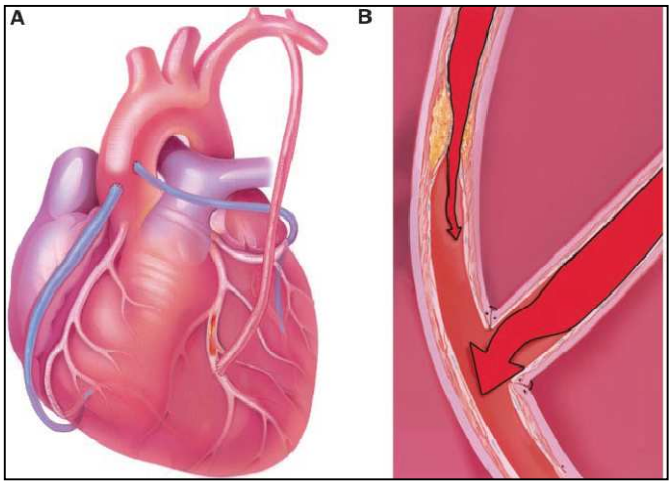

Figure 2.3. Coronary artery bypass grafts. A vein is removed from the leg and/or an artery from inside the chest wall (A) to provide a new path for blood flow from the aorta to the heart muscle (B) [11]

In stent implantation procedure the goal is to hold the artery in an open state by providing mechanical support to the vessel walls. Therefore, narrowing of the vessel and stenosis are tried to be prevented. Firstly, balloon angioplasty is performed to the blocked artery and the stent, tightly placed on the special angioplasty balloon, is directed to the blocked location. The balloon is then inflated; the stent is stretched and implanted into the blockage site of the artery. After stent implantation, the balloon is deflated and taken out. So the stent stays consistently in place to hold the artery open [14].

However, the recovery of the disease is generally not satisfying and produces drawbacks that cause repetition of the blockage in the vessel and long-term safety. Therefore, the researchers are still going on for creating a new and complete solution and the consequences of the studies prove that stent implantation to the vessel for preventing occlusion is one of the ideal treatment for CAD.

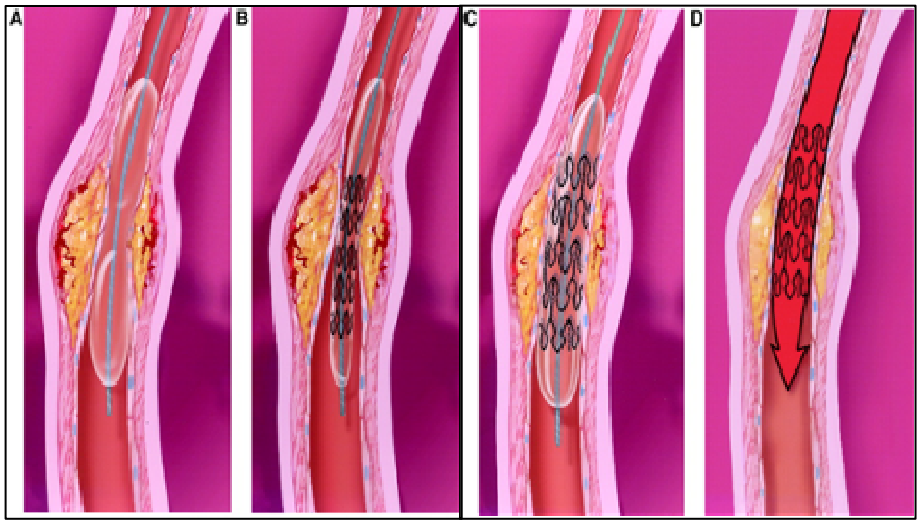

Figure 2.4. Balloon angioplasty and stenting. A balloon is guided in the coronary artery narrowing and inflated (A). The stent is directed to the narrowed location (B). When the balloon is inflated, the stent opens and settles in the arterial wall (C). The balloon is deflated and taken out. The stent remains permanently in place, providing mechanical support to hold the artery open (D). Within a few weeks, healing process begins and the new tissue covers the stent contour [11]

2.4. STENTS, TYPES AND PROPERTIES

A stent is a mesh tube, which is utilized to treat the narrowed or weakened arteries in the body. The first clinical application with a metal stent was conducted in 1986 by Sigwart et al [15] and since 1986 various types of stents like bare metal stents, drug eluting coated metal stents, polymer stents and drug eluting coated polymer stents have been developed to be used in percutaneous coronary interventions [14].

2.4.1 Bare Metal Stents

Bare metal stents (BMS), which are the first-generation stents, have beneficial characteristics such as formation of scaffold in the vessel, prevention of acute collapse, dissection and late vessel modeling and decrease in restenosis ratio. However, the disadvantages of the bare metal stents as pre-acute thrombosis, formation, risk of restenosis and bleeding, corrosion and impairment in the vessel. In bare metal stent design, physical

and chemical characteristics are required to be considered distinctly. These important characteristics are metallic properties, design and chemistry, biocompatibility, bulk and surface properties. Metallic properties are evaluated in stent design because of the elasticity and plasticity of the material. These two properties are significant for deployment of the stent in the vessel and prevention of recoil of the vessel. Considering bulk and surface characteristics, design and chemistry of the stent, corrosion possibility is the most critical parameter. Therefore, these properties are attentively regulated in stent design to prevent corrosion. These regulations are generally formation of metal oxide film on the surface of the metal stent or increase of chromium and titanium amounts in the metal alloy. Also regarding biocompatibility, the metal alloy used in metal stents is chosen from non-immunogenic, non-thrombogenic and non-inflammatory materials. Especially immunogenic response to the material is tried to be prevented in the body. Through these characteristics, there are various metals utilized in stent design and mostly the best results are provided from stainless steel, nitinol and tantalum. All these three materials have both benefits and deficits [3, 4].

As a summarized statement, stainless steel is represented as 316L, which has reduced C content (0.03% weight). The alloy is composed of 60-65% iron, 17-18% chromium and 12-14% nickel. Chromium is used to decrease corrosion possibility and contribute the strength and hardness of the material. 316L is highly biocompatible and it is widely used for stent design. Still considering thrombogenicity, it has limited biocompatibility. Nitinol is the other metal alloy used for stent design. It is composed of 55% nickel and 45% titanium and it is corrosion-resistant. Nitinol is highly biocompatible, but if there is nickel leakage in the material, then immunogenic response risk occurs. Tantalum is also used as stent material. It is an inert material and biocompatible. Tantalum has good mechanical properties and it is resistant to degradation. Although tantalum seems an ideal material for stent design, through the studies, it has the risk for stent thrombosis and therefore it is still under investigation [4].

Different researches have been performed to compare these metals. Scott et al. [16] compared two identical coil stents made up of tantalum and stainless steel in baboon arteriovenous shunts and porcine coronary arteries. There was no difference in platelet

deposition and fibrin accumulation. Sheth et al. [17] investigated differences of the thrombosis rates and vessel injuries of rabbit carotid arteries after implantation of slotted tube nitinol stents and stainless steel slotted tube. The results showed that the nitinol stents caused less thrombosis and vessel injury. Barth et al. [18] applied three different stents in dog peripheral arteries and it was shown that tantalum Strecker stents produced higher neointima formation than stainless steel Wallstent and Palmaz stents. In addition, the alterations on the bare metal stent surfaces increase the stent performance. Sheth et al. [19] showed that mechanical polishing of the surfaces of nitinol and stainless steel stents resulted reduction in thrombus formation compared to unpolished stents.

2.4.2 Drug Eluting Permanent Stents

These metal alloys have been widely used in CAD but the disadvantages of the bare metal stents lead the researchers to utilize drug release systems and polymers in stent design. Drug eluting coated metal stents decrease the ratio of restenosis and neointimal formation. Still they have the deficits of hypersensitivity reactions, late thrombosis and delayed endothelization risks. There are different coating materials and systems used in drug eluting coated metal stents for drug release mechanisms [20]. In drug eluting coated metal stents (DES), the metal stent body is covered with a material that is used to release the anti-proliferative agent. The coating material is chosen from a polymer either biodegradable or not. DES was approved by FDA after their clinical trials proved that they are superior to BMS. The results showed that neointimal formation, restenosis and major adverse cardiac events rates of DES are lower compared to BMS. In DES concept, these superiorities are provided by the local delivery of the anti-proliferative agent to the stenotic region. By anti-proliferative drug elution, cell proliferation, especially vascular smooth muscle cell proliferation, is blocked. So that, fibrosis, thrombus and neointima formation after stent implantations are prevented. However, endothelization is also suppressed. The first successful drug incorporated to the DES system was sirolimus in Cypher stent, approved in U.S. in 2003. Sirolimus is an immunosuppressant and anti-proliferative drug, also known as rapamycin. After Cypher stent, paclitaxel eluting Taxus stent was developed and approved by FDA in 2004. Since then, various types of DES were constructed with different drugs and agents as heparin and everolimus and different coating materials. But

as a generalization, it can be asserted that the most effective outcomes are provided from paclitaxel and sirolimus [21-24]. Baur et al. [22] demonstrated that paclitaxel and sirolimus coated biodegradable stents, when placed in a culture of smooth muscle cells obtained from human coronary atherosclerotic plaque, produced severe destruction of cytoskeletal components of the cells, suggesting a possible strategy for *in vivo* use, assuming the problems of inflammation and radial strength can be overcome. They inhibit vascular smooth muscle cell proliferation and migration, reduce neointima formation, and prevent restenosis and inflammatory reaction.

Table 2.1. Comparison of critical parameters for paclitaxel and sirolimus, EC; endothelial cells, SMC; Smooth muscle cells, ⟺ ; equal distribution, ⇧; accumulation [23]

	Sirolimus	Paclitaxel
Inhibition of SMC proliferation	++	++
Inhibition of SMC migration	++	+
Inhibition of EC proliferation	++	++
Immunosuppresive properties	++	(+)/(-)
Pro-apoptotic effects	(+)	++
Therapeutic range	Wide	Narrow
Vascular wall distribution	⟺	⇧ Adventitia
Pattern of restenosis	Focal	50% Proliferative
Impact on late in stent lumen loss	++	+

As mentioned above, the goal of the drug eluting stents is the local delivery of antirestenotic drugs to the coronary vessel wall and the vessel lumen. There are different mechanisms used to release these antiproliferative agents. These are diffusion, swelling followed by diffusion and degradation or erosion [25]. Diffusion occurs through pores in the polymer matrix by passing between polymer chains. Also reservoir systems are used and diffusion takes place from a membrane or film. In swelling, through the swelling of the material, polymer mesh size is increased and the drug is released. Finally in degradation, the drug is released by the degradation of the polymer [25].

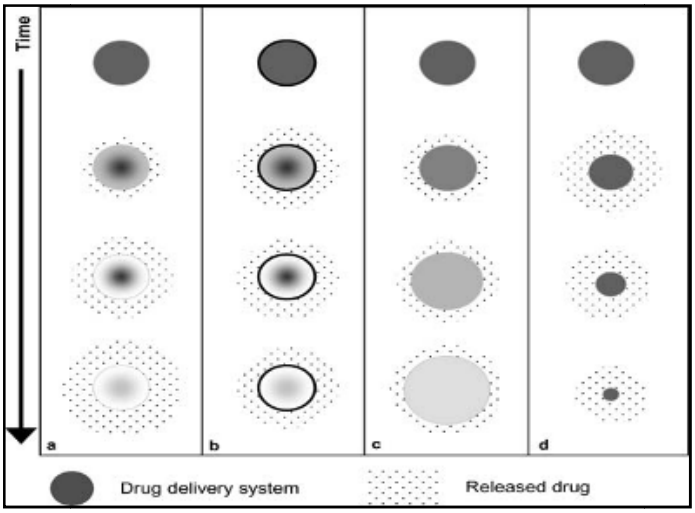

Figure 2.5. Drug eluting mechanisms. Diffusion; through pores in the polymer matrix by passing between polymer chains (a). Reservoir systems; by diffusion and combined with a film or membrane (b). Swelling systems; by swelling increase in polymer mesh size and drug release (c). Biodegradation or erosion; drug release through degradation of polymer (d) [25]

For incorporating the drugs to the stents, different mechanisms are used. The drug can be directly incorporated in the polymeric material and release by diffusion through degradation or the drug can be incorporated in a polymeric material which is used to coat the stent and by the degradation of coated material and diffusion, the release of drug occurs. The materials that can be used to coat the bare stents are PU, PDMS and PET, which are biostable polymers and PLLA, PGA, PCL, PHBV and PBS that are biodegradable polymers [4]. All of these polymers have different characteristics and advantages. PET (Polyethylene terepthalate) is a biostable and biocompatible polymer. Its elastic modulus is between 1 to 5 GPa and has good mechanical properties. But PET can cause chronic foreign body reactions and intense proliferative neointimal response [4]. PU (Polyurethane) film is utilized to cover the stent body in order to minimizing biocorrosion of the metal (stainless steel or nitinol), and providing a homogeneous surface for surface treatment and incorporation of various eluting drugs to prevent platelet aggregation while supporting endothelialization. PU formulations cover an extremely wide range of stiffness, hardness, and densities. They have relatively good biocompatibility. Although PU has good physical and mechanical properties and physiological acceptability, inherent blood

compatibility of PU is insufficient. So a variety of surface and bulk modifications are required to enhance hemocompatibility of PU [27, 28]. Poly dimethyl siloxane (PDMS) is also used in coating metallic stent bodies in order to supply drug release. PDMS has several advantages such as biocompatibility, chemical inertness, optical transparency, flexibility, and gas permeability. However, the most significant disadvantage of PDMS is hydrophobicity. So it requires surface treatments in order to be used in biomedical applications [29]. Besides these biostable polymers, biodegradable polymers are also utilized in stent coating for drug release. PLGA (Poly-L-glycolic acid) is biodegradable aliphatic polyester. It has semi-crystalline structure with an elastic modulus 5 GPa to 7 GPa and it is suitable for load bearing applications. PLGA is widely used for stent coatings [28, 30]. PCL (Polycaprolactone) is also biodegradable polyester. Its elastic modulus is nearly 0.4 GPa and its melting point is 60°C. PCL is highly biocompatible and it is degraded in nearly 24 months in the body [30, 31]. PLLA (Poly-L-lactic acid) is thermoplastic, biodegradable aliphatic polyester, which is widely used for stent coating. Its elastic modulus is 3GPa and its melting point is between 173°C to 178°C. PLLA is also highly biocompatible like PCL and degraded nearly in 24 months [31]. As detailed information for PLLA, it is fully degraded in the body and causes minimal tissue response. Nevertheless, this characteristic is changeable due to PLLA's molecular weight. If it has low molecular weight, its degradation rate will increase and it will cause high tissue response. If it has high molecular rate, then degradation time will decrease and it will cause minimal tissue response [32]. The strength and elasticity of PLLA are adjustable and it is suitable for drug release mechanisms. Although PLLA is highly advantageous, there are still some disadvantages of the material [33]. Because of incomplete endothelization, late thrombosis can occur. Fragmentation of the polymer can cause particle migration and lastly occurrence of neointimal formation and fibrosis can result stenosis or occlusion [32, 33].

