VANCOMYCIN CONTAINING PLLA DELIVERY SYSTEM FOR BONE TISSUE BIOCOMPATIBILITY AND TREATMENT OF IMPLANT RELATED CHRONIC OSTEOMYELITIS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

VANCOMYCIN CONTAINING PLLA DELIVERY SYSTEM FOR BONE TISSUE BIOCOMPATIBILITY
AND TREATMENT OF IMPLANT RELATED CHRONIC OSTEOMYELITIS

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Osteomyelitis is an infection of bone or bone marrow, usually caused by pyogenic bacteria. It can cultivate by hematogen way or it can cultivate by the help of local soft tissue infection. Osteomyelitis often requires prolonged antibiotic therapy and surgery. But for therapy; antibiotic must reach to effective dose in the bone. So that; for prevention and treatment of osteomyelitis controlled antibiotic release systems can be used. These systems have been developed to deliver antibiotics directly to infected tissue. As a carrier material; polymers are widely use. Polymer can be biodegradable or non biodegradable. The advantage of biodegradable polymers is; you do not need a second surgery for the removal of the carrier material from the body.

In this study; vancomycin loaded PLLA/TCP composites were developed and characterized to treat implant related chronic osteomyelitis in experimental rat osteomyelitis model. Some of the composites were prepared by coating the vancomycin loaded composites with PLLA to observe the difference between the

coated and uncoated composites. Also, some composites were developed free from the vancomycin to determine the biocompatibility of the composite for the bone tissue. The coating extended the release of the vancomycin up to 5 weeks and changed the surface morphology of the composites. According to the cell culture studies, vancomycin loaded PLLA/TCP composites promoted cell adhesion, cell proliferation and mineralization so; the composite was biocompatible with bone tissue. Radiological and microbiological evaluations showed that vancomycin loaded and coated vancomycin loaded PLLA/TCP composites inhibited MRSA proliferation and treat implant related chronic osteomyelitis.

Keywords: Antibiotic, osteomyelitis, PLLA, vancomycin, bone, polymer, MRSA, in vivo, drug delivery systems, controlled release, biodegradable polymer

ÖZ

VANKOMİSİN İÇEREN PLLA TAŞIYICI SİSTEMİNİN KEMİK DOKUSUNDAKİ BİYOUYUMLULUĞU VE İMPLANT İLİŞKİLİ KRONİK OSTEOMİYELİT TEDAVİSİNDE KULLANIMI

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Osteomiyelit; piyojenik bakteriler tarafından kemikte ya da kemik iliğinde oluşturulan bir enfeksiyon hastalığıdır. Hematojen yolla bir bakteriyemi sonrasında sekonder olarak gelişebildiği gibi yakındaki yumuşak dokudan infeksiyonun yayılması ile de meydana gelebilir. Osteomiyelit tedavisi antibiyotik tedavisinin yanında cerrahi tedaviyi ve hastanın durumunun düzeltilmesini ve altta yatan hazırlayıcı faktörlerin ortadan kaldırılmasını kapsar. Ancak tedavide antibiyotiğin kemikte etkili düzeye ulaşabilmesi önemlidir. Bu yüzden osteomiyelitin önlenmesi ve tedavisinde kontrollü antibiyotik salım sistemleri kullanılabilir. Böylece antibiyotik doğrudan ve kontrollü olarak hastalıklı dokuya göndermektedir. Taşıyıcı materyal olarak polimer sıkça kullanılmaktadır. Polimer; biyobozunur ya da biyobozunmaz olabilir. Biyobozunur polimerlerin avantajı; vücuda yerleştirildikten sonra çıkartılabilmeleri için ikinci bir operasyona gerek duymamalarıdır.

Çalışmada, deneysel sıçan osteomyelit modelinde oluşturulan implant ilişkili kronik osteomiyelitin tedavisi için vankomisin içeren PLLA/TCP kompozitleri geliştirilmiştir. Kompozitlerden bazıları PLLA ile kaplanarak, kaplamalı ve kaplamasız kompozitler arasındaki fark gözlenmiştir. Ayrıca, kompozitlerin bir bölümü kemik dokusuyla biyouyumluluğu araştırabilmek için vankomisinsiz hazırlanmıştır. Kaplamanın vankomisin salımını 5 haftaya kadar uzattığı ve kompozitlerin yüzey morfolojisini değiştirdiği gözlemlenmiştir. Hücre kültürü çalışmalarına göre; vankomisin içeren kompozitler, hücre yapışması, çoğalması ve mineralizasyon sağlayarak kemik dokusu ile biyouyumludurlar. Radyolojik ve mikrobiyolojik değerlendirmelere göre vankomisin içeren ve vankomisin içeren kaplamalı kompozitler MRSA çoğalmasını engelleyerek implant ilişkili kronik osteomyeliti tedavi etmektedir.

Anahtar Kelimeler: Antibiyotik, osteomiyelit, PLLA, vankomisin, kemik, polimer, MRSA, in vivo, biyouyumluluk, ilaç taşıyıcı sistemler, kontrollü salım, biyobozunur polimer

To my dear uncle

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This research is approved by Hacettepe University Animal Ethics Comittee (2007/65-1)

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ABBREVIATIONS

VC	Vancomycin Loaded PLLA/TCP Composite
CVC	Coated Vancomycin Loaded PLLA/TCP Composite
VUC	Vancomycin Free PLLA/TCP Composite
MRSA	Methicillin Resistant Staphylococcus Aureus
PMMA	Polymethyl methacrylate
e.g.	Exempli gratia (For example)
PLLA	Poly-L-Lactic Acid
PLA	Polylactide acid
PGA	Polyglycolide acid
TCP	Tricalcium phosphate
β-TCP	Beta-Tricalcium Phosphate
DEXA	Dual-energy X-ray Absorptiometry
SEM	Scanning Electron Microscopy
HPLC	High Performance Liquid Chromatography
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
ELISA	Enzyme Linked Immunosorbent Assay
METU	Middle East Technical University
MSC	Human Bone Marrow originated Mesenchymal Stem Cells
PBS	Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
UV	Ultraviolet
ml	Mililiter
nm	Nanometer
mm	Milimeter
mcg	Microgram

mg/kg	Miligram per kilogram
kV	Kilovolt
mAs/s	Miliamperesecond per second
CFU/cm^3	Colony Forming Unit per cubic meter

CHAPTER 1

INTRODUCTION

Osteomyelitis is the infection of bone that, suppressed several bone components causing bone destruction and necrosis (1). Osteomyelitis is classified as acute or chronic. Acute osteomyelitis is a short term condition of the disease while chronic osteomyelitis is the long term condition (2). The infection is usually caused by pyogenic bacteria, mycobacteria or fungi (3). The delivery of the microorganisms to the bone tissue might take place during direct contamination, as in trauma and/or surgery, or during haematogenous or contiguous spread. Osteomyelitis is one of the toughest infections to treat, as the bacteria can adhere to the surface of the biomaterial and form a biofilm in biomaterial centered osteomyelitis. The biofilm makes pathogens resistant towards systematically administered antibiotics. Also, in immunocompromised patients nonpathogenic bacteria can form the biofilm and nonpathogenic bacteria may become resistant to antibiotics (4). Conventional and systemic antibiotic therapy may not effect the bacteria through the formed biofilm.

In the treatment of osteomyelitis, pharmacokinetic difficulties of antibiotic therapy have to overcome and surgery may require. During treatment; the antibiotic must reach a curative dose in the bone. Conventional and systemic antibiotic therapy may not treat osteomyelitis unless, the higher doses of antibiotic is given. On the other hand, the higher doses of antibiotics may cause toxic and side effects. In this case, controlled drug delivery systems can be used.

Controlled drug delivery systems have been developed to deliver antibiotics directly to infected tissue without harming the other body systems. They are constructed by a carrier material and the drug. The carrier material must be biocompatible with both the loaded drug and the biological environment and the loaded drug must be at a desired level for the controlled release. As a carrier material biodegradable polymer can be used.

Biodegradable polymers are suitable for the manufacturing of medical devices or delivery systems. They have excellent biocompatibility and biodegradability and safe to use. These types of polymers do not require a second surgery for the removal from the body and preferred for drug delivery systems.

CHAPTER 2

BACKGROUND INFORMATION

2.1 Osteomyelitis

Osteomyelitis is the most common deep infection associated with orthopaedic surgery (5). It may appear after the orthopaedic surgery as a result of bacterial biofilm formation in implant which leads the bone destruction (6). The infection is usually caused by pyogenic bacteria, especially by Methicillin Resistant Staphylococcus Aureus (MRSA). The delivery of the bacteria to the bone is done by hematogenous seeding, contiguous spread of infection or by direct inoculation (7). When an infection occurs; chronic inflammatory cells will develop and wound will become unable to supply an effective response towards inflammatory. As the infection progresses, bacteria colonize within the biofilm. Antibiodies try to get rid of the infection but they are ineffective in the presence of the biofilm. The periosteal abscess becomes larger and periosteum peels off from the shaft of the bone causing sequestrum. The abscess helps the development of sinus tract and extruding the sequestrum (8). The like hood of osteomyelitis is increased if an implant is present because; the bacteria are also adhered to the surface of the implant and form a biofilm (9). In vitro studies show that; staphylococcus which attached to implant surfaces, change their metabolism and become resistant to antibiotics (10). So, the implant should be removed before the treatment. Osteomyelitis is treated by surgical debridement, soft tissue coverage, hyperbaric oxygen treatment and administration of antibiotics (11). If the antibiotics are administrated by prolonged systemic administration, the treatment will last 4 to 6 weeks (5). During the prolonged systemic antibiotic administration, only small doses of antibiotics can reach to the infected area and side effects may occur (28). Instead of systemic antibiotic administration, local antibiotic delivery systems can be used to treat osteomyelitis. Polymethyl methacrylate (PMMA) was a

biocompatible polymer used for local drug delivery systems. It had been used as carrier material for the drug delivery systems since 1970s. The disadvantage of using PMMA as a carrier material was PMMA needed a second surgery for the removal from the body because of its nonbiodegradable property (13). To overcome the disadvantage, biodegradable polymers were used as carrier material.

2.2 Controlled Drug Delivery Systems

In the past decades, a drug can only be administrated to the body by intravenous or oral pathway. With these methods the plasma levels of the drug is whether high or low in the body. The high plasma levels may cause toxicity and low plasma levels are not enough for the treatment (14). To overcome these issues, controlled drug delivery systems have been developed. In controlled drug delivery systems, the active compound is released from the biomaterial in a predesigned way. "The release of the drug may be constant or cyclic over a longer period, or it may be triggered by environmental events including pH, temperature and ionic strength" (15). The controlled drug delivery systems keep the drug level at a desired range so; low drug plasma levels and toxicity can be overcome. Springer et al. states that; the delivery of a high concentration of antibiotics in a localized area is thought to be safer than systemic administration of intravenous antibiotics in such doses (16). The figure of plasma drug concentration versus time profile of a drug when administered orally as compared to a sustained release drug delivery system is given in Fig 2.1 (14).

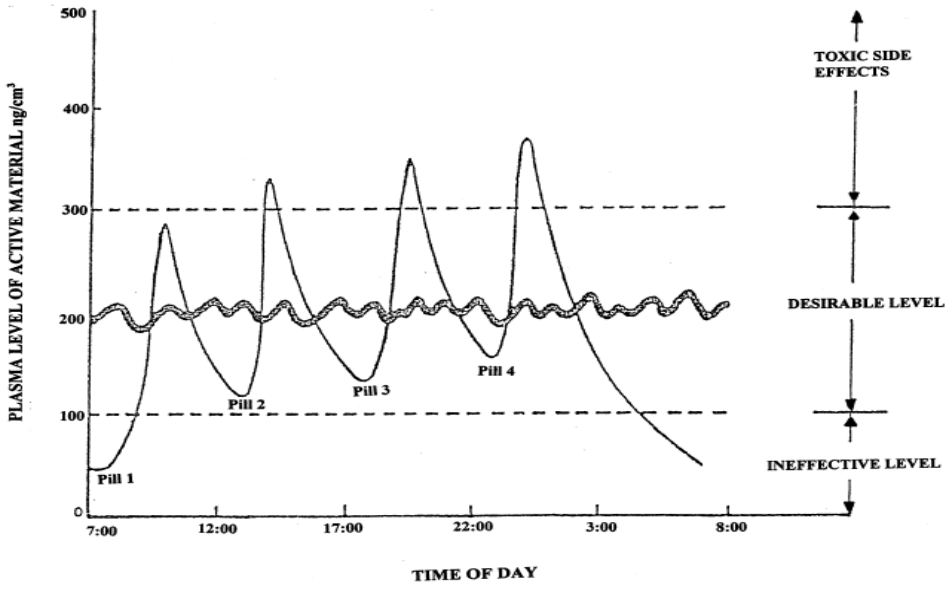


Fig 2.1 The figure of plasma drug concentration versus time profile of a drug when administered orally as compared to a sustained release drug delivery system

A controlled drug delivery system is consisted of a carrier material and the drug. Polymers and ceramics can be used as carrier materials for controlled drug delivery systems.

In a controlled drug delivery system, drug can release either by; diffusion, chemical reaction or solvent activation and transport mechanism (17). The schematic drawing of the three mechanisms is given in Fig 2.2 (18).

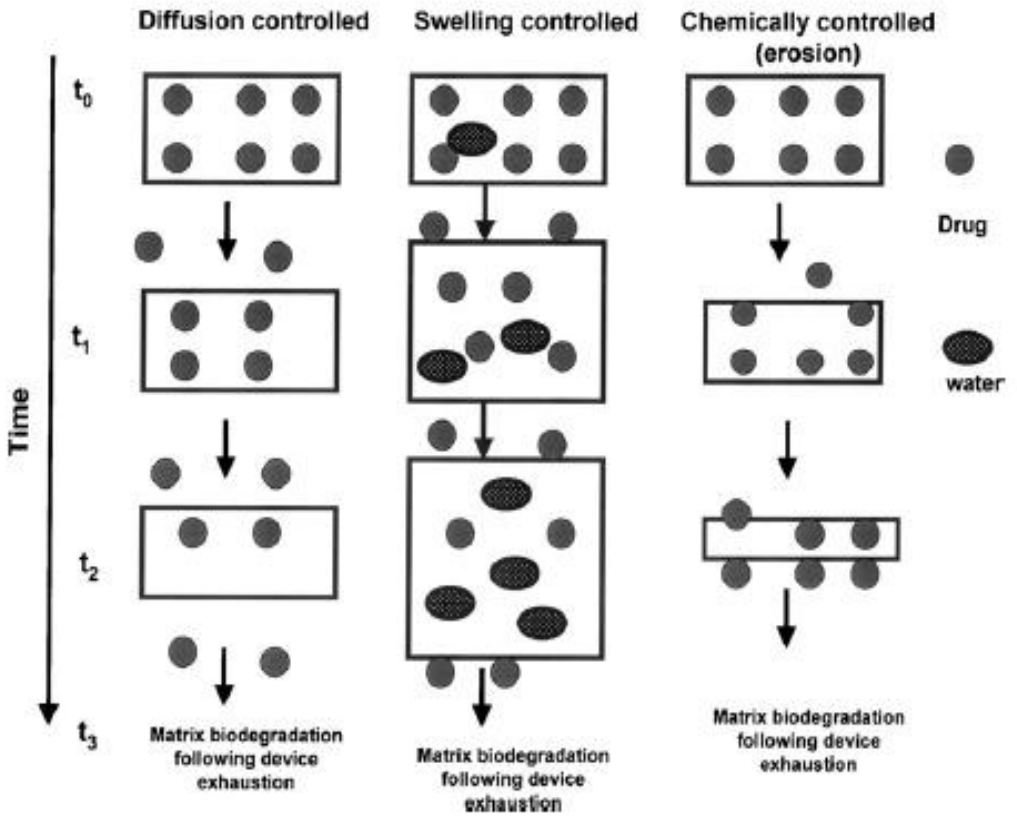


Fig 2.2 A schematic drawing illustrating the three mechanisms for controlled drug release from a polymer matrix.

2.2.1 Diffusion Controlled Drug Delivery Systems

In diffusion controlled drug delivery systems, drug diffuses through the carrier material (18). A diffusion controlled drug delivery system can either be a reservoir type or a matrix type. In reservoir type, the drug is surrounded by a barrier. In matrix type, drug is homogenously distributed through the polymer (17). Also a combination of these two mechanisms can be done. The schematic drawings of three types of polymer-based diffusion controlled drug delivery devices are given in Fig 2.3 (18).

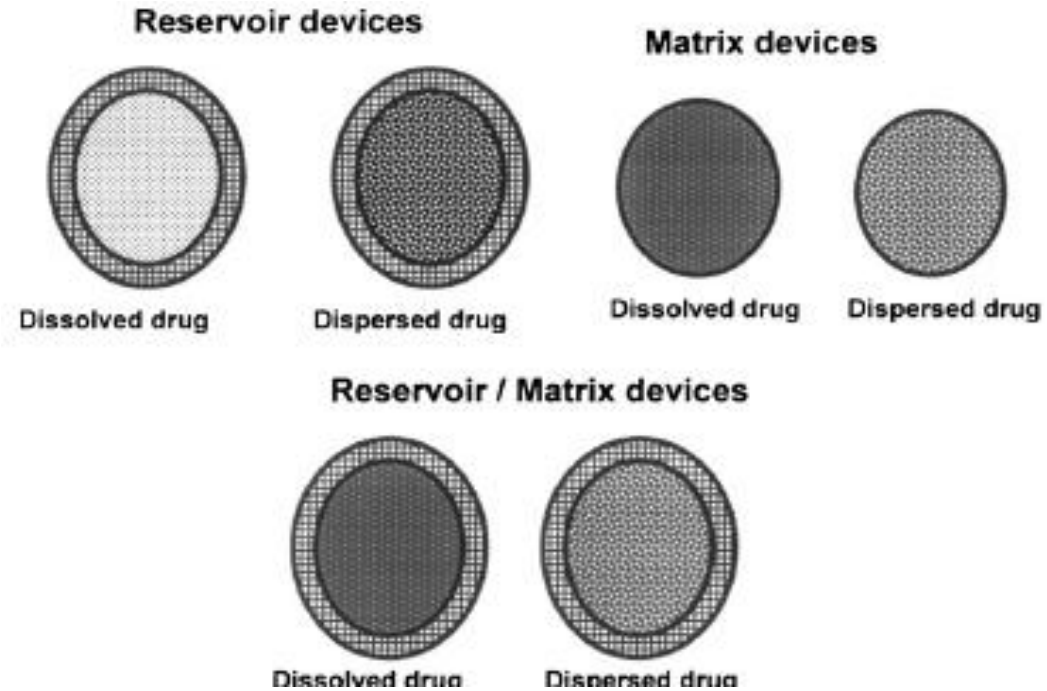


Fig 2.3 The schematic drawings of reservoir and matrix diffusion controlled drug delivery systems

Bajpai et al. states that; a matrix device is easy to formulate and gives a higher initial release rate than a reservoir device and can be made to release at a nearly constant rate (18).