Table 2.2. Structural properties of biodegradable polymers [34]

Typical properties of polymers	Crystallinity	Tensile Strength (MPa)	Tensile modulus (GPa)	Approximate time for complete resorption
PGA	Semi-crystalline	60 to 80	5-7	Six to 12
PGA-co-TMC	Semi-crystalline	60	2,4	12 to 15
85:15 PDLLA/GA	Amorphous	40 to 50	2	Six to 12
PLLA	Semi-crystalline	60 to 70	3	More than 36
PCL	Semi-crystalline	20 to 25	0,4	More than 36

PHBV (Polyhydroxybutrate-co-hydroxyvalerate) belongs to polyhydroxyalkanoate polymers. They are natural, thermoplastic polyesters. PHBV is a biodegradable polymer but the degradation time depends upon the microbial activity of the environment, the surface area of the item, temperature, pH, molecular weight, and crystallinity. The values of elastic modulus and tensile strength for PHBV are 3.5 to 1 GPa and 31 to 20 MPa respectively. Also it was proved by numerous studies that PHBV is highly biocompatible and it has low toxicity. PHBV is a suitable polymer to be used in tissue engineering applications and for stent coating [28, 36]. Examining PBS (Polybutylene succinate), only a few works have been reported [35]. PBS is chemosynthetic aliphatic polyester. It is biodegradable and crystalline. Elastic modulus of PBS is 0.6 GPa and it’s melting point is 114°C. PBS is highly biocompatible as PLA and PCL (Table 2.3). However, it has low miscibility with some polymers such as PHBV [35].

Table 2.3. Structural properties of biodegradable polyesters;
PLA, PCL and PBS [36].

	Tm/Tg (°C)	E(MPa)
PLA (Polylactic Acid)	180/60	3,000
PCL (Polycaprolactone)	60/-60	400
PBS (Polybutylene succinate)	114/-32	600

Besides these potential coating materials, there are various examples of DES and different materials have been used as a platform for local drug delivery. In Taxus stent (Boston Scientific), the metal stent body is coated with the Translute that is a hydrocarbon-based copolymer. Besides Taxus, phosphorylcholine (PC) is used as coating in Endeavor stent (Medtronic). In both of these stents, the drug is released by diffusion mechanism. In Cypher stent (Cordis), PEVA-PBMA (Polyethylene-co-vinyl acetate- Poly n-butyl methacrylate) polymer blend is utilized. The studies prove that both Taxus and Cypher stents have shown significant reduction of restenosis in clinical trials and in the field [19]. In addition to these stents, in second generation Palmaz-Schatz stents, heparin has been directly bound covalently to the metal surface of the stent body. Heparin acts both as a coating and an antiproliferative agent. The results have shown that heparin coating are associated with a significant reduction in acute thrombus formation [37, 38].

2.4.3 Biodegradable Polymeric Stents

As it was described above, by development of drug eluting stents, several disadvantages of bare metal stents have been eliminated. However, there are still some drawbacks of drug eluting coated metal stents. Therefore, researchers have focused on fully polymeric stents that are biodegradable and provide local drug delivery. In polymeric stents, one of the main concerns is the mechanical characteristics of the stent body. Polymeric stent body should supply sufficient mechanical strength in order to hold the artery in an open state. On the other hand, various properties of the polymer used in stent design are distinctly significant. The stent material should be biocompatible. Degradation time of the polymer should be equal to healing rate. It should be deployed to the vessel safely and provide anchorage. When these characteristics are satisfied, drug eluting polymeric stents become superior to the other stents. These stents are highly biocompatible and degraded after healing. Furthermore, degradation time can be controlled and fully degradation of the implanted stent offers possibility for renewing the operation in the condition of repetition of the disease.

Materials utilized in polymeric stent design are generally the same with the coating materials, but the biodegradable polymers are chosen. The main polymers are PLLA, PDLLA, PCL and PLGA. Their characteristics have been discussed above in drug eluting metallic stents. These polymers have been tried in different studies and some of them have been tested clinically [29, 33]. One of the major polymeric stent is Igaki-Tamai stent. It is a biodegradable, self-expanding stent made of PLLA monofilament with a zigzag helical coil design. The stent is coated with PCL for tranilast and paclitaxel elution. The trials of Igaki-Tamai stent prove that stent thrombosis and inflammation are fully prevented, minimal neointimal hyperplasia is observed and restenosis rate is decreased to 10.5% within 6 months [39, 40].

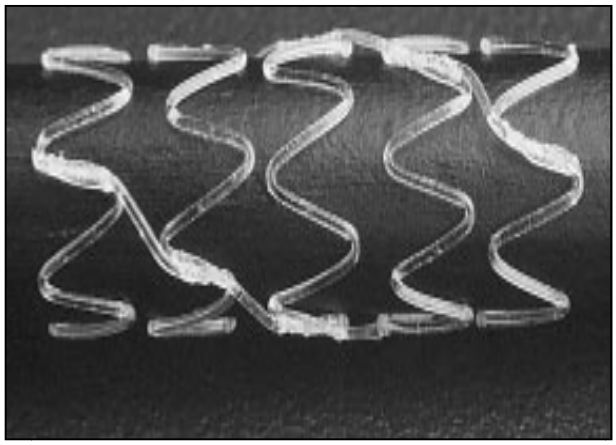

Figure 2.6. Igaki-Tamai Stent [40]

One other polymeric stent REVA is made up of tyrosine-derived polycarbonate, which is bioresorbable. It has a special slide and lock mechanism and sustained biocompatibility. Paclitaxel has been used for local drug delivery. The results show that it generates normal healing response with a normal foreign body reaction at the tissue/material interface and no acute and/or chronic inflammation is formed. Besides, it limits the occurrence of localized restenosis and re-narrowing of vessels [41, 42].

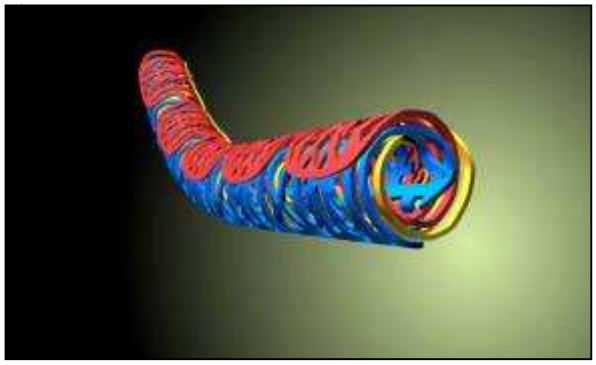

Figure 2.7. REVA stent, special slide & lock design [43]

Among these stents, BVS stent has been invented by Guidant. BVS is constructed from PLLA and coated with PDLLA for everolimus release. Clinical trials of the stent have proven that intrastent neointimal hyperplasia is reduced, late stent thrombosis is prevented and the rate of major adverse cardiac events is lowered [44].

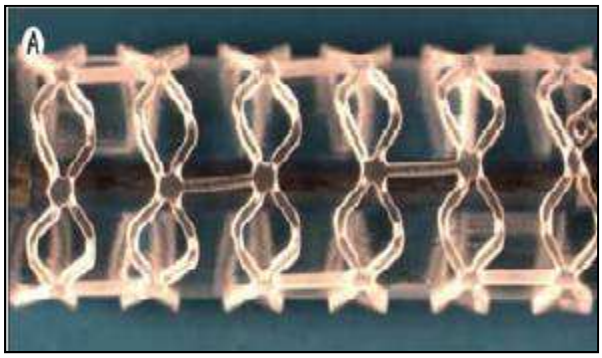

Figure 2.8. BVS stent [44]

Recently, clinical trial of a new biodegradable stent ISAR-TEST-4 has been completed [45]. This trial was accepted as the largest randomized study of drug eluting biodegradable stents. The stent material was a mixture of biodegradable polymer and natural resin. Besides, rapamycin was used as an anti-proliferative agent. In this trial, bioerodable ISAR-TEST-4, Cypher stent and Xience stent were compared. Xience is a non-erodable polymeric stent eluting everolimus. The stents were implanted to 2603 patients and 12-month results were obtained. Through the outcomes of the study, stent thrombosis percentage was lowered by ISAR-TEST-4 and restenosis ratios of all three stents were almost the same. Researchers concluded about the trial that, the advantages of ISAR-TEST-4 were also similar with the permanent stents and ISAR-TEST-4 was non-inferior to the other two leading permanent stents. Furthermore, by longer follow up studies, potential safety and efficacy superiorities of biodegradable stents may be proven [45].

2.5. *IN VITRO* STUDIES IN CAD

As was described in the first part, the main problems after stent implantation are thrombosis formation, risk of restenosis and bleeding, corrosion and impairment in the vessel. Besides, there are hypersensitivity reactions, late thrombosis and delayed endothelization risks. In order to eliminate these problems and reduce these risks, *in vitro* cell studies were performed during the ideal stent design experiments. Generally VSMCs (Vascular Smooth Muscle Cells) and HUVEC (Human Umbilical Vein Endothelial Cells) are used for the evaluation of the stent material and the drug incorporated to the stent. Stent

materials and coatings are interpreted by cell proliferation and biocompatibility assays. On the other hand, the anti-proliferative agents incorporated to the stent are considered by apoptosis assays. In addition to these evaluations, thrombogenicity of the stent material is tested for prevention of thrombosis after stent implantation.

Different approaches and researches have been performed concerning these experiments. Indolfi et al. investigated the effect of biodegradable methacrylate (MA) coating on VSMC proliferation and apoptosis. Cell proliferation was tested by bromodeoxyuridine incorporation (Table 2.4). Besides, cell death was analyzed by Annexin-V/propidium iodide detection. The results showed that there was a significant decrease in VSMC growth in MA group compared to the control group. Also, cell apoptosis increased in a MA dose-dependent manner compared to control VSMCs. So it was concluded in the article that, MA is a potential anti-proliferative agent which can be incorporated drug eluting coated metal stents [46].

Table 2.4. Cumulative data of MA-coating effects on VSMC proliferation *in vitro* assessed by BrdU incorporation [46]

	Control	Stent	MA (0,3 mL)	MA (1,5 mL)	MA (3 mL)
0 h	20±4	22±3	23±4	22±3	20±5
24 h	50±7	48±3	31±3	25±4	22±2
48 h	78±9	73±8	43±4	29±2	24±3

In another study, Vaishnav et al. examined the effectiveness of paclitaxel-loaded stents by immobilization with a biodegradable polymer, to inhibit cell proliferation. HUVECs were utilized in this study to compare the drug loaded and unloaded stents. In this research, mainly apoptosis and necrosis were studied to prove that paclitaxel does not result damage to the surrounding tissue. Annexin-V/propidium iodide assay was performed as in the previously described study. It was shown that, HUVEC attached on drug loaded stents like unloaded stents however; cell death progressed by time. Besides, all cells were apoptotic, not necrotic. Moreover, platelet adhesion on drug loaded stents was investigated.

Through the results, platelet adhesion was found minimal on polymer coated drug loaded stent compared to the unloaded stent. It was concluded that, paclitaxel significantly inhibited endothelial cell proliferation and blood compatibility of the stent was improved by drug incorporation to the polymer coating [47].

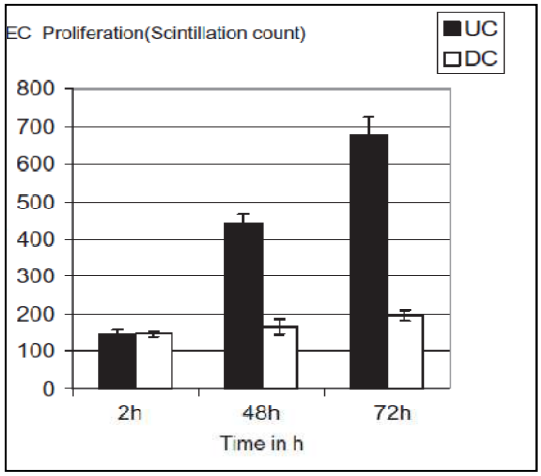

Figure 2.9. EC proliferation on uncoated and drug loaded stents. Adhesion and proliferation were quantified by 3H-thymidine uptake of the cells on devices at various time points (2 h – adhesion, 48 and 72 h – proliferation) [47]

Concerning thrombogenicity and hemocompatibility of the stent materials, various studies have been performed. There are different techniques that were applied in order to evaluate thrombogenicity. Tabrizian et al. [48] investigated hemocompatibility of chitosan (CH)-based membranes that are used for stent coatings. Heparin and sodium hyaluronate (HA) were incorporated to the membranes in order to reduce platelet adhesion, improve thromboresistance and prevent thrombosis. Thrombogenicity assay was performed to the samples and the results proved that there were 50.1% reduction for CH–PEO–Heparin samples and 63% reduction for CH–PEO–HA samples in platelet adhesion.