2.2.2 Chemically Controlled Drug Delivery Systems

In chemically controlled drug delivery systems, drug is released by degradation of the carrier material by water or a chemical reaction. Also, the drug can be linked to the carrier material with unstable bonds that can be degrading by water or a chemical reaction and release the drug (18).

2.2.3 Solvent- Activated Controlled Drug Delivery Systems

In solvent activated mechanism, the drug can be released either by swelling of the carrier material or by osmotic effect in which external water enters the system and gives out the drug (17).

2.3 Biomaterial

"A biomaterial is a material; intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body" (19). "Biomaterials must be biocompatible, nontoxic or noncarcinogenic, relatively inexpensive, reproducible and easy to fabricate. They must have adequate mechanical properties, chemical properties, service life and bioactivity. Also; their physical properties must match with the tissue they replaced or they are implanted in" (20).

"Biomaterials can be classified as bioinert, bioresorbable, or bioactive according to tissue responses.

i. Bioinert refers to any material that once placed within the human body has minimal interaction with its surrounding tissue, e.g. stainless steel, titanium, alumina, ultra high molecular weight polyethylene.

ii. Bioactive refers to a material, which upon being placed within the human body interacts with the surrounding bone and in some cases, even with the soft tissue, e.g. synthetic hydroxyapatite, glass-ceramic.

iii. Bioresorbable refers to a material that upon placement within the human body starts to dissolve (resorbed) and is slowly replaced by advancing tissue (such as bone), e.g. tricalcium phosphate, and polylactic-polyglycolic acid copolymers" (21).

2.4 Poly-L-Lactic Acid (PLLA)

Polymers are easily fabricated materials that can be used as sutures, stents and drug delivery systems in biomedical applications (22). Polymers can be found as nonbiodegradable or biodegradable. Nowadays, biodegradable polymers are much useful than the nonbiodegradable polymers because they do not need a second surgery for the removal from the body.

"Poly (alpha (α)-hydroxy acids), mainly polylactide acid (PLA) and polyglycolide acid (PGA) are the most widely known synthetic polymers used for the production of degradable biomaterials" (Fig 2.4) (23). Lactic acid can be found in D and L stereo isomeric forms. Also D, L-PLA racemic form is available (23). Cohn et al. states that; the polymers derived from the optically active D and L monomers are semi crystalline materials, while the optically inactive D, L-PLA is always amorphous (23). For biomedical applications, L isomer of lactic acid, poly-L-lactic acid (PLLA) is preferred because; it can metabolized in the body (24). Because of its biocompatibility and safety PLLA can be used as implantable reservoirs for sustained release drug delivery (25).

Fig 2.4 Structure of PLA

2.4.1 Degradation of Poly Lactic Acid and Copolymers

"Poly lactic acid degrades to its monomer, lactic acid, which is a normal metabolite of the human body" (26). Poly lactic acid (PLA) and PLA copolymers degrade by hydrolysis of their ester bonds (24). The monomer, lactic acid, is then incorporated into the tricarboxylic acid cycle. "These natural metabolites are ultimately converted to water and carbon dioxide through the action of enzymes in the tricarboxylic acid cycle and are excreted via the respiratory system" (15). The schema of the metabolic degradation is given in Fig 2.5 (27). "The degradation process is completed in four steps:

i. Water penetrates the amorphous region of the polymer and disrupts the secondary forces;

ii. Cleavage of the covalent bonds in the polymer backbone begins by hydrolysis and, as hydrolysis proceeds, more and more carboxylic end groups may autocatalyse the hydrolysis reaction;

iii. Significant mass loss begins to occur by massive cleavage of the backbone covalent bonds;

iv. The polymer loses weight" (23).

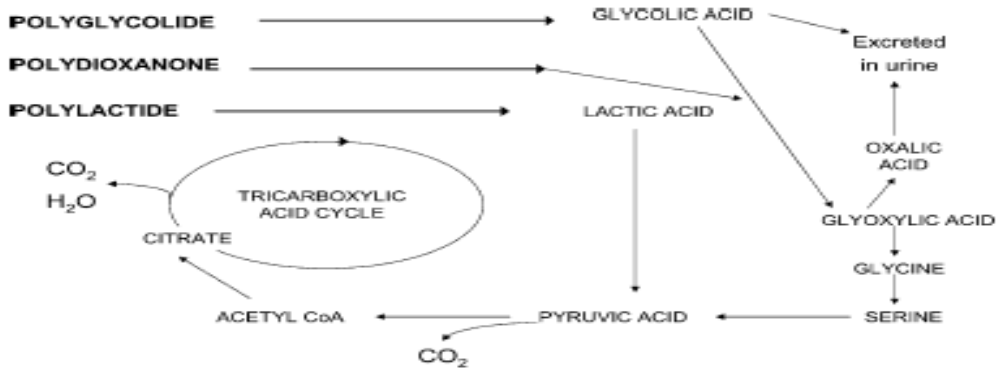


Fig 2.5 The metabolic degradation schema of polyglycolide, polydioxanone and polylactide

During the degradation process; the biodegradable material can release by-products. These products can be catalysts, initiators and solvents (Fig 2.6) (15).

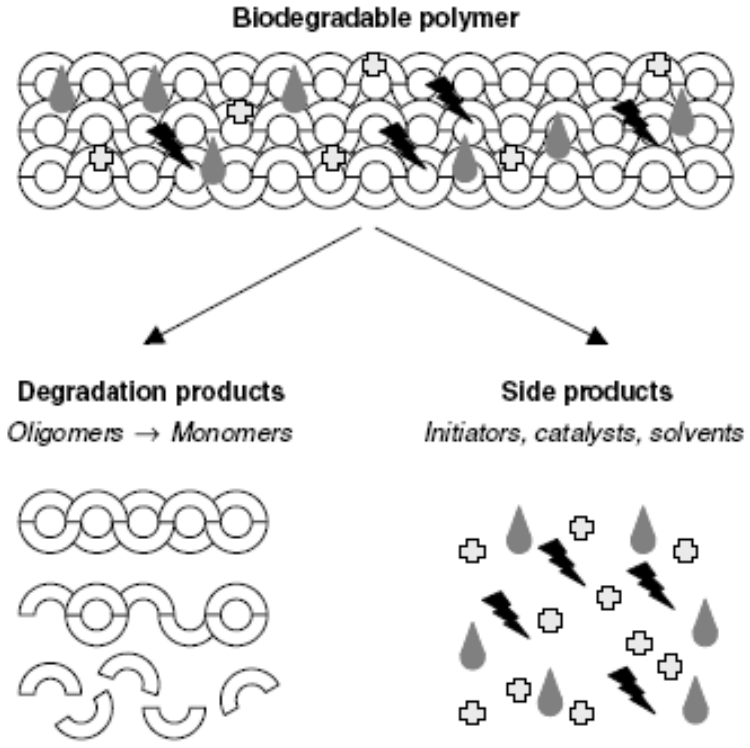


Fig 2.6 The figure of degradation productions and by-products

2.5 Beta-Tricalcium Phosphate (β-TCP)

Tricalcium phosphate (TCP) is one of the most biocompatible reabsorbable synthetic hard tissue implant material (14). It has $Ca_3(PO_4)_2$ formula. TCP can be found as in alpha or beta crystal form. It can be used for bone healing and also as drug releasing system when it is loaded with the drug (14). TCP has similar chemical composition to the mineral phase of bone, thus, it can be clinically use for substituting the bone (12). Many reports have appeared in the literature showing that ceramic materials of beta-tricalcium phosphate (β-TCP) and hydroxyapatite (HA) can be used in implantable drug delivery systems for local antibiotic treatment of bone infections. "Beta-tricalcium phosphate has higher solubility and faster resorption kinetics than hydroxyapatite under physiological conditions" (28). On the other hand; tricalcium phosphate systems degrade so fast and it is difficult to maintain the drug release (29). As a ceramic material, TCP can break easily and can only overcome low stress levels. This property is limited the application areas of the ceramics. However; biodegradable polymers can overcome much higher stress levels. So, composite materials of calcium phosphate and biodegradable polymers have been developed to overcome the disadvantage of the ceramics (30).

2.6 Bone

Bone is a stiff skeletal material composed of calcium phosphate, water and type 1 collagen (31). It supports and protects the body and also functions like a storage as it can store; mineral, fat and growth factors. Bone has a passive role in movement as the muscles on them stretch and relax (32). It consists of cells which are; bone-lining cells, osteoblasts, osteocytes and osteoclasts.

Bone-lining cells cover the surface of the bone like a sheet to control the ion exchange between body and the bone. Osteoblasts function for the blood formation and they derived from bone-lining cells. Osteocytes are the cells that

are found in the body of the bone. They play role in the formation of bone and calcium homeostasis (32). Osteoclasts are the bone destroying cells (31).

In a human body there are 206 bones with five different types. These bones are long bones, short bones, flat bones, irregular bones and sesamoid bones (32). Long bones are mainly faced with bone infections.

Long bone has periosteum, a connective tissue layer, on the outer structure. The outer structure is a compact bone whereas the deeper parts are spongy bone containing red bone marrow. In the inner part of the long bone there is medullar cavity containing yellow bone marrow (Fig 2.7) (33).

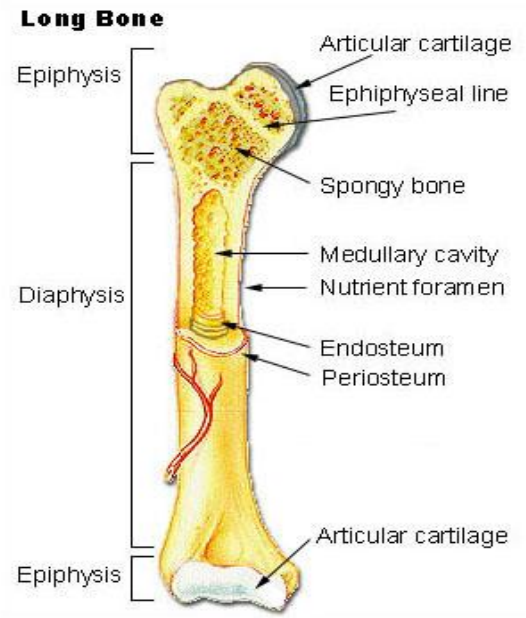


Fig 2.7 Structure of the long bone

2.7 MRSA (Methicillin Resistant Staphylococcus Aureus)

Staphylococci are gram positive bacteria with 0.5-1.5 μm diameter. They are non-motile and non-spore forming bacteria grow by aerobic respiration or by

fermentation. Staphylococcus aureus and Staphylococcus epidermidis are the most characterized strains of staphylococci. Wilkonson states that staphylococci are tolerant to the high concentrations of salt and high temperatures. Staphylococci are found on the skin and in the nasopharynx of the human body naturally (34). Staphylococci have high affinity to bone. They can rapidly induce osteonecrosis and resorb bone matrix (10). Methicillin resistant Staphylococcus Aureus (MRSA) is a well known bacteria associated with implant related bone infections. MRSA is resistant to the beta-lactam antibiotics which are including penicillin and cephalosporins (35). It is hard to treat bacterial infections with MRSA because MRSA is capable of forming a biofilm. MRSA can elaborate prostagladins and active osteoclasts to resorb bone (8). It forms a biofilm by producing an exopolysaccharide matrix, glycocalyx (36), and embedded into a layer of slime by the help of hydrophobic, Van der Waals and electrostatic interactions (10). Biofilm is formed by adhesion of bacteria to surface and cell-cell adhesion (34). It can act as an interaction gel between bacteria of the same or different species and also as an ion exchange resin for nutrition (8). Biofilm formation makes bacteria become more resistant towards antibiotics and it becomes difficult to treat the infection in a conventional way.

2.8 Vancomycin

Vancomycin is glycopeptide antibiotic produced from Amycolatopsis orientalis (Fig 2.8) (37). It shows its bactericidal effect by inhibiting the cell wall biosynthesis with binding to the cell wall precursors (38). Vancomycin is active against Gram positive bacteria (37). It can be used by the patients who have allergy towards penicillin and cephalosporin (38). Vancomycin has side effects such as, nephrotoxicity, ototoxicity, poor venous tolerance (39) and red man or red neck syndrome (37). To diminish the side effects, vancomycin can be administrated with controlled drug delivery systems. Vancomycin is generally used for the treatment of osteomyelitis, caused by MRSA (11).

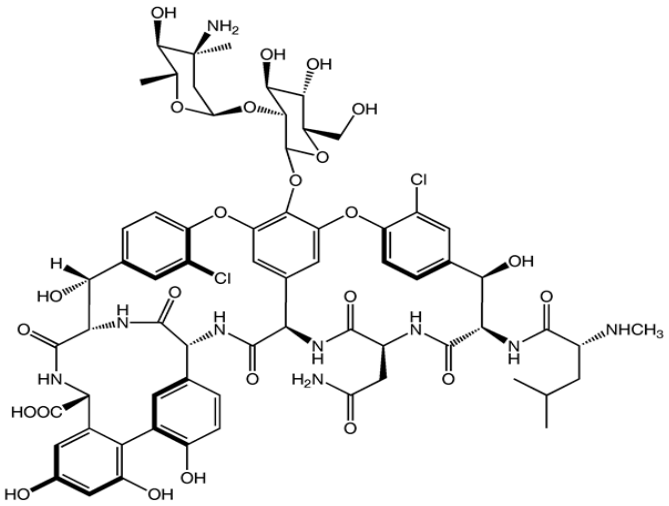


Fig 2.8 The structure of vancomycin

2.9 Recent Studies

Öztürk et al. have studied alendronate enhanced antibiotic-impregnated bone grafts in the treatment of osteomyelitis. Experimental tibial osteomyelitis was formed in the rats tibia with the inoculation of Staphylococcus aureus.Experimental groups have received plain bone grafts, vancomycin-loaded bone grafts, vancomycin-loaded bone grafts and systemic alendronate, alendronate-impregnated bone grafts, vancomycin and alendronate-impregnated grafts. The results of the study were evaluated by swab cultures, radiology, quantitative computed tomography, dual-energy X-ray absorptiometry (DEXA) and histopathology. The results showed that vancomycin release was influenced by the time used for impregnation and the pH of the medium. It was not affected by increasing the surface area of the graft. Both bone mineral content and bone mineral density were higher in the alendronate treated groups. Bone density increase was achieved in the presence of antibiotic application. An increase in the osteoblastic activity has been observed in the alendronate and antibiotic administered groups. As a result, systemic application of alendronate provided improvements in terms of bone density and bone graft integration. Also, local

application of alendronate had stronger effects, but interfered with the infection control process (40).

Gitelis and Brebach have studied biodegradable antibiotic-impregnated implant for the treatment of the chronic osteomyelitis. Calcium sulphate was used as a carrier material; vancomycin and tobramycin were used as drugs. Drugs were mixed with the sodium chloride diluent separately. After that; the antibiotic solutions were mixed with the calcium sulphate powder and the paste was placed into the silicone mold. In the study, six consecutive patients with chronic osteomyelitis were treated with both systemic antibiotics and local antibiotics with biodegradable calcium sulphate implants at the same time. Five patients had Staphylococcus aureus infection, while one patient had polymicrobial infection. Tobramycin loaded implants were used in five patients with S.aureus infection, and tobramycin and vancomycin loaded implants were used in one patient with polymicrobial infection. As a result, all the implants were degraded in the body and bone repair occurred. The bone repair was the lowest in the patient with polymicrobial infection so, polymicrobial infection was difficult to treat. In conclusion, local antibiotic delivery with calcium sulphate was effective for infection control and bone repair (41).

Korkusuz et al. have studied antibiotic-calcium hydroxyapatite composites for the treatment of experimental implant related osteomyelitis. 150 female Spraque Dawley rats were operated and inoculated with Staphylococcus Aureus. Stainless steel implants were inserted in to the medullar cavity of tibia to construct implant related osteomyelitis. The rats were divided into two groups, each containing 75 rats. First group was used to compare the effect of antibiotic-calcium hydroxyapatite composites with the parenteral antibiotic therapy. 25 animals received antibiotic-calcium hydroxyapatite composites, each containing 5 mg of gentamicin sulphate powder, 25 animals were treated with intraperitoneal injection of gentamicin sulphate for five weeks, and 25 rats received no treatment. In second group, antibiotic-calcium hydroxyapatite composites were compared

with calcium hydroxyapatite and surgical debridement with and without the implantation of antibiotic-impregnated acrylic bone cement. In 25 animals, only surgical debridement was performed while in other 25 animals surgical debridement was followed with the implantation of gentamicin loaded acrylic bone cement. The rest of 25 animals, calcium hydroxyapatite blocks without gentamicin was implanted in the infection area. Each week, 5 animals were killed from each group the results were assessed by radiography, histopathology, bacteriology, and scanning electron microscopy (SEM). The results showed that, gentamicin impregnated calcium hydroyapatite produced twenty times higher concentrations than intraperitoneal injections, and 2.5 times higher concentrations, for 1.2 times longer, than did the acrylic bone-cement drug-delivery system (42).