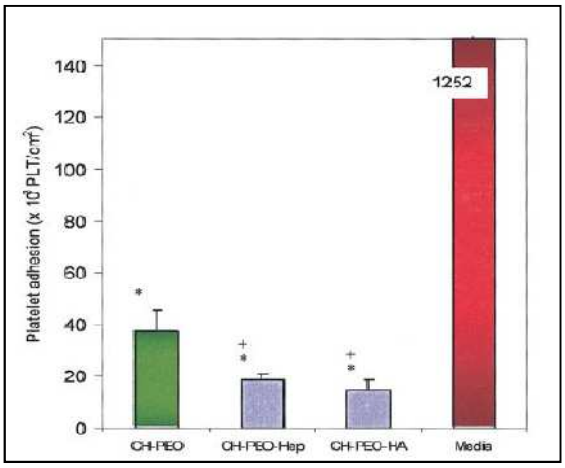

Figure 2.10. Platelet adhesion on CH–PEO and heparin/HA complexed CH–PEO (CH PEO–Hep and CH–PEO–HA) membranes. Damaged arteries (Media) were used as controls [48]

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Cell Line

HUVEC (Human Umbilical Vein Endothelial Cells) and Swiss 3T3 fibroblast cells were kindly provided by Assoc. Prof. Gamze Torun Köse.

3.1.2. Tissue Culture

Dulbecco's Modified Eagle Medium Glutamax 1g/L (GIBCO-10567-014, USA)
DPBS without Ca^{+2} and Mg^{+2} 10X (PAN Biotech-P04-37500, Germany)
Trypsin EDTA 5%-10X (GIBCO 15400-054, USA)
Fetal Bovine Serum- FBS (GIBCO 10108-165, USA)
Dimethyl Sulfoxide- DMSO (Applichem-A3006, 0100, Germany)
Gelatin from bovine skin Type B (Sigma-G9391, USA)
Penicillin-Streptomycin (P/S) solution (GIBCO- 15140-122, USA)

3.1.3. Preparation of Polymeric Films

Poly-L- lactide- PLLA, M_n 59,100 (Fluka- 93578, USA)
Poly-L- lactide- PLLA, M_n 50,400 (Fluka-94829, USA)
Poly butylene succinate extended with 1,6-diisocyanatohexane-PBS (Sigma-Aldrich-448028, USA)
Polycaprolactone-PCL (Sigma-Aldrich- 440752, USA)
Dichloromethane (Riedel-34856, Germany)
Paclitaxel, >99.5% (LC Laboratories, P-9600, USA)

3.1.4. Cell Proliferation Assay

MTS- CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega-G5421, USA)
Calcein AM Assay- LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen-L3224, USA)

3.1.5. Scanning Electron Microscope Analysis

Glutaraldehyde solution (25%) (Sigma-Aldrich, G5882, USA)
Cacodylic acid sodium salt trihydrate (AppliChem, A2140, 0100, Germany)

3.1.6. Apoptosis Assay

Caspase-3 Apoptosis Detection Kit (Santa Cruz, sc-4263 AK, USA)
Propidium iodide solution (Sigma-Aldrich, P4864, USA)

3.1.7. Thrombogenicity Assay

Citrate-Dextrose Solution (ACD) (Sigma-Aldrich, C3821, USA)
Sodium chloride (NaCl) (Sigma-Aldrich, S7653, USA)
Calcium Chloride ($CaCl_2$) (Sigma-Aldrich, C1016, USA)

3.2. METHODS

3.2.1. Culture of Swiss 3T3 Fibroblast Cells

Swiss 3T3 fibroblast cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) Glutamax (1g/L) containing 10% FBS and 1% P/S solution. Cells were grown in T-75 flasks with 13 mL medium and incubated in CO_2 incubator at 37 °C, 5% CO_2. When the cells reached confluency, they were detached from flasks by incubating in trypsin-EDTA solution for 5 min. After incubation, the cells were centrifuged at 1500 rpm for 5 min and cell pellet was resuspended in growth medium. The cells were cultivated in CO_2

incubator at 37 °C, 5% CO_2. The cells were splitted by this trypsinization method every 2 days.

3.2.2. Culture of HUVECs

Human umbilical vein endothelial cells (HUVEC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) Glutamax (1g/L) containing 10% FBS and 1% P/S solution in T-25 flasks coated with gelatin (0,5%) and incubated in CO_2 incubator at 37 °C, 5% CO_2 until they reach confluency. They were usually splitted every 3-4 days by trypsinization.

3.2.3. Preparation of Polymeric Films

Polymeric films from different biodegradable polymers (PLLA,~1.0 dl/g and PBS) at different blend ratios were formed by solution-casting method. Polymer blend (0,3 g) was weighed and dissolved in 30 mL dichloromethane by sonication (bath sonicator, PCI, India). The solution was then poured in glass molds and allowed to evaporate overnight at room temperature. Polymeric films were then kept for one hour in a vacuum oven. Then the films were cut in equal pieces for cell seeding.

3.2.4 . Preparation of Drug Eluting Films

For preparation of drug eluting films, 21 mg paclitaxel was added to polymer blend (0,1g polymer/10mL solution) of PLLA ~0.5 dl/g and PCL before sonication.

3.2.5. Seeding of Cells onto Polymeric Films

The polymeric films are cut in equal pieces for *in vitro* experiments. They were sterilized in %70 ethanol for overnight at 4 ^{o}C. Then, they were washed with DPBS for 3 times and dried under laminar flow cabinette (Telstar, Bio-II-A, Spain).

Confluent monolayers of cells (HUVEC and 3T3) were harvested by Trypsin/EDTA and the cells were concentrated by centrifugation at 1500 rpm for 5 min and resuspended in medium for seeding. Cells were counted by Trypan blue staining. Aliquots of 20 μL of

cell suspension (2.10^4 cells/ film) were seeded onto the top of films placed in the wells of 24-well plate. The matrices were left undisturbed in an incubator for 2 h to allow the cells to attach to the matrix. Then, 1 mL of medium was added into each well. Medium was changed every other day.

3.2.6 Proliferation of Cells on Polymeric Films by MTS

Cell seeded films were incubated for 1, 7, and 14 days in the CO_2 incubator at 37 ^oC. Cell Titer 96® non-radioactivity Cell Proliferation (MTS) assay was used to determine the cell density onto the polymeric films. MTS reagent (1:5, MTS solution-DMEM low glucose medium, 200 µL) was added to each well of the 24-well plate and incubated for 120 min at 37 ^oC in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader (Bio-Tek, EL x 800, USA). All experiments were performed three times.

3.2.7 Analysis of Cells on Polymeric Films by Calcein AM Cell Viability Assay

Cell proliferation on polymeric films was visualized by Calcein AM cell viability assay. After seeding the cells onto polymeric films, they were incubated for 1, 7, and 14 days in the CO_2 incubator at 37 ^oC. Calcein AM (1µM) and Ethidium Homodimer (2µM) solution were added to each well of the 24-well plate and incubated for 30 min at 37 ^oC in a CO_2 incubator. After 30 min of incubation, samples were visualized by fluorescence microscope (TE 200 Inverted Microscope, Nikon Eclipse, Japan).

3.2.8 Scanning Electron Microscope Analysis

Polymer-cell samples were prepared as was described in a previous section. At the end of 7 and 14 days culture period, samples were fixed with 2,5% glutaraldehyde in 0,1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4 ^oC. They were washed and refrigerated in 0,1 M of sodium cacodylate buffer (pH 7.4). Before SEM observation, samples were freeze-dried for 8 h and coated with gold. SEM was carried out in a Carl Zeiss instrument (EVO, Germany).

3.2.9 Apoptosis Assay

In order to detect cell death depending on paclitaxel eluting films, 80.000 cells/mL were seeded into each well of 6-well plates. The cells were incubated for 2 h for cell attachment to the plate. Paclitaxel including films were sterilized by UV in laminar flow cabinet for 2 h and placed in wells including cells. As a control group, polymeric films, sterilized by 70% ethanol for overnight at $4^{o}C$ and placed in wells including cells were used. As a negative control, cells incubated only in culture medium were studied. At the end of 1, 3 and 7 day culture period, the cells were trypsinized and 1µL DEVD-AFC component of Caspase-3 Apoptosis Detection Kit in DPBS was added to cell suspension and incubated for 15 min. After incubation, cell death was detected by flow cytometer (BD Facscalibur, USA).

3.2.10 Thrombogenicity Assay

Thrombogenicity and platelet adhesion of the polymeric films were determined by thrombogenicity assay, also called as Kinetic Model. The polymeric films were weighed and placed in each well of 24-well plates. Acid citrate dextrose solution was added into fresh human blood (170 µL ACD in 1.6 mL fresh blood) and 200 µL ACD solution was put onto samples. To start clot formation, 20 µL of $CaCl_2$ (0.1 M) solution was added to each sample and mixed. After 1 h of incubation at room temperature, 5 mL of distilled water was added to end the clot formation. The clot formation was fixed with formaldehyde solution (5 mL, 37 %) with 5 min incubation. Finally, samples were washed with distilled water, dried between tissue paper and weighed.

4. RESULTS AND DISCUSSION

4.1. PREPARATION OF POLYMERIC FILMS

In order to test the biocompatibility, cytotoxicity and thrombogenicity of the stent body and drug eluting stent coating polymers, polymer blend films (1% of PLLA, PCL and PBS) were prepared. Different compositions were tried and appropriate blends were chosen for *in vitro* experiments which were listed in Table 4.1. For drug eluting films (stent coating), the blend preparation differed from the stent body by using low molecular weight PLLA and paclitaxel as shown in Table 4.2.

Table 4.1. Polymeric Films for Stent Body

Type	**Blends**					
PLLA-PBS	90:10	85:15	80:20	75:25	70:30	60:40
PBS-PLLA	90:10	85:15	80:20	75:25	70:30	60:40
PBS-PCL	90:10	80:20	75:25	60:40		
PLLA-PBS-PCL	90:5:5	60:20:20	50:25:25			

Table 4.2. Polymeric Films for Stent Coating

Type	**Blends**			
PLLA-PCL (for drug delivery)	95:5	90:10	85:15	80:20

PLLA with different molecular weights (M=101,700 and 67,400 g/mol) were used for stent body and stent coating, respectively. PLLA with high molecular weight (M=101,700 g/mol) were chosen for the stent body since slow degradation rate (within 12 months) is expected to keep the stent intact for this period of time. On the other hand, higher degradation rate (within 1 month) is required for drug release from the stent coating to obstruct stenosis and plaque formation. Because of that reason, PLLA with low molecular weight (M=67,400 g/mol) was chosen as a stent coating polymer.

4.2. PROLIFERATION OF CELLS ON POLYMERIC FILMS BY MTS ASSAY

As was mentioned in the section 4.1, different polymeric blends were prepared and tested by MTS assay in order to determine biocompatibility of the films and decide which polymer compositions would be used for the rest of the experiments. For that purpose, various combinations of polymeric films were prepared and 3T3 Swiss fibroblast cells were seeded on these blends.

Figure 4.1 represents the cell proliferation on PLLA-PBS, PBS-PLLA and PBS-PCL blends at the day 1, 7 and 14. In all compositions, as the incubation time increases there was an increase in cell proliferation. It is obvious that PLLA-PBS and PBS-PLLA blends showed better results compared to the PBS-PCL blends. Initial cell attachment onto the PBS-PCL blend surfaces at day 1 was lower than that of the other blends. Depending on this result, it was decided to continue with PLLA-PBS and PBS-PLLA blends with various compositions.

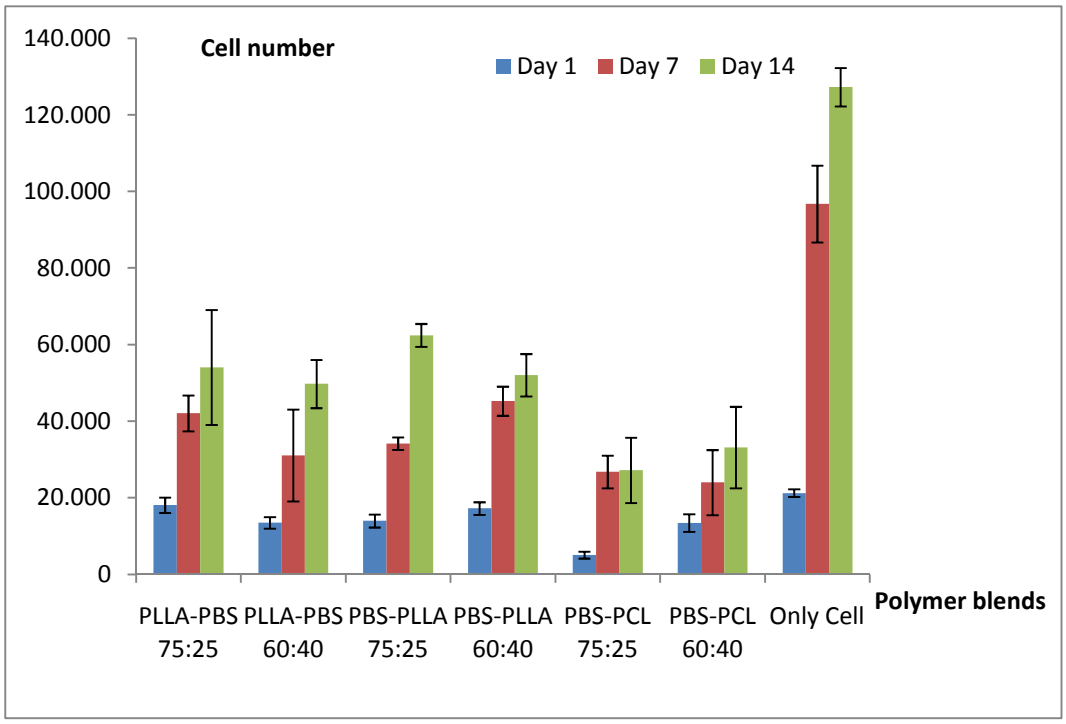

Figure 4.1. Swiss 3T3 fibroblasts growth determination on PLLA-PBS, PBS-PLLA and PBS-PCL films with different compositions at the end of 1, 7 and 14 days of incubation by MTS assay. OC samples do not contain polymer films. Initial cell number was 20.000 cells/ well

As was mentioned in the sections 3.2.5 and 3.2.6, HUVECells were also seeded on polymeric films and cell proliferation on films were determined at the end of 1, 7 and 14 days of incubation by MTS assay.