Liu et al have been studied the in vivo release of vancomycin from biodegradable beads. As a carrier material, poly (D,L)-lactide-co-glycolide with a ratio of 50:50 and as a drug vancomycin was used. Polymer and drug was mixed and compressed into 8 mm diameter beads. Beads were placed in an oven for sintering at 55˚C. The in vitro release of vancomycin from the antibiotic beads was determined by the elution method. For the in vivo release of the vancomycin, a rabbit animal model was designed and performed. A bony cavity was surgically made at the left and right distal femur of five New Zealand rabbits. A polymethylmethacrylate spacer was placed in the bone cavity and wound was closed. After two weeks, the wound was reopened, spacer was removed and vancomycin loaded biodegradable beads were inserted into the cavity. The vancomycin concentrations of in vitro and in vivo studies were determined by high performance liquid chromatography (HPLC) assay. The activity test of the released vancomycin on Staphylococcus Aureus in bone cavities was determined by antibiotic disc diffusion method. As a result, antibiotic provided serum concentrations above breakpoint sensitivity for 4 to 6 weeks and not producing toxic serum concentrations so, compression-sintering method could be used to manufacture antibiotic beads to provide a potentially useful and safe method to treat or prevent surgical infections in vivo (43).

Saito et al. have been studied Slow-Releasing Potential of Vancomycin-Loaded Porous Hydroxyapatite Blocks Implanted into Methicillin Resistant Staphylococcus Aureus Osteomyelitis. In the study, five patients with chronic osteomyelitis due to Methicillin Resistant Staphylococcus Aureus infection were treated with implantation of vancomycin loaded hydroxyapatite blocks. Vancomycin releases from the blocks and the bactericidal activity of the vancomycin remaining in the blocks were evaluated with an in vitro experiment. The concentrations of vancomycin were assayed with high performance liquid chromatography (HPLC). As a result, vancomycin had been rapidly released from the hydroxyapatite blocks by 3 months, 72% by 1 month, and 90% by 3 months. The releasing potential was maintained for at least 12 months. At 18 months vancomycin remained in the hydroxyapatite blocks but, block could not release vancomycin and vancomycin changed its form (44).

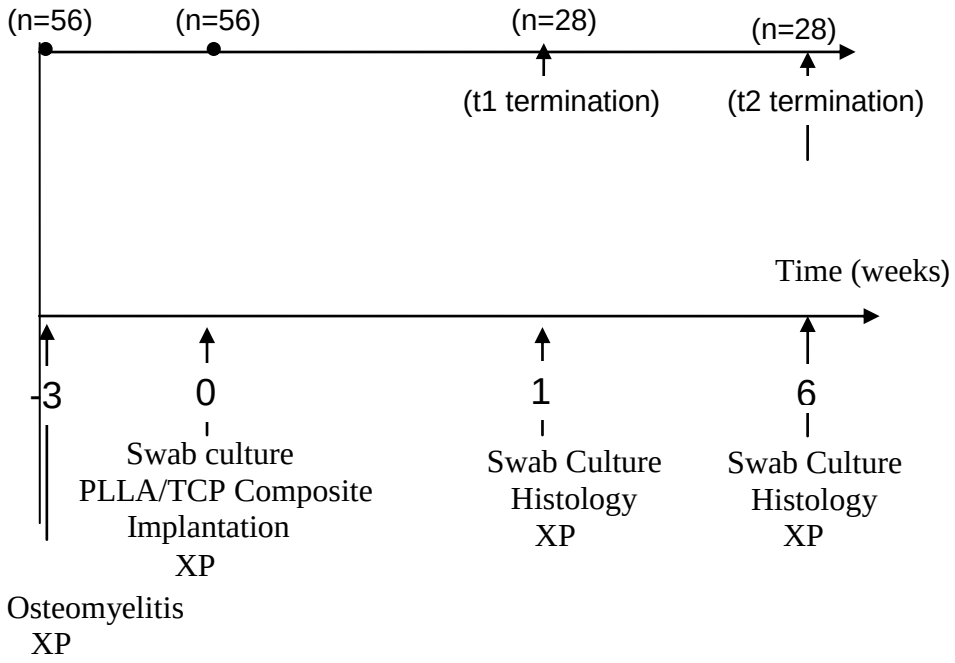
2.10 Design of the Study

A prospective longitudinal randomized controlled study was designed to search the treatment of implant related chronic osteomyelitis and biocompatibility of the vancomycin loaded PLLA/TCP composite delivery system for bone tissue. At the first stage, the material was developed and characterized due to its surface topography, pore size distribution, apparent density and surface area. At the second stage, in situ studies were done to determine vancomycin release from PLLA/TCP composites with both elution method and high performance liquid chromatography (HPLC) assay. At the third stage, in vitro cell culture and microbiological studies were performed. Cell culture studies were performed to search the biocompatibility of PLLA/TCP composite for bone tissue with cell adhesion, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cell proliferation and mineralization assays. The microbiological studies were performed for both before and during the in vivo studies to confirm the bactericidal activity of the PLLA/TCP composites. At the fourth stage, in vivo study was done and implant related chronic osteomyelitis was induced in 56 male

Spraque Dawley rats with Methicillin Resistant Staphylococcus Aureus ATCC 2592 strain.

The independent variable was the groups. In control group, implant related chronic osteomyelitis was not developed and PLLA/TCP composite was not implanted. In biocompatibility testing group, rats were implanted with vancomycin free PLLA/TCP composites without developing implant related chronic osteomyelitis. For the treatment groups, the rats were induced with implant related chronic osteomyelitis and implanted with vancomycin free PLLA/TCP composite, vancomycin loaded PLLA/TCP composite and coated vancomycin loaded PLLA/TCP composite.

The results were evaluated with swab cultures and radiology which were the dependent variables of the study (Fig 2.9).



XP= Radiography; n=number; t=time

Fig 2.9 The graph of animal model design

2.11 Research Questions

The research questions of the study were:

Will vancomycin be delivered form the PLLA/TCP composite for a month to inhibit MRSA proliferation?
Will the PLLA/TCP composite be biocompatible with bone cells in in vitro conditions?
Will the PLLA/TCP composite effectively treat implant related osteomyelitis in an animal model?

2.12 Hypotheses

The hypotheses of the study were:

The vancomycin will be delivered from the PLLA/TCP composite for a month to inhibit MRSA proliferation.
The PLLA/TCP composite will be biocompatible with bone cells in in vitro conditions.
The PLLA/TCP composite effectively treat implant related osteomyelitis in an animal model.

2.13 Aims of the Study

The aims of the study are to develop a PLLA/TCP composite structure that can release vancomycin to treat implant related chronic osteomyelitis and be biocompatible with the bone tissue.

CHAPTER 3

EXPERIMENTAL

3.1 Materials

3.1.1 Polymers

Poly-L-lactic acid (PLLA) (Resomer L 209 S) was purchased from Boehringer Ingelheim (Germany). PLLA is a white to off-white granulate with -($C_6H_8O_4$)n molecular formula. PLLA melts at 174.94 ˚C and has 2.6 - 3.2 dl/g inherent viscosity.

3.1.2 Drugs

Vancomycin (1.0 g) was obtained from Abbott (France). The active substance was Vancomycin hydrochloride. Vancomycin is a white crystalline powder with $C_{66}H_{75}Cl_2N_9O_{24}$ molecular formula. It has 1449.25 $gmol^{-1}$ molecular weight and decomposes before melting.

3.1.3 Ceramic

β-tricalcium phosphate (β-TCP) was kindly provided by Prof. Dr. Muharrem Timuçin, Middle East Technical University Metallurgical and Materials Engineering Department. β-TCP is a white amorphous powder with $Ca_3(PO_4)_2$ molecular formula. It has 310.18 $gmol^{-1}$ molecular weight and liquefies under high pressure. β-TCP is similar to bone structure and can be resorbed by bone.

3.1.4 Chloroform

Chloroform was purchased from J.T.Baker (USA). Chloroform is a colorless liquid with $CHCl_3$ molecular formula. It has 119.4 $gmol^{-1}$ molecular weight and melts at -63.5˚C.

3.2 Methods

3.2.1 Material Development and Characterization

3.2.1.1 Preparation of the Vancomycin/TCP Mixture

80 mg vancomycin was mixed with 920 mg de-ionized water to prepare 8% vancomycin solution. 720 mg β-TCP was added to this solution and dried at 37˚C for 12 hours. During the drying process, all the water was evaporated and finally a powder like structure was obtained that contains 10% vancomycin and 90% β-TCP.

3.2.1.2 Preparation of the PLLA/TCP Composites

3% (w/w) PLLA solution was prepared by using 80 mg PLLA and 2670 mg chloroform. The solution was mixed with vancomycin/TCP mixture which had 720 mg β-TCP (90%), 80 mg vancomycin (10%). Small sphere shapes were obtained from the paste manually and they were dried at room temperature for 24 hours to evaporate the chloroform. By this method, a total number of 267 vancomycin loaded PLLA/TCP composites were obtained.

123 of the vancomycin loaded PLLA/TCP composites were coated with thin a polymer film by the dip coating method. A 3% PLLA solution was prepared and vancomycin loaded PLLA/TCP composites were dipped into this solution for 10

seconds. After, polymer/TCP composites were dried at room temperature for 24 hours.

3.2.1.3 Preparation of PLLA/TCP Composites For Testing Biocompatibility

3% (w/w) PLLA solution was prepared by using 80 mg PLLA and 2670 mg chloroform. 720 mg β-TCP was added to the solution. Small sphere shapes were obtained from the paste manually and they were dried at room temperature for 24 hours to evaporate the chloroform. 134 PLLA/TCP composites were obtained by this method. These PLLA/TCP composites were prepared for testing biocompatibility and did not contain any vancomycin.