In Figure 4.2, cell proliferation on PLLA-PBS films were shown and the results show that all PLLA-PBS blends were biocompatible and cells grew happily onto them throughout 7 days of incubation. The cell growth favored most on polymeric blends PLLA-PBS 75:25, 80:20 and 90:10. In addition, there was an over-all decrease in cell number at the end of day 14. This situation can be explained by high proliferation of cells throughout 14 days of incubation. The cells began to die after day 7 due to insufficient place for cells to grow and contact inhibition. The same behavior was observed in the control samples (only cells) in which no polymeric films were used.

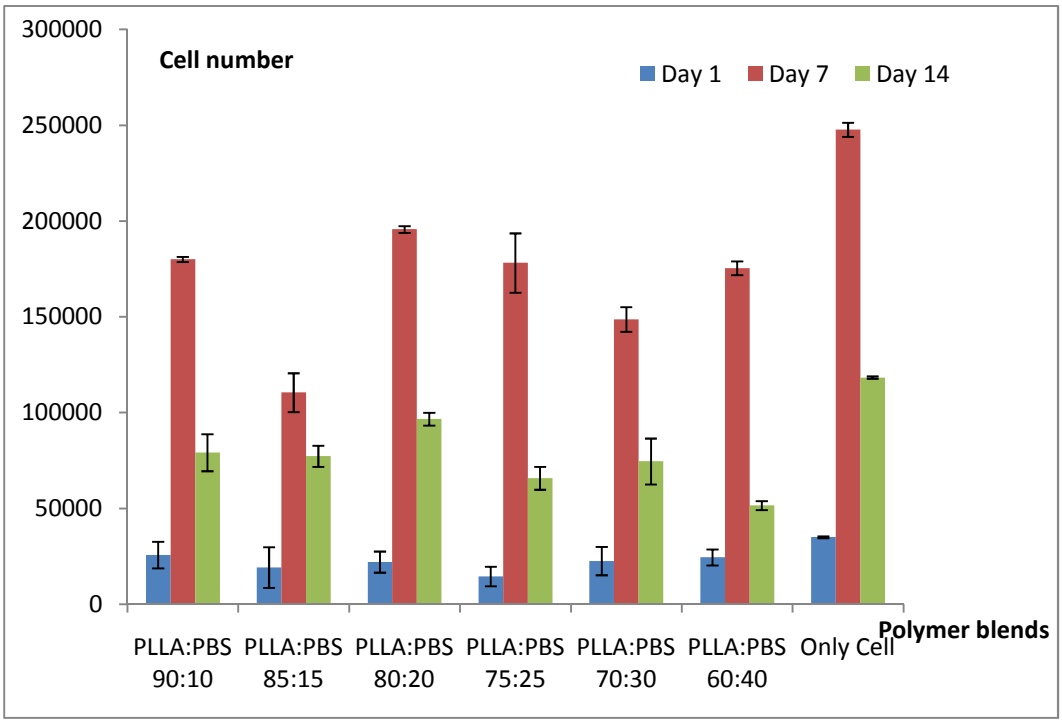

Figure 4.2. HUVECells growth determination on PLLA-PBS films with different compositions at the end of 1, 7 and 14 days of incubation by MTS assay. OC samples do not contain PLLA-PBS films. Initial cell number was 20.000 cells/ well

In Figure 4.3, cell proliferation on PBS-PLLA films were shown and the results show that all PBS-PLLA blends were all biocompatible. However, compared to the PLLA-PBS blends, although the same number of cells was seeded on to the films initally, lower cell number was determined at the end of day 7. The cell growth favored most on polymer blends PBS-PLLA 80:20 and 75:25. In addition, there was an over-all decrease in cell number at the end of day 14. This situation can again be explained by insufficient place for cells to grow and contact inhibition. Besides, it can be caused due to the disparity between the cells and the polymer. The same behavior was observed in the control samples (only cells) in which no polymeric films were used.

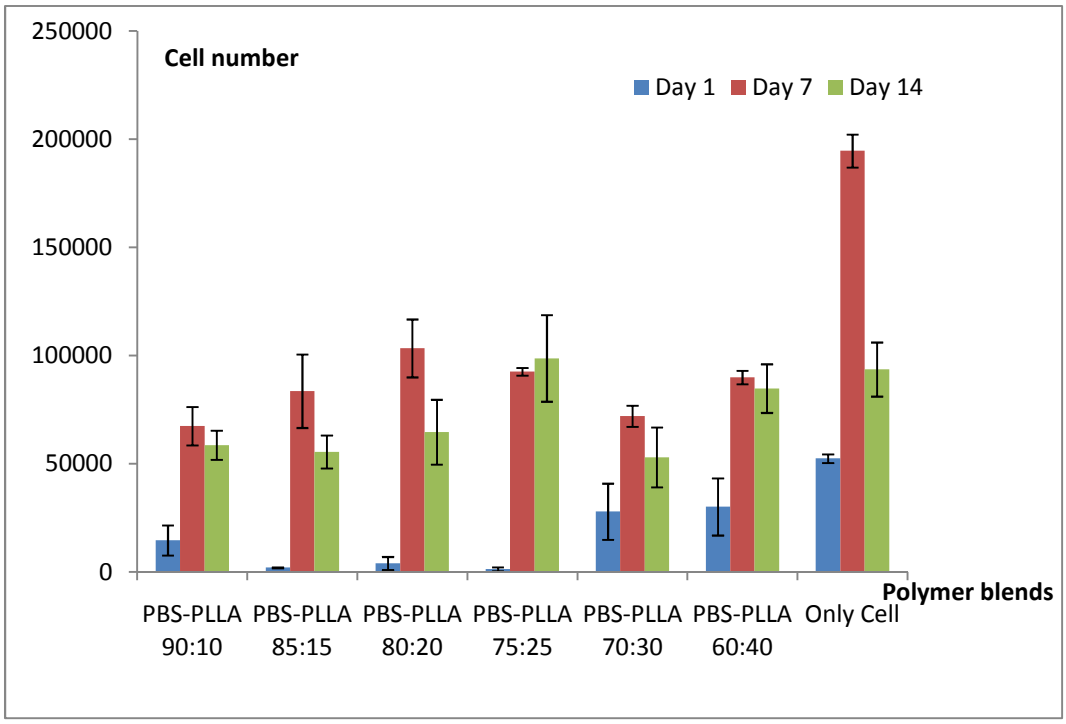

Figure 4.3. HUVECells growth determination on PBS-PLLA films with different compositions at the end of 1, 7 and 14 days of incubation by MTS assay. OC samples do not contain PBS-PLLA films. Initial cell number was 20.000 cells/well

In Figure 4.4, cell proliferation on PLLA-PCL films were shown. The results showed that all of the polymeric films mainly have the same cell number at the defined time intervals. Cell growth rates of the PLLA-PCL blends were almost the same with the control (OC). The least standard deviation was observed in PLLA-PCL 85:15 blend and also this film can be asserted to have the most consistent cell proliferation.

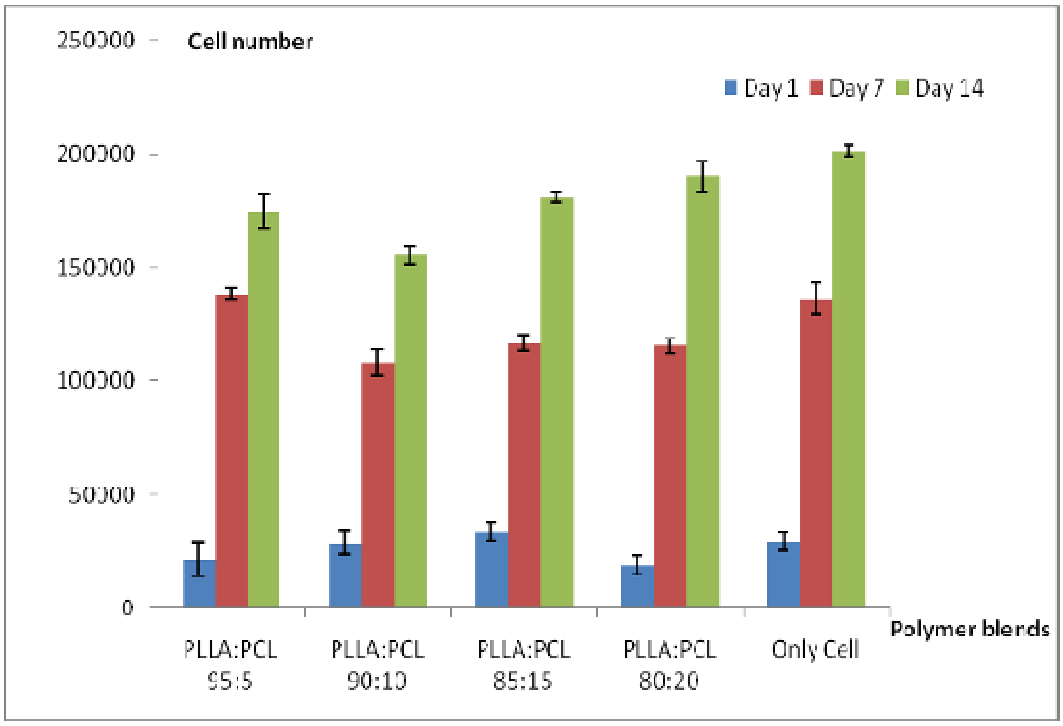

Figure 4.4. HUVECells growth determination on PLLA-PCL films with different compositions at the end of 1, 7 and 14 days of incubation by MTS assay. OC samples do not contain PLLA-PCL films. Initial cell number was 20.000 cells/ well.

Even though the same number of cells (20.000 cells/ well) were seeded onto all blends; PLLA-PBS, PBS-PLLA and PLLA-PCL, cell growth rate on PLLA-PBS was observed higher than those of both PBS-PLLA and PLLA-PCL.

The biocompatibility of the polymer is the most significant criteria and concerning biocompatibility, the most desirable biodegradable polymer is PLLA which has been used in numerous studies of tissue engineering and biomedical applications [33, 39, 40, 49]. Cell proliferation tests of PLLA have proved that the polymer does not cause cytotoxicity and it is highly biocompatible [39, 40]. However, these properties are directly affected with the molecular weights and crystallinity of the polymer. Researchers assert that low molecular weight PLLA is more cytotoxic than high molecular weight PLLA due to faster degradation and sudden change in pH [32]. The results in this project support this information. Two different PLLA (1.0 dl/g and 0.5 dl/g) were used for the stent design. For stent body, PLLA ~1.0 dl/g and PBS blends were used to provide slow degradation and

high biocompatibility. For stent coating, PLLA ~0.5 dl/g and PCL blends were used to supply faster degradation and prevent cellular adhesion. In this study, the MTS results showed that, high molecular weight PLLA blends (Figure 4.2) induced more cell growth (130.000-200.000 cells/mL at day 7) than the low molecular weight PLLA blends (Figure 4.4) (100.000 -150.000 cells/mL at day 7).

In PLLA ~1.0 dl/g and PBS blends, cell growth rate of PLLA-PBS was higher than that of PBS-PLLA and PLLA-PCL blends. Since more cell attachment was expected in stent body, PLLA-PBS blends were more suitable material with respect to cell proliferation. On the other hand, for stent coating less cell attachment was desired. Cell growth rate data of PLLA-PCL blends gave us expected results for this part of the stent.

4.3. ANALYSIS OF CELLS ON POLYMERIC FILMS BY CALCEIN AM CELL VIABILITY ASSAY

In order to visualize cell viability and cell death on polymeric films, HUVECs were seeded on films of different polymer blends. The viable cells were stained with green fluorescence of Calcein AM. The death cells were stained with red fluorescence of Ethidium Homodimer. This technique is based on qualitative analysis.

In this study, Figure 4.5 represents cell viability imaging on PLLA-PBS blends. As the time passes cell viability increased in all sets. Higher cell viability was observed on the polymer blends PLLA-PBS 80:20 and 90:10. Besides, there was an increased cell death on PLLA-PBS 60:40 polymer blend at the day 14.