3.2.1.4 Scanning Electron Microscopy (SEM)

Surface topography of the vancomycin loaded and coated vancomycin loaded PLLA/TCP composites were analyzed by using JEOL JSM 6400 (Jeol Ltd, USA) Scanning Electron Microscope located in METU Metallurgical and Materials Engineering Department under 2000 magnification. The PLLA/TCP composites were fixed on supports and coated with gold film to obtain a conducting surface.

3.2.1.5 Pore Size Distribution

Pore size distribution of the vancomycin loaded, coated vancomycin loaded and vancomycin free PLLA/TCP composites were analyzed by using Quantachrome Polemaster 60 mercury porosimeter located in METU Central Laboratory. Pore size distribution of the PLLA/TCP composites was obtained by mercury intrusion data. Mercury porosimeter had low pressure (up to 50 psi) and high pressure (up to 60000 psi) modes. Vancomycin loaded, coated vancomycin loaded and vancomycin free PLLA/TCP composites were analyzed under 50 psi pressure because of the possibility of their disintegration under high pressure.

3.2.1.6 Apparent Density

Apparent density of the vancomycin loaded, coated vancomycin loaded and vancomycin free PLLA/TCP composites were analyzed by using Quantachrome Polemaster 60 mercury porosimeter located in METU Central Laboratory. At first, sample reservoir was measured then PLLA/TCP composite was added to the reservoir and measured again. On the other hand, mercury that would force to enter the composite was measured. Finally, reservoir, PLLA/TCP composite and mercury were measured all together and apparent density measurements were done by volume-density measurement option in mercury porosimeter software.

3.2.1.7 Surface Area

Surface area of the vancomycin loaded, coated vancomycin loaded and vancomycin free PLLA/TCP composites were analyzed by using Quantachrome Autosorb 6 Single Beam BET Surface Characterization Device located in METU Central Laboratory. The PLLA/TCP composites were preheated to 30˚C to avoid water and foreign particles from the PLLA/TCP composites before the analysis and to protect the PLLA/TCP composites from thermal process. Following that; surface area analysis were done by the nitrogen adsorption in the single beam BET surface characterization device.

3.2.2 In situ Studies

3.2.2.1 Vancomycin Release from PLLA/TCP Composites

The release studies were done with de-ionized water instead of serum as, there was no significant difference between them. The calibration curve for vancomycin was obtained by dissolving 1, 2, 4, 6, 8, 10, 12 and 14 mg vancomycin in 20 ml de-ionized water, separately. The solutions were analyzed spectrophotometrically

by using Hewlett Packard 8452A Single Beam Diode Array Spectrometer. Vancomycin showed a maximum absorption at 280 nm in UV spectrum.

An in situ elution method was employed to determine the amount of vancomycin released from PLLA/TCP composites. Vancomycin loaded and coated vancomycin loaded PLLA/TCP composites were immersed in 20 ml de-ionized water at pH 7.0. They were stored at 37˚C for 6 weeks. In the first day and the weeks followed, the solutions were drawn. The drawn solutions were analyzed spectrophotometrically at 280 nm in order to determine the amount vancomycin release. After every measurement, the solution medium was withdrawn and replaced with equal volumes of fresh de-ionized water.

3.2.2.2 High Performance Liquid Chromatography (HPLC) Assay

HPLC assay was utilized to obtain more accurate released vancomycin concentrations than spectrophotometric analysis. The solutions of elution method were used for the assay. HPLC assay was conducted in Hacettepe University Faculty of Pharmacy Analytical Chemistry Department. Symmetry C8, 3.9 cm x 150 mm HPLC column was used with Shimadzu SCL-10 AVP (Shimadzu,Japan). The mobile phase was composed of 0.01 mol heptanesulphonic acid (Fisher Scientific Ltd, USA) and acetonitrile (Mallinnckrodt, USA) (85/15, v/v). The flow rate was 1.4 ml/min and absorbency was monitored at 280 nm.

3.2.3 In vitro Studies

3.2.3.1 Cell Culture Studies

Vancomycin loaded and coated vancomycin loaded PLLA/TCP composites were studied for their biocompatibility for bone tissue. Vancomycin free PLLA/TCP composites could not study for biocompatibility as the composites were crumbled in the cargo pack after gamma sterilization. Cell adhesion, MTT cell proliferation and mineralization assays were done with human bone marrow originated

mesenchymal stem cells (MSC) (passage 4) and Saos-2 type cells. MSC were multipotent cells which had ability to differentiate into osteoblasts (46). Saos-2 type cells were human osteosarcoma cells that possessed several osteoblastic features and could be useful as a permanent line of human osteoblast like cells (47). The study was conducted with two types of cells to observe the differences in the responses. For every cell a control group was used because, all the studies were done at the same time. For the control group, cell wells without PLLA/TCP composites were used.

For cell adhesion assay, 462000 cells for both cell types were used. The cells were incubated with PLLA/TCP composites and growth medium in 6 well plates for the first day. In every 3-4 days the medium was replaced. In the third and seventh days, wells were washed with PBS and 1 ml Trypsin/EDTA was applied. In the Thoma lamina; cell's vitality and cell count were evaluated with Trypan blue.

For MTT cell proliferation assay, 27000 cells for both cell types were used. The cells were incubated with PLLA/TCP composites and growth medium in 96 well plates for the first day. In every 3-4 days the medium was replaced. In the third and seventh days, MTT was added into the wells and after 4 hours incubation, reading was done with the ELISA machine.

For mineralization studies, 375000 cells for both cell types were used. The cells were incubated with PLLA/TCP composites and growth medium in 12 well plates for the first day. In every 3-4 days the medium was replaced. When the cells reached to %50-60 confluence, differentiation medium was added. In the tenth and twenty first days, one of the wells was dyed with Alizarin Red, while the other two wells were analyzed with Quantichrom Calcium Assay Kit.

3.2.3.2 Microbiological Analysis

3.2.3.2.1 Microbiological Analysis Before In vivo Studies

Microbiological analysis was performed before in vivo studies to confirm the bactericidal activity of the vancomycin loaded PLLA/TCP composites. For the analysis, vancomycin loaded, coated vancomycin loaded PLLA/TCP composites and BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs (USA) were used as control. Aseptic conditions were created during the study. Methicillin Resistant Staphylococus Aureus (MRSA) (strain: ATCC 2592) was obtained from Ankara Numune Hospital Microbiology Department. MRSA was diluted with 1 ml distilled water and 0.1 ml of this bacterial suspension was spread on to the agar plate. The test disc was placed on the right side of the plate while placing the PLLA/TCP composite on the left side of the plate (Fig 3.1).

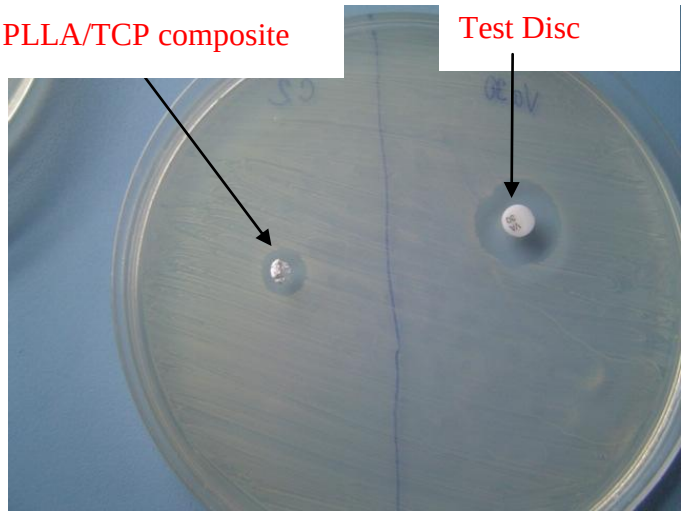


Fig 3.1 Placing of the test disc and the PLLA/TCP composite

PLLA/TCP composites were wetted with 0.1 ml of water and plates were incubated at 37˚C for four days. Both test discs and PLLA/TCP composites created inhibition zones. The zone diameters were measured for the first, second

and fourth days by using inhibition zone reading scale. The diameters were evaluated according to the zone diameters of the test disc (Table 3.1).

Table 3.1 The zone diameters of the test disc

Antibiotic	Disc Potency	Zone Diameter(mm)		
		Resistant	Intermediate	Sensitive
Vancomycin	30 µg	≤ 9	10-11	≥ 12

3.2.3.2.2 Microbiological Analysis During In vivo Studies

Surgery was performed for 56 healthy rats to reconstruct implant related chronic osteomyelitis. 3 weeks after surgery, operated tibiae were swabbed with the sterile test stripes to confirm osteomyelitis. The test stripes were streaked onto blood agar plates. The plates were incubated at 37 ˚C for 12 hours and the presence or absence of growth in the media was recorded.

Before terminating the in vivo studies, operated tibiae were swabbed with the sterile test stripes to quantify bacteria in the bone marrow. The test stripes were streaked onto blood agar plates and plates were incubated at 37 ˚C for 12 hours. The presence or absence of growth in the media was recorded

3.2.3.3 Radiography

For radiography the Siemens Multix-C x-ray device was used. Before examination, the rats were anaesthesized by 0.5 mg/kg ketamine. Anteroposterior and lateral extremity graphies were obtained at 46 kV and 2.5 mAs/s. The beam tube was 1 meter away from the bone samples. Agfa Crurix x-ray films (Agfa,

Germany) and Agfa Crurix 60 automatic developing machine (Agfa, Germany) were used.