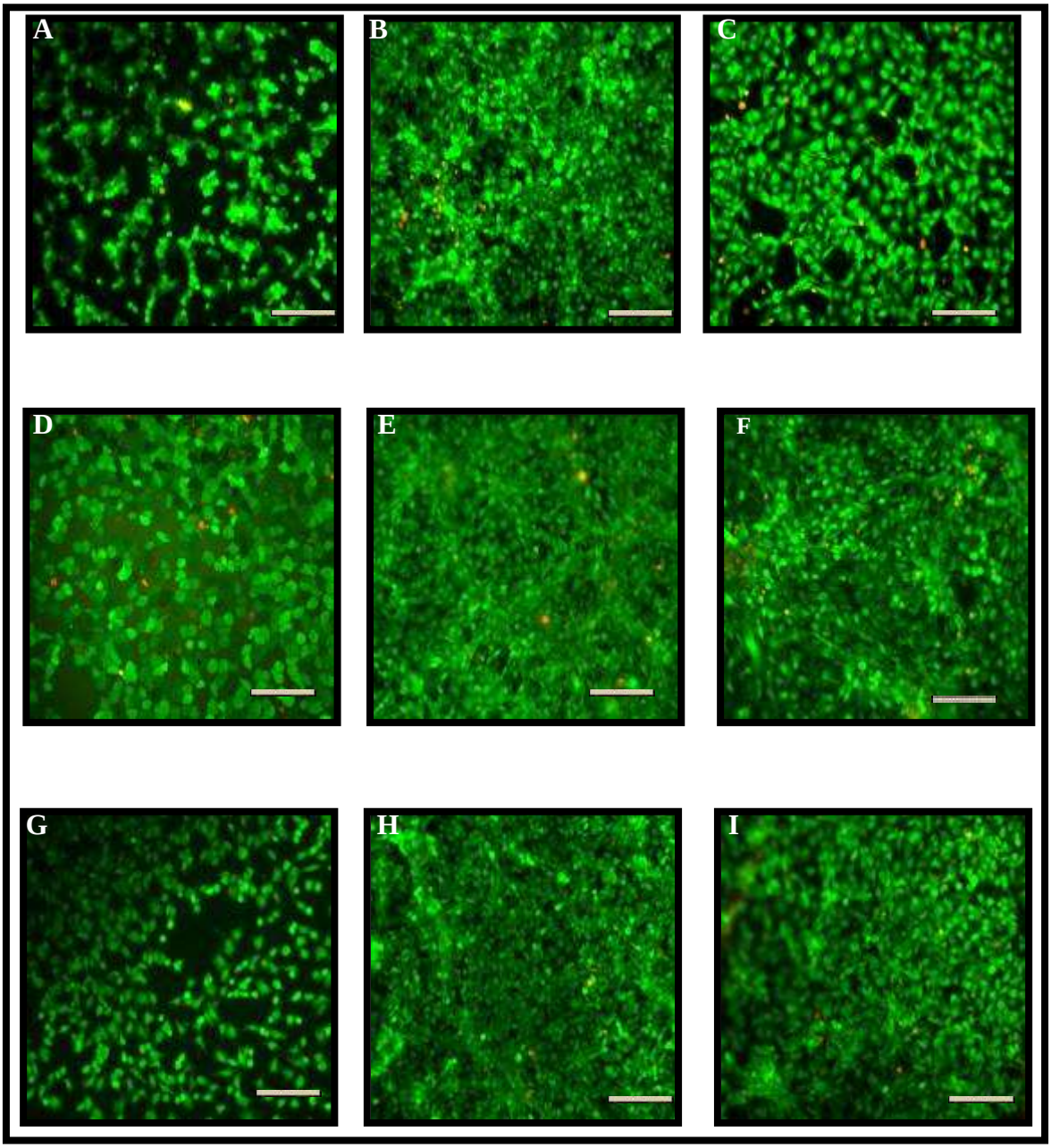

Figure 4.5. Cell viability imaging on PLLA-PBS blends at the end of 1, 7 and 14 days with Calcein AM assay. Initial cell number was 20.000 cells/ film. Scale Bar: 100µm (10 X)

Day 1 (A) 90:10, (D) 85:15, (G) 80:20, (J) 75:25, (M) 70:30, (P) 60:40

Day 7 (B) 90:10, (E) 85:15, (H) 80:20, (K) 75:25, (N) 70:30, (R) 60:40

Day 14 (C) 90:10, (F) 85:15, (I) 80:20, (L) 75:25, (O) 70:30, (S) 60:40

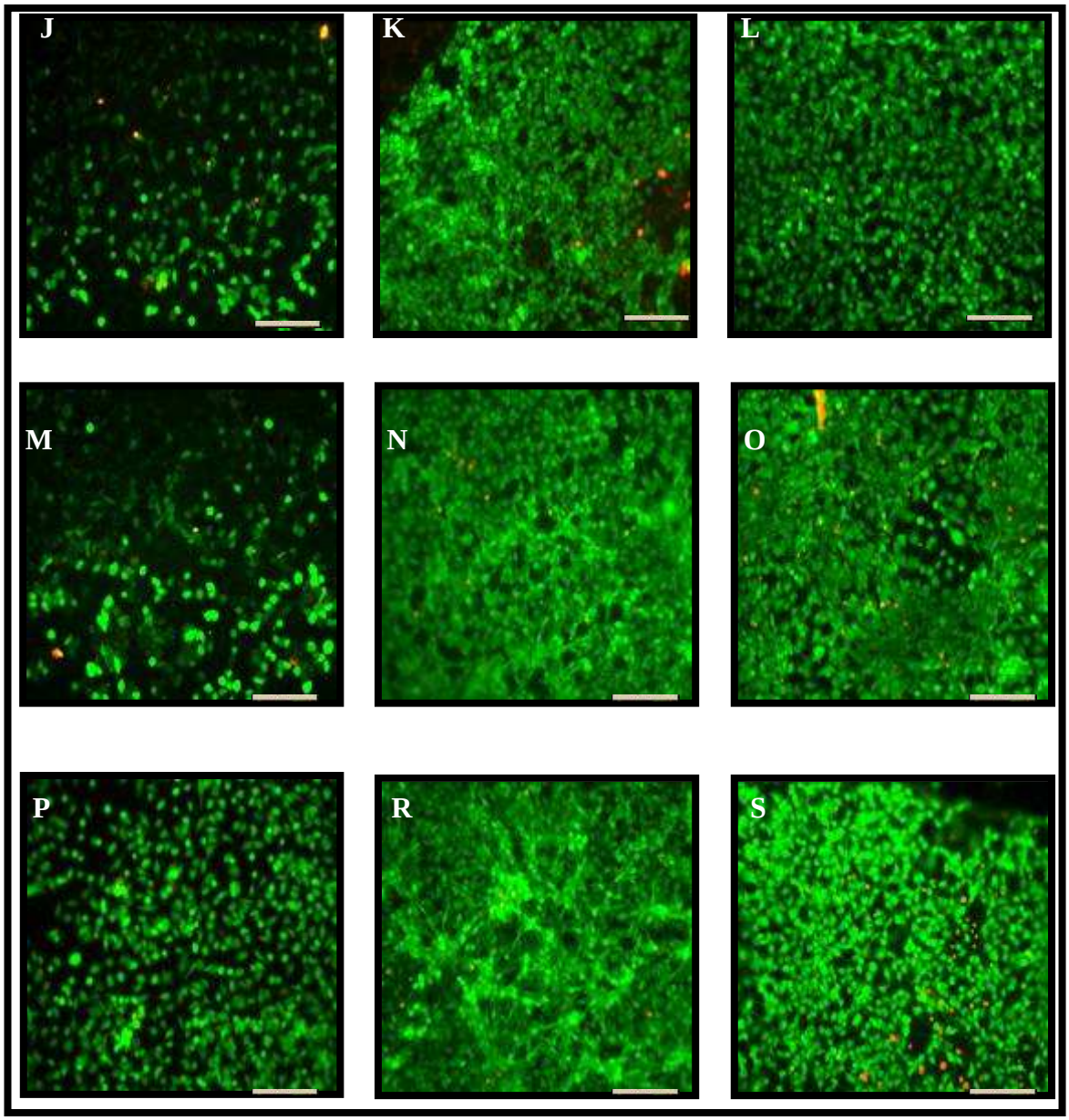

Figure 4.5. (continue) Cell viability imaging on PLLA-PBS blends at the end of 1, 7 and 14 days with Calcein AM assay. Initial cell number was 20.000 cells/ film. Scale Bar: 100µm (10 X)

Day 1 (A) 90:10, (D) 85:15, (G) 80:20, (J) 75:25, (M) 70:30, (P) 60:40

Day 7 (B) 90:10, (E) 85:15, (H) 80:20, (K) 75:25, (N) 70:30, (R) 60:40

Day 14 (C) 90:10, (F) 85:15, (I) 80:20, (L) 75:25, (O) 70:30, (S) 60:40

Figure 4.6 shows cell viability imaging on PBS-PLLA blends throughout 14 days of incubation. At the end of 14 days of incubation, it was observed that cell number was generally decreased.

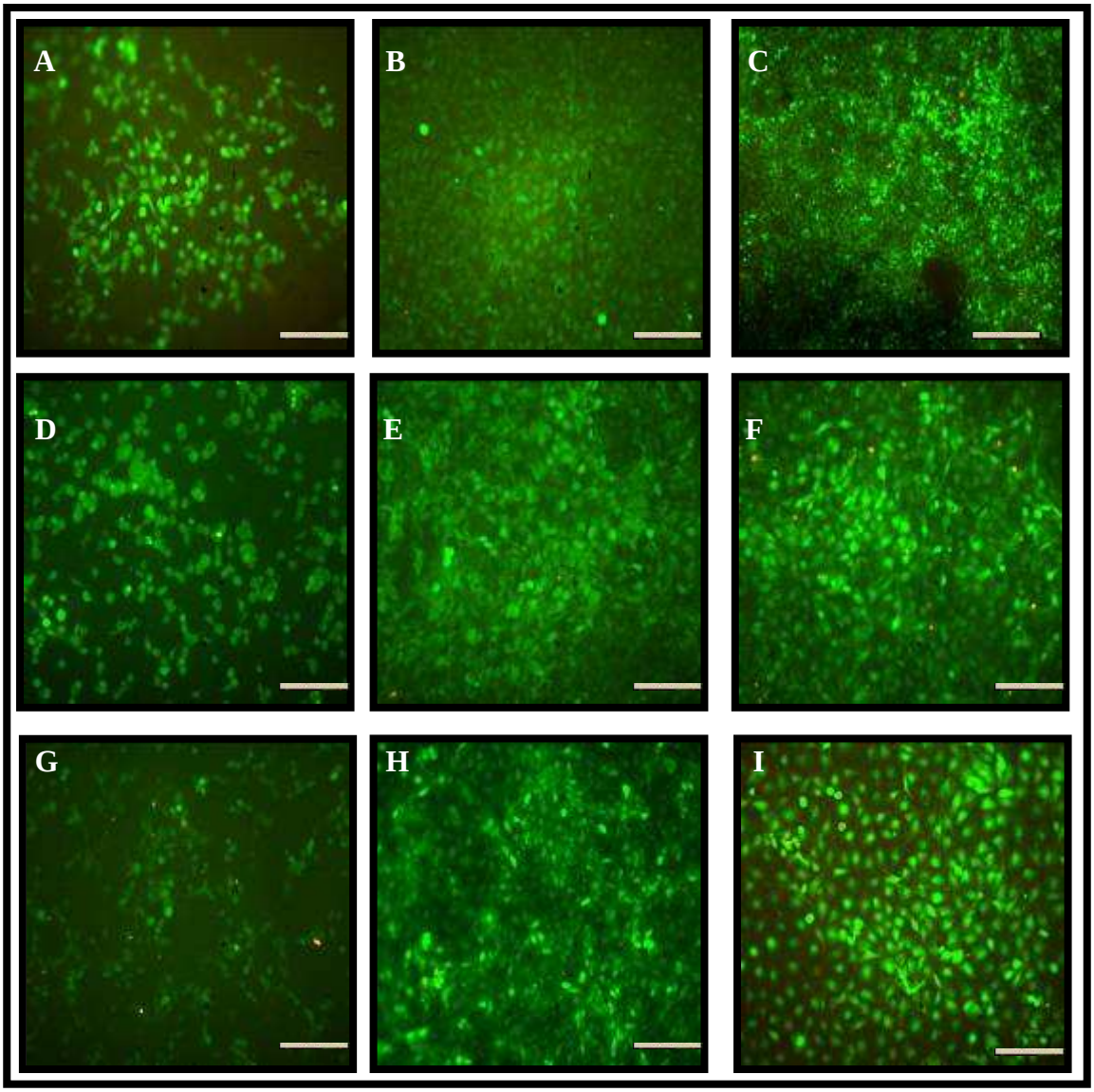

Figure 4.6. Cell viability imaging on PBS-PLLA blends at the end of 1, 7 and 14 days with Calcein AM assay. Initial cell number was 20.000 cells/ film. Scale Bar: 100µm (10 X)

Day 1 (A) 90:10, (D) 85:15, (G) 80:20, (J) 75:25, (M) 70:30, (P) 60:40

Day 7 (B) 90:10, (E) 85:15, (H) 80:20, (K) 75:25, (N) 70:30, (R) 60:40

Day 14 (C) 90:10, (F) 85:15, (I) 80:20, (L) 75:25, (O) 70:30, (S) 60:40

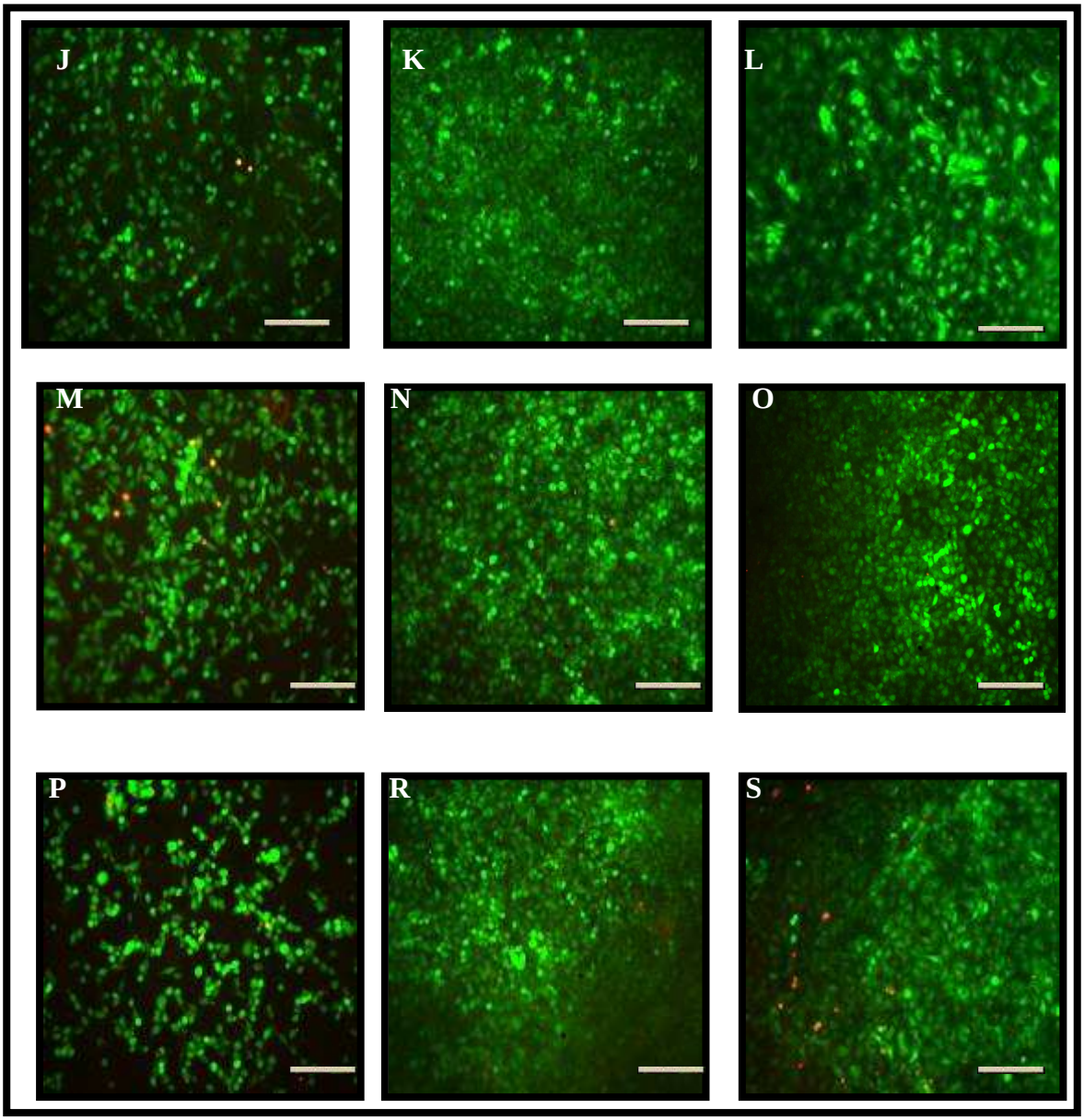

Figure 4.6. (continue) Cell viability imaging on PBS-PLLA blends at the end of 1, 7 and 14 days with Calcein AM assay. Initial cell number was 20.000 cells/ film. Scale Bar: 100µm (10 X)

Day 1 (A) 90:10, (D) 85:15, (G) 80:20, (J) 75:25, (M) 70:30, (P) 60:40

Day 7 (B) 90:10, (E) 85:15, (H) 80:20, (K) 75:25, (N) 70:30, (R) 60:40

Day 14 (C) 90:10, (F) 85:15, (I) 80:20, (L) 75:25, (O) 70:30, (S) 60:40

In both PLLA-PBS and PBS-PLLA blends (Figures 4.5-4.6), as can be observed in the figures, cell number began to decrease after day 7 and more cell death was observed on all of the polymer films at the day 14 due to contact inhibition. This result also supports the MTS data of PLLA-PBS and PBS-PLLA films.

4.4. SCANNING ELECTRON MICROSCOPE ANALYSIS

HUVECs were seeded on to polymeric films and the morphologies of cells, polymers and cell-polymer interactions were analyzed by SEM in order to observe cell presence and cell alignment on films. Figure 4.7 represents cell-polymer relationship of cell seeded PLLA-PCL films with different compositions throughout 14 days of incubation. In both blends, cell morphologies became more aligned and comfortable on the films as time passes.

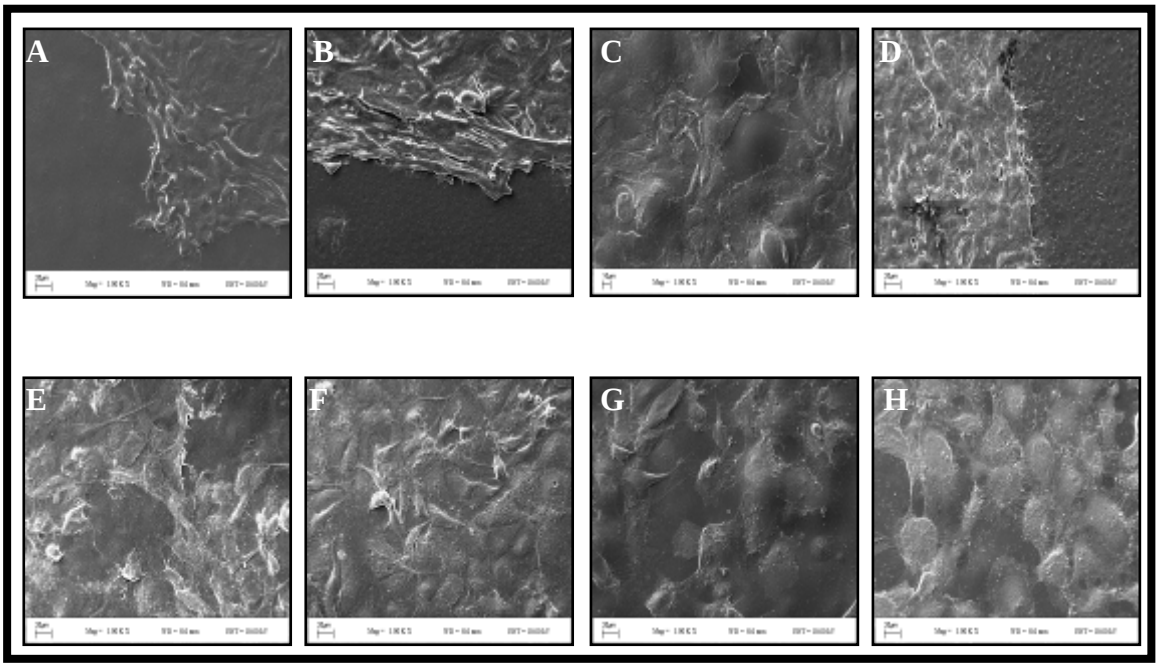

Figure 4.7. SEM micrographs of cell seeded PLLA-PCL films with different compositions at the end of 7 and 14 days of incubation (1000 X);
7 days : (A) 95:5, (B) 90:10, (C) 85:15, (D) 80:20;
14 days : (E) 95:5, (F) 90:10, (G) 85:15, (H) 80:20

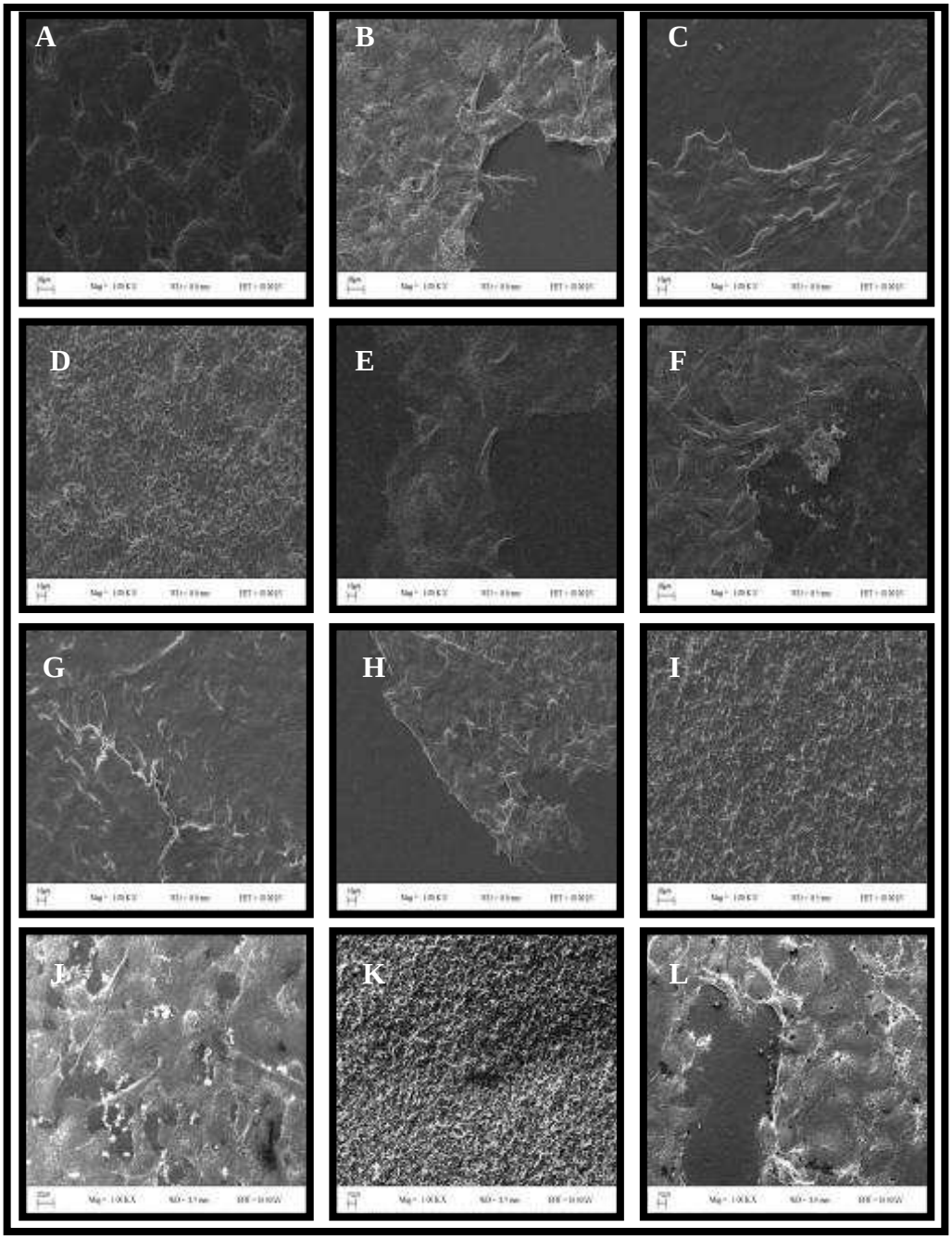

Figure 4.8. SEM micrographs of cell seeded PLLA-PBS films with different compositions at the end of 7 and 14 days of incubation (1000 X);
7 days : (A) 90:10, (B) 85:15, (C) 80:20, (G) 75:25, (H) 70:30, (I) 60:40;
14 days : (D) 90:10, (E) 85:15, (F) 80:20, (J) 75:25, (K) 70:30, (L) 60:40.

Figure 4.8 represents cell attachment behavior of cell seeded PLLA-PBS films with different compositions throughout 14 days of incubation. Cell morphologies were more aligned and flattened at the end of 14 days of incubation.

Figure 4.9 represents cell-polymer interaction of cell seeded PBS-PLLA films with different compositions throughout 14 days of incubation. Aligned and relaxed cell morphologies were also observed in this set.