Radiological evaluation was done by two impartial orthopaedists who had no prior information about the experiment design and groups. Examiners evaluated the graphies two times by a week break independent from each other with the criteria given below. In the case of disagreement, mean values for both examiners were taken for scoring. The radiological evaluation criteria were; (1) periosteal reaction, (2) diaphyseal widening, (3) osteolysis, (4) bone deformation, (5) sequestrum formation, (6) joint effusion, (7) soft tissue swelling. Parameters 1 to 4 were graded as, (0) absent, (1) mild, (2) moderate, (3) severe and parameters 5 to 7 were graded as, (0) absent and (1) present. The results were separately evaluated for each parameter and no total score was calculated (45) .

3.2.4 In vivo Study

3.2.4.1 Animal Model

An experimental implant related chronic osteomyelitis model was done in rat tibia. 56 male Spraque Dawley rats with approximately 350 gr body weight were used. The in vivo studies were done with 5 different experiment groups and with 2 different time intervals which were 1 and 6 weeks (Table 3.2).

Table 3.2 The experimental groups

Group Name	1 week	6 weeks
Control (no osteomyelitis)	n=6	n=6
Vancomycin free PLLA/TCP composite (VUC) without osteomyelitis	n=6	n=6
Vancomycin free PLLA/TCP composite (VUC) with osteomyelitis	n=6	n=6
Vancomycin loaded PLLA/TCP composite (VC) with osteomyelitis	n=5	n=5
Coated vancomycin loaded PLLA/TCP composite (CVC) with osteomyelitis	n=5	n=5

The healthy rats were anaesthetized intra peritoneal by 0.2 ml ketamine chloride (Ketasol, Richterpharma, Austria) and 0.1 ml xylasine (Alfazyne, Alfasan, The Netherlands) injection. The left hind leg was shaved and cleaned by iodine solution (Polyod, Drogsan, Turkey). The proximal tibia was exposed from its anterior and drilled through with a 3.2 mm burr (Dremel,USA) until the bone marrow appeared (Fig 3.2).

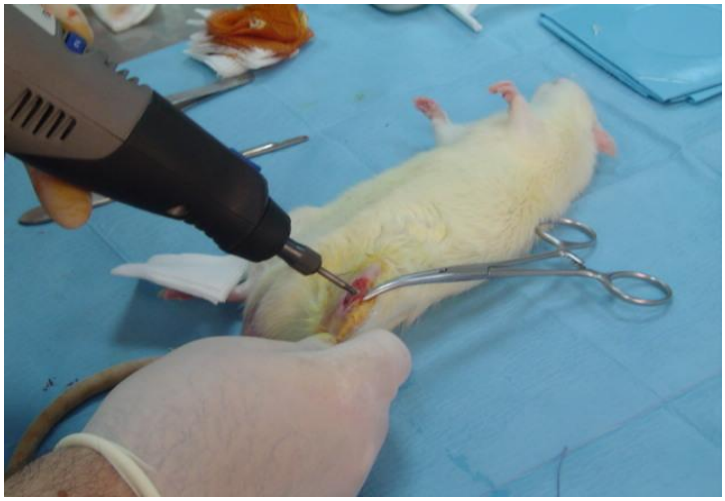


Fig 3.2 Photograph of opening and drilling the tibia

The hole was washed with sodium chloride solution (Eczacıbaşı,Turkey) (Fig 3.2). 0.1 ml Methicillin Resistant Staphylococus aureus (MRSA) (strain: ATCC 2592) suspension ($1x10^6$ CFU/cm^3) was injected into the hole together with 1x1 mm titanium sprinkles. The hole was closed with bone wax to prevent bacterial leakage (Fig 3.4).

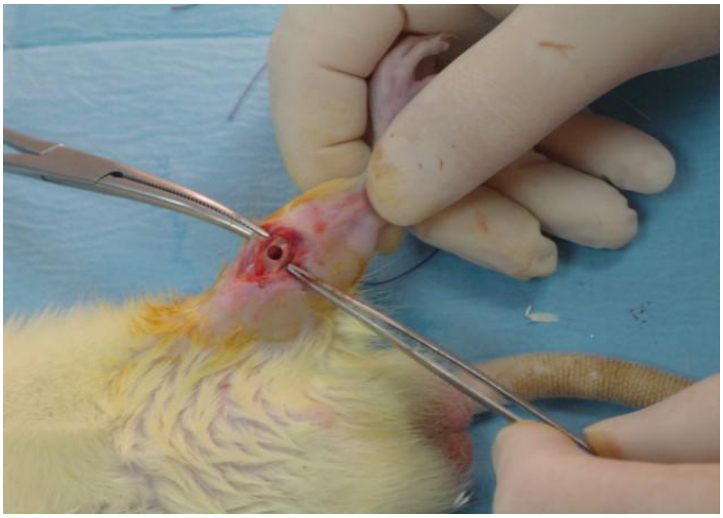


Fig 3.3 Photograph of the hole in the tibia

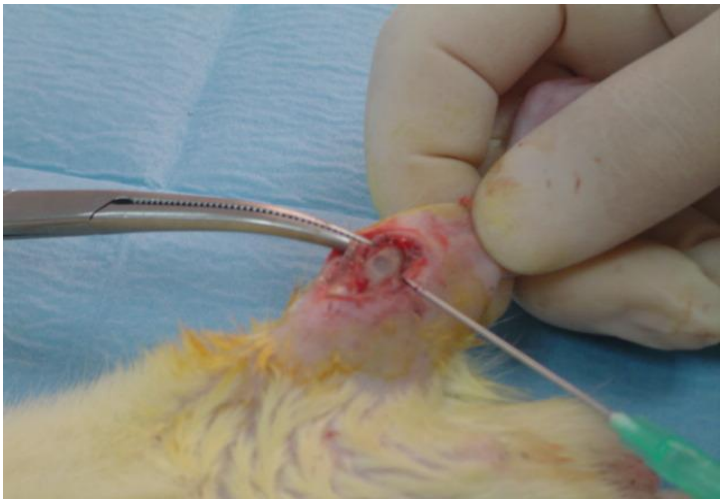


Fig 3.4 Photograph of closing the bone with bone wax

The operation area was sutured with 2.0 silk sutures (Doğsan, Turkey), cleaned and sprayed with an antibacterial film spray (Opsite, Smith&Nephew, England). The rats were put into cages allowing their free movement and fed with regular diet. Three weeks after the inoculation process, the x-rays of the rats were taken and microbiological analyses were utilized to confirm osteomyelitis. In the next phase, VC, CVC and VUC were implanted into the osteomyelitis developed zones according to the experimental groups (Fig 3.5).

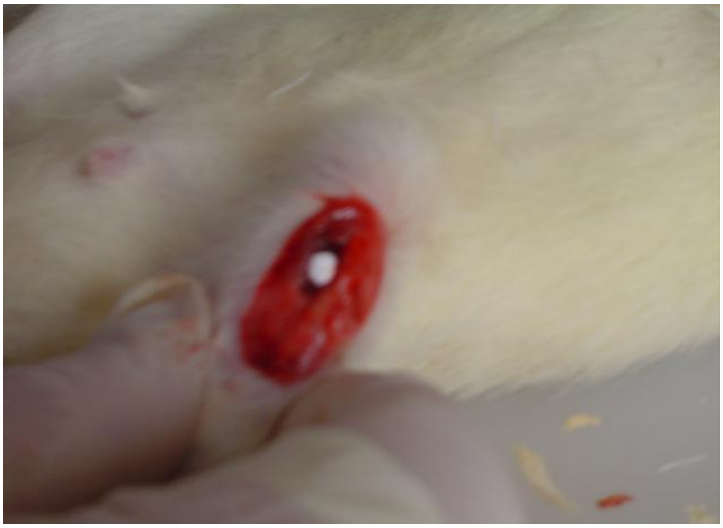


Fig 3.5 Photograph of implanting the PLLA/TCP composite

In another group, VUC were implanted into the rats without developing osteomyelitis to search the biocompatibility of the composite.

In the next 1 and 6 weeks, the implants were removed with the bone tissue around.

The rats were terminated by developing cerebral infarct with the high dosage ketamine and xylasine anesthesia. The bone tissues with implants were taken into 10% formaldehyde solution prepared by phosphate buffer for future histological analyses.

CHAPTER 4

RESULTS

4.1 Material Development and Characterization

4.1.1 Scanning Electron Microscopy (SEM)

Both VC and CVC had porous structure. The surface of VC was rough, covered with the β-TCP and vancomycin powder (Fig 4.1) while, CVC had a network like structure free from β-TCP and vancomycin powder (Fig 4.2). The pore size of CVC was 3.5±1.9 μm with range between 0.8-4.8 μm.