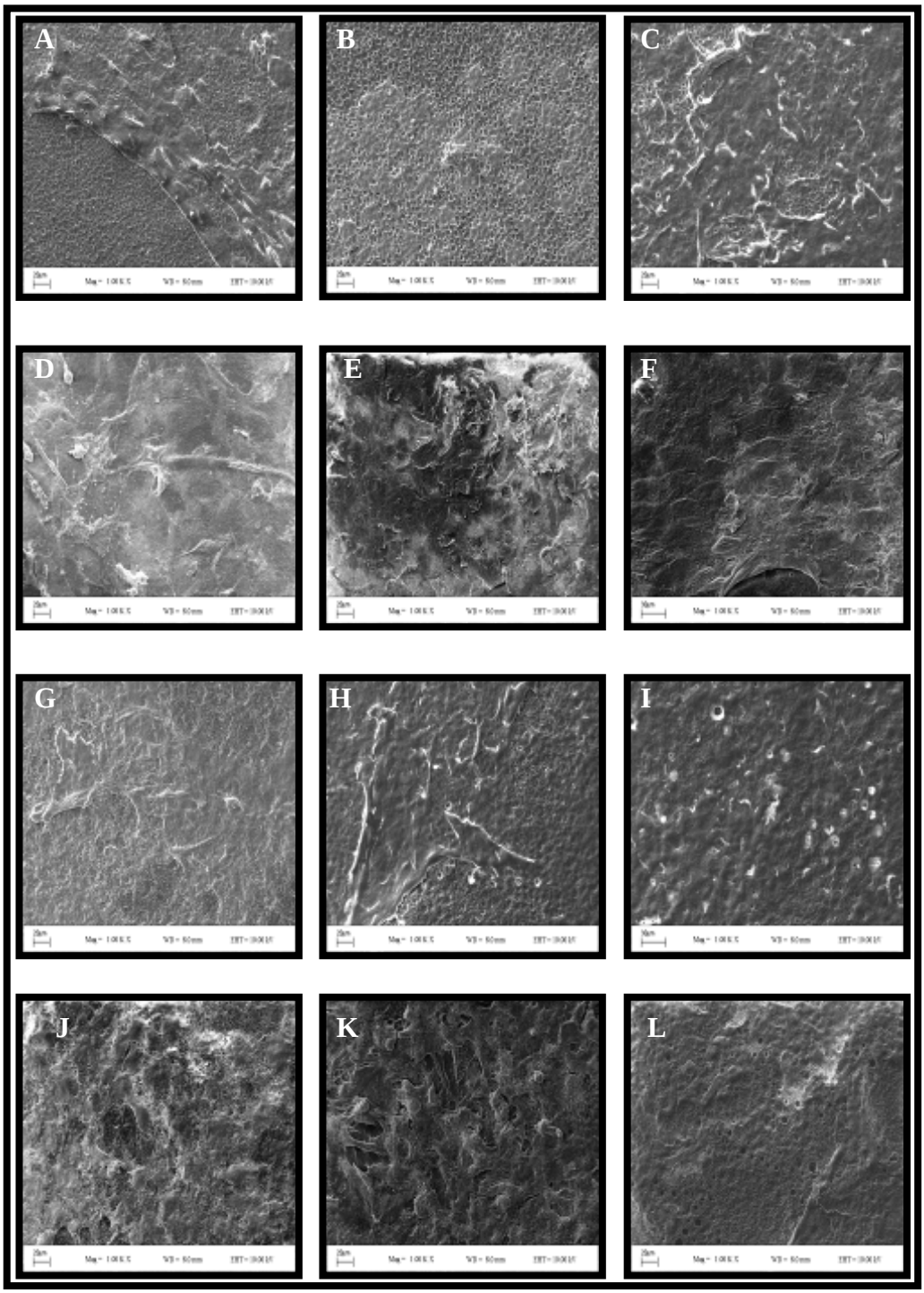

Figure 4.9. SEM micrographs of cell seeded PBS- PLLA films with different compositions at the end of 7 and 14 days of incubation (1000 X);
7 days :(A) 90:10, (B) 85:15, (C) 80:20, (G) 75:25, (H) 70:30, (I) 60:40;
14 days : (D) 90:10, (E) 85:15, (F) 80:20, (J) 75:25, (K) 70:30, (L) 60:40

4.5 APOPTOSIS ASSAY

In order to determine the apoptotic effect of paclitaxel release from different compositions of PLLA-PCL films, apoptosis assay was performed. The cells were incubated with drug loaded PLLA-PCL films for 1, 4 and 7 days.

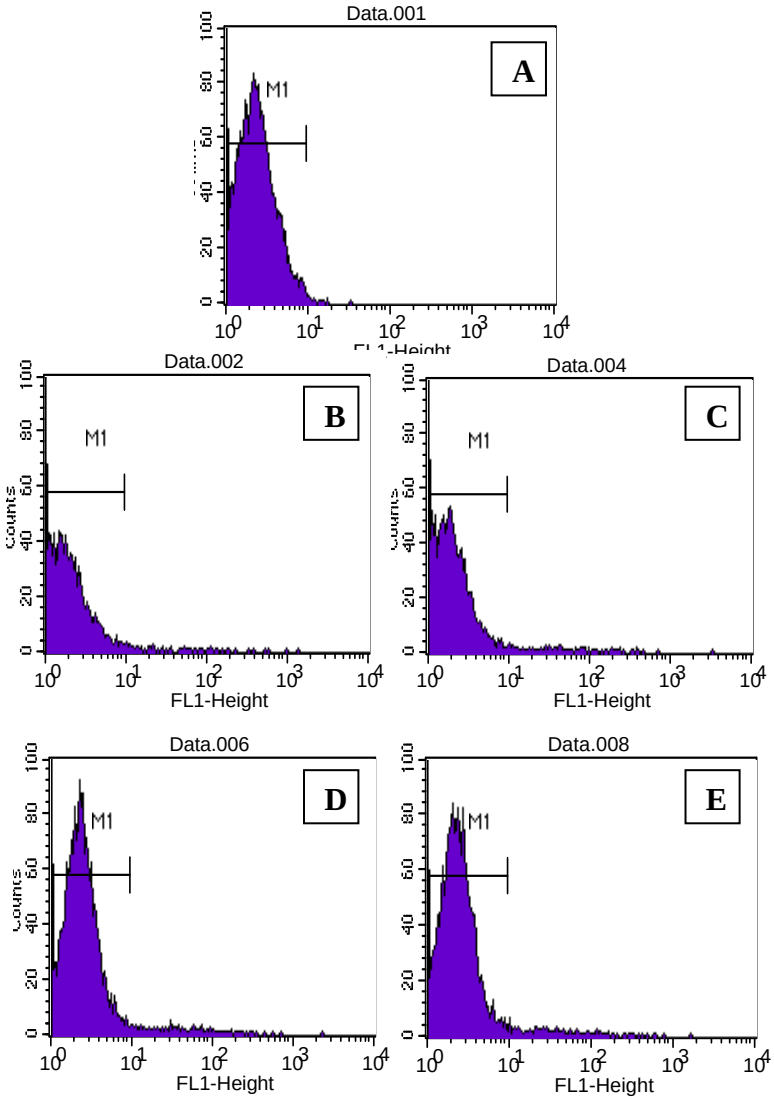

Figure 4.10. Flow cytometer histograms, apoptosis ratios of HUVEC due to paclitaxel elution from PLLA-PCL blends at the day 1; (A) Control, (B) 95:5, (C) 90:10, (D) 85:15, (E) 80:20

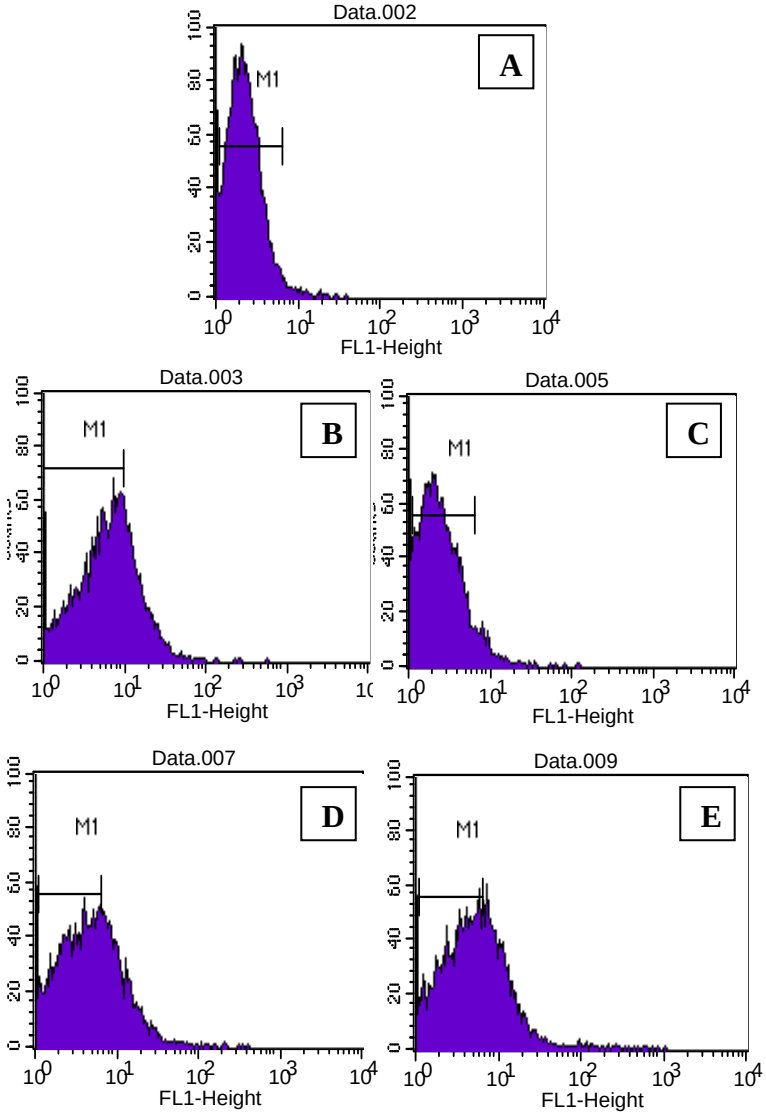

Figure 4.11. Flow cytometer histograms, apoptosis ratios of HUVEC due to paclitaxel elution from PLLA-PCL blends at the day 4; (A) Control, (B) 95:5, (C) 90:10, (D) 85:15, (E) 80:20

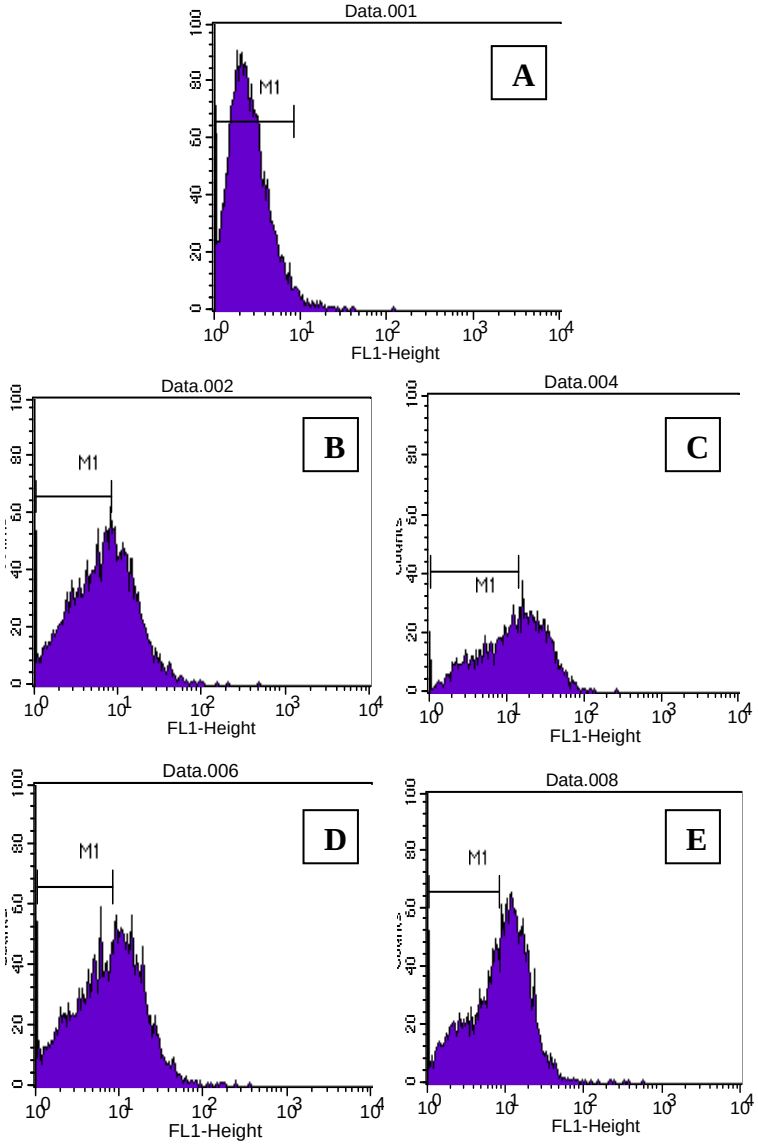

Figure 4.12. Flow cytometer histograms, apoptosis ratios of HUVEC due to paclitaxel elution from PLLA-PCL blends at the day 7; (A) Contro,l (B) 95:5, (C) 90:10, (D) 85:15, (E) 80:20

Table 4.3. Cell viability percentages of HUVECs due to paclitaxel elution from PLLA-PCL blends at the day 1, 4 and 7

Cell viability (%)	Day 1	Day 4	Day 7
OC	99,68	88,43	92,27
95:5 wt pxt	98,86	74,06	57,11
95:5 control	99,72	80,03	88,24
90:10 wt pxt	98,08	79,41	53,82
90:10 control	99,92	85,39	86,95
85:15 wt pxt	96,86	59,79	49,99
85:15 control	99,86	88,61	89,74
80:20 wt pxt	97,02	59,12	42,63
80:20 control	99,87	91,51	90,64

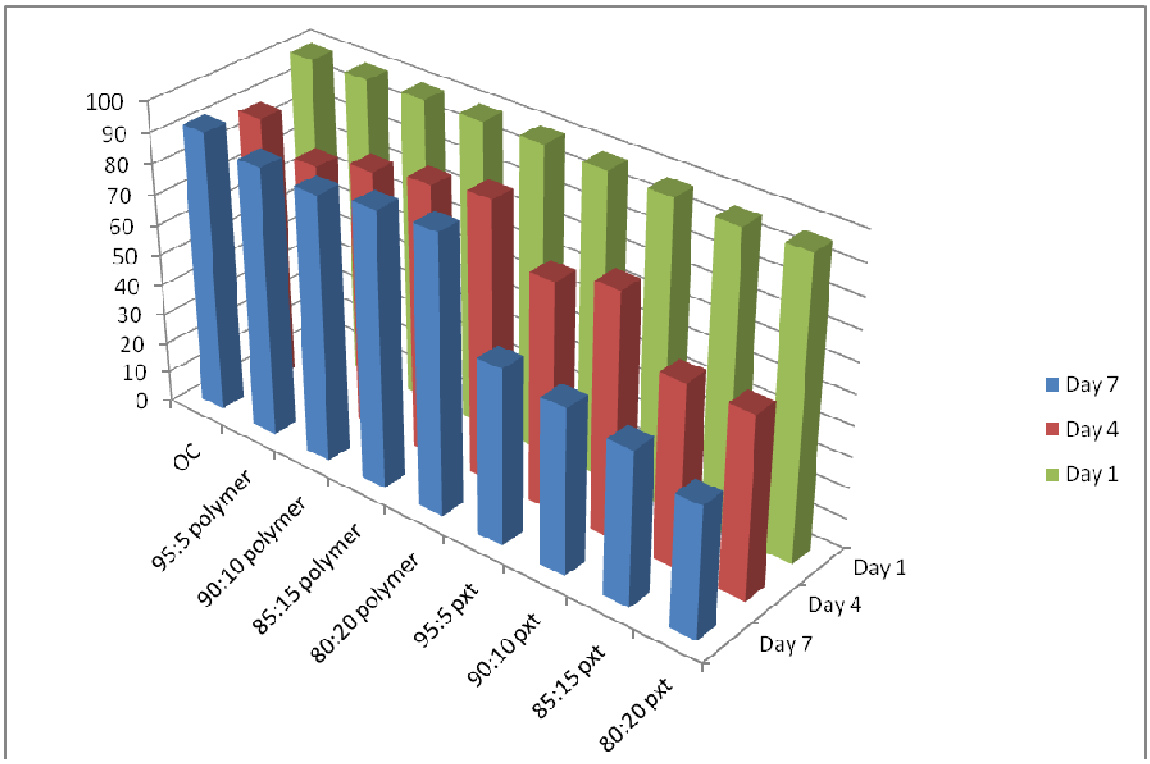

Figure 4.13. Cell viability percentages of HUVECs due to paclitaxel elution from PLLA-PCL polymeric films at the day 1, 4 and 7

In figure 4.10-4.12 flow cytometer histograms of PLLA-PCL blends at the day 1, 4 and 7 were shown. Table 4.3 and Figure 4.13 summarize all of these results. It was observed that there was an increase in cell death in all of the contents of PLLA-PCL films throughout 7 days of incubation (Figure 4.13).

PCL is a biodegradable polymer that is used for stent coating. However, PCL is generally used in blend compositions in stent design because it does not supply sufficient mechanical strength to support the vessel and has lower biocompatibility compared to the other polymers such as PLLA, PLGA and PBS. However, PCL is still a preferred material which mainly provides faster degradation when utilized in the blends. For these reasons, in this study, PCL was used in stent coating, with low weight ratio in the blends [31, 50].

The results of this study proved that efficient drug release from stent coating can be supplied by using PLLA-PCL blend. The biocompatibility of the unloaded PLLA-PCL blends was shown by MTS assay. In the mean time, apoptotic effect of paclitaxel loaded PLLA-PCL blends was shown by Caspase-3 assay and flow cytometry results. HUVECs were incubated with different ratios of PLLA-PCL blends loaded with paclitaxel and apoptosis percentages of cells were detected through 7 days of incubation. According to the results, cell death was increased as PCL ratio increase in the blends. In the 80:20 PLLA-PCL blend, nearly 53% decrease in cell viability was observed comparing to its control due to paclitaxel release at the end of day 7. Meanwhile, 35%, 38% and 44% decreases were determined in cell viability in 95:5, 90:10 and 85:15 PLLA-PCL blends, respectively. As was mentioned in section 1.4.2, there are various drug release mechanisms. In our design, paclitaxel was released by diffusion and biodegradation. Therefore, it may show that degradation accelerated by the PCL percent in the blend since more pxt is released, leading to more cell death.

There are numerous studies using paclitaxel and sirolimus as an anti-proliferative drug. The aim is always supplying cell death in order to prevent restenosis and neointima formation. However, the incorporated dosage and release rate are crucial. Late endothelization and inflammation reactions mostly occur by anti-proliferative drug release in the vessel.

Axel et al. [24] investigated the effect of paclitaxel on monocultures of SMCs and co-cultures with human arterial endothelial cells. Nonstop paclitaxel exposure resulted a complete and prolonged inhibition of the cells up to 14 days. In a subsequent *in vivo* study, local paclitaxel delivery to the carotid arteries of rabbits after induction of an atherosclerotic plaque caused significant decrease in the extent of the stenosis. However, there were no supplementary information about the inflammatory reactions and late endothelization risks.

Some studies have shown that some synthetic polymers, biodegradable or non-biodegradable, resulted in an important inflammatory and proliferative tissue response [51-52]. However, our results showed that stent coating polymeric blend of PLLA-PCL was biocompatible. According to MTS data and SEM analysis, strong cellular attachment was observed and cell number on polymeric films increased by time.

4.6. THROMBOGENICITY ASSAY

In order to determine the thrombogenicity of the various polymeric blends, the polymeric films were treated with fresh human blood and weight differences of the films before and after the blood treatment were obtained. The weight differences were originated from platelet, leukocyte and thrombus residuals adhesions on polymeric films.

Figure 4.14 represents the weight differences and thrombosis ratio of PLLA-PBS blends after blood treatment. The highest thrombosis ratio was obtained with PLLA-PBS 85:15 and 70:30 blends meaning that more blood cells were attached onto the film surfaces. On the other hand, PLLA-PBS 75:25 and PLLA-PBS 80:20 blends showed the least thrombosis ratios.

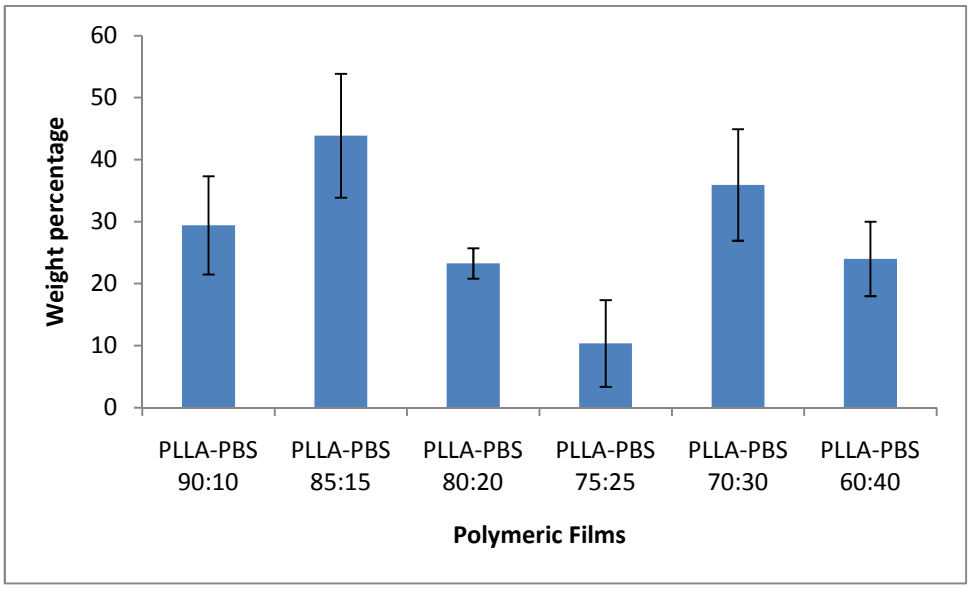

Figure 4.14. Thrombosis ratios of PLLA-PBS polymeric blends; weight difference percentages of polymeric films after blood treatment were indicated in the y-axis.

Figure 4.15 represents the weight differences and thrombosis ratio of PBS-PLLA blends after blood treatment. Blends of PBS-PLLA 90:10, 85:15, 80:20, 75:25 and 60:40 had almost the same thrombosis ratio which was higher than that of PBS-PLLA 70:30 blend that showed the least thrombosis ratio. This shows that there was a less blood cell adhesion on to the surface of PBS-PLLA 70:30 film.

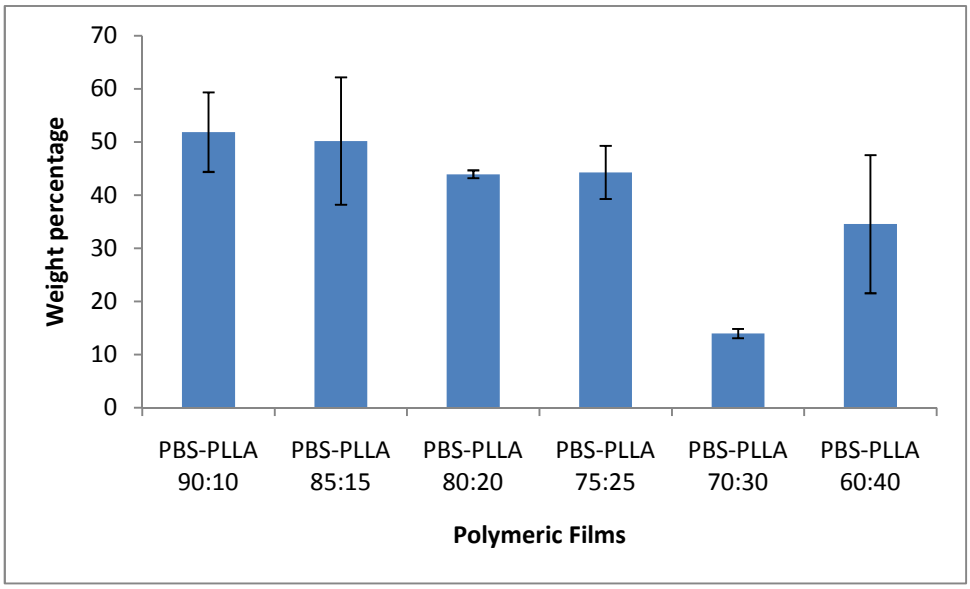

Figure 4.15. Thrombosis ratios of PBS-PLLA polymeric blends; weight difference percentages of polymeric films after blood treatment were indicated in the y-axis.

Concerning hemocompatibility, there are several studies on thrombogenicity, platelet and leukocyte adhesions to the stent materials [47, 48, 53, 54]. Prasad et al. [47] reported that polymer coating significantly reduced platelet and leukocyte adhesion to stent material. However, drug loaded and unloaded polymers showed similar platelet adhesion.

In our study, both PLLA-PBS and PBS-PLLA blends were tested through thrombogenicity properties. The results showed that there was not a trendline between different compositions of PLLA-PBS blends. But the least weight differences were obtained from the PLLA-PBS 80:20 and 75:25 blends. This situation can be explained due to surface characteristics of the blends.

On the other hand, there was a trendline between the compositions of the PBS-PLLA blends. Weight differences decreased by the increment of PLLA ratio in the blends which means hemocompatibility of PLLA is suitable for using the polymer as a stent material.

5. CONCLUSION AND RECOMMENDATIONS

5.1. CONCLUSION

In this study, the aim was to investigate the *in vitro* convenience of drug eluting biodegradable polymeric stent to the human body for the treatment of coronary artery disease. *In vitro* results of the polymeric materials and the drug loaded stent coating lead us to decide the appropriate compositions of the polymeric blends and drug release duration. Besides, it can be assumed with these *in vitro* results that when the stent will be tested for *in vivo* investigations and clinical trials in the future experiments, it may provide significant outcomes for the use against CAD.

As it was discussed in the text, the main parameter that should be considered in stent design is the biocompatibility of the stent material. Therefore, the biocompatibility of the stent body and stent coating polymer was tested by MTS assay quantitatively. Besides, MTS results were supported by Calcein AM assay and SEM analysis. All these experiments were complementary to each other. According to the results; PLLA-PBS polymeric blends were more suitable for stent body. Especially PLLA-PBS 90:10, 80:20 and 75:25 seem more favorable. For the stent coating, biocompatibility of the PLLA-PCL blends were proved by MTS assay and cell attachment on the blends was also shown by SEM analysis. PLLA-PCL resulted less cell attachment compared to PLLA-PBS and PBS-PLLA blends as was desired for stent coating.

In addition to biocompatibility, cytotoxicity of the drug loaded stent coating polymer is crucial. Drug loaded in stent coating should provide apoptosis of VSMCs, fibroblasts and inflammatory cells in order to prevent restenosis and neointima formation. However, the antiproliferative drug release should be completed within 1 month so that healing process and endothelization will not be prevented. For that purpose, apoptotic effect of stent coating PLLA-PCL blends was tested by Caspase-3 apoptosis assay. The results proved that drug release and degradation of the polymeric blend accelerated by the increment of PCL ratio. Drug loaded in PLLA-PCL 80:20 blend caused more cell death

compared to the other blends through 7 days of incubation and this blend was chosen as a suitable composition for stent coating polymeric blend.

Considering hemocompatibility, PLLA-PBS and PBS-PLLA blends were tested by thrombogenicity assay. These blends which were evaluated for stent body, should lead less blood cell adhesion in order to prevent restenosis and thrombus formation in the vessel. The least blood cell attachment to the polymeric films was detected in the PLLA-PBS 80:20 and 75:25 and PBS-PLLA 70:30 blends.

Finally, PLLA-PBS 80:20 and 75:25 blends were selected as the most appropriate compositions for stent body. In addition, PLLA-PCL 80:20 blend was chosen as stent coating polymer composition. As a conclusion, selected polymeric blends and compositions have high potentials for the construction of drug eluting biodegradable polymeric stents.

5.2. RECOMMENDATIONS

For future studies, the design and the morphology of the stent will be determined and the stent will be constructed from the chosen polymeric blends with the preferred design. Then, drug eluting polymeric stents will be implanted to the rabbit arteries in order to investigate the *in vivo* convenience and functionality by histological analysis. After animal experiments, clinical trials will be performed and the final product will be developed.

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