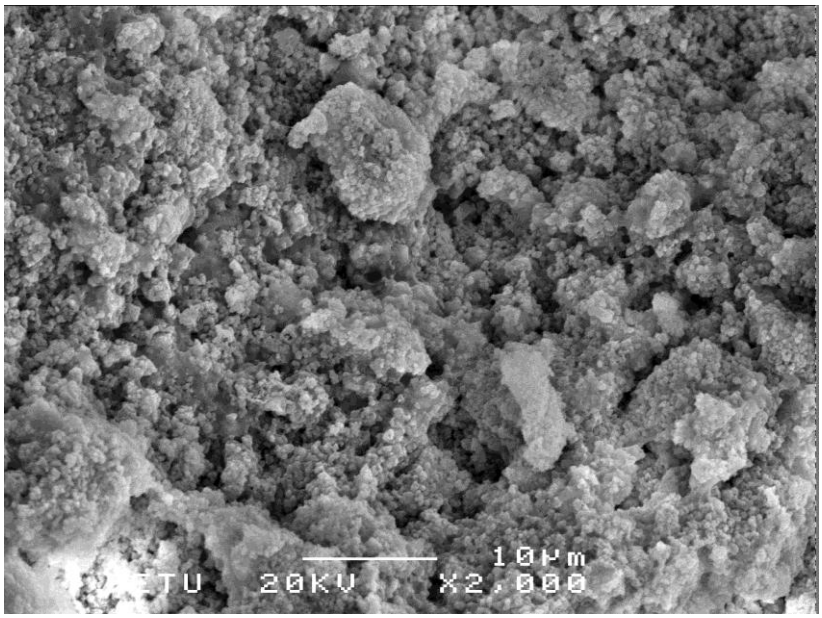


Fig 4.1 SEM micrograph of VC

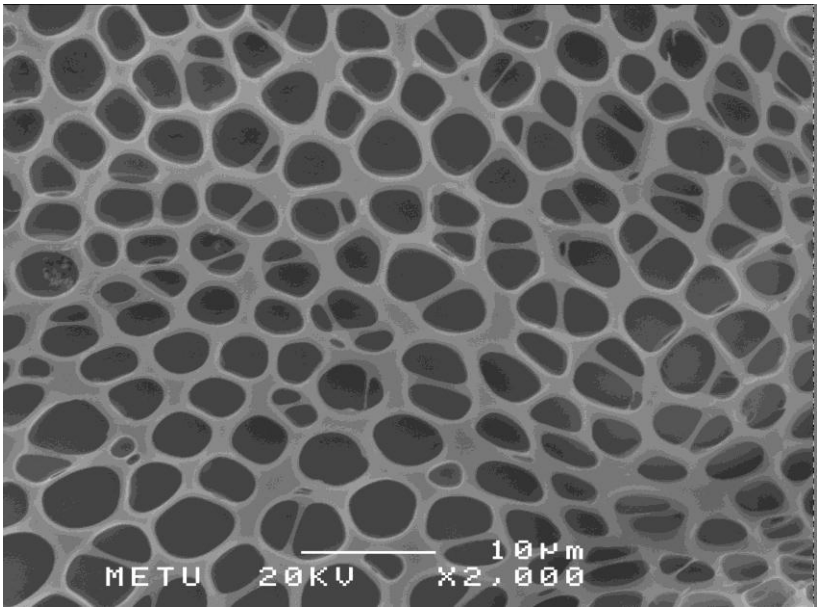


Fig 4.2 SEM micrograph of CVC

4.1.2 Pore Size Distribution

The normalized internal volume of the pores was 0.005±0,002 cm^3/g with the range between 0.0003-0.009 cm^3/g and same for all composites (Fig 4.3, 4.4, 4.5).

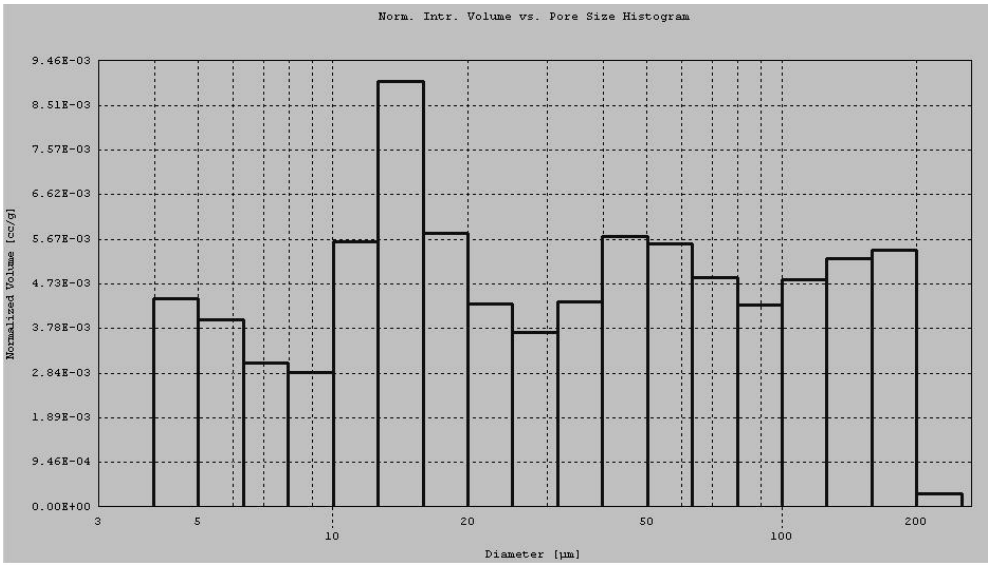


Fig 4.3 Pore size distribution of VUC

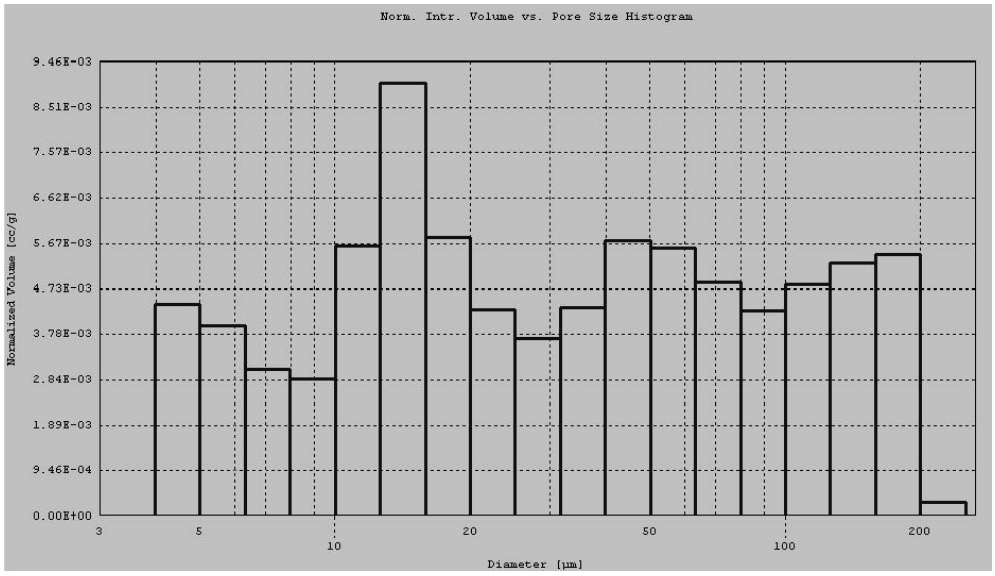


Fig 4.4 Pore size distribution of VC

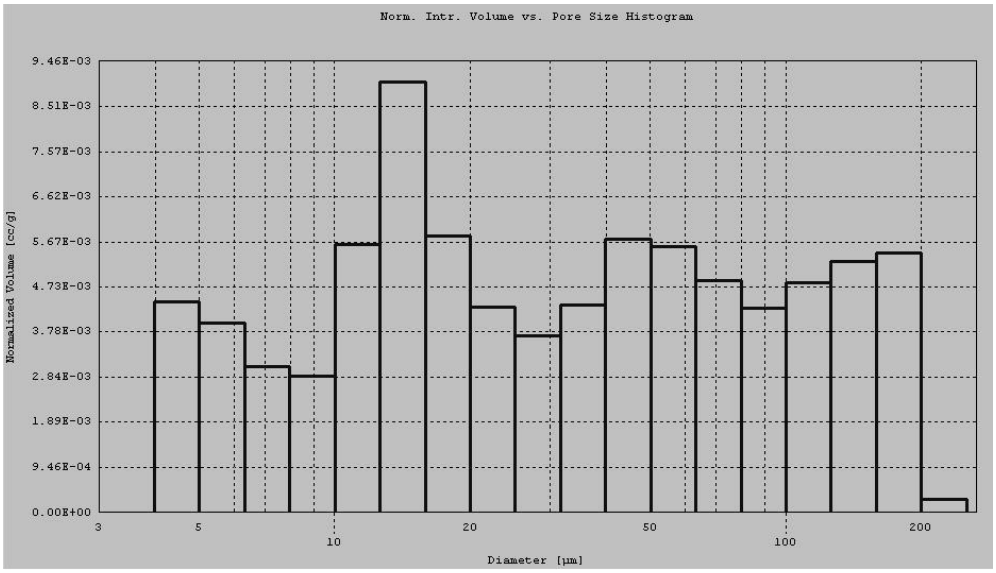


Fig 4.5 Pore size distribution of CVC

4.1.3 Apparent Density

The apparent densities of CVC, VC and VUC were 1.18 g/cm^3, 1.19 g/cm^3 and 1.12 g/cm^3.

4.1.4 Surface Area

VC had 3.40 m^2/g, CVC had 3.68 m^2/g and VUC had 4.77 m^2/g surface area.

4.2 In situ Studies

4.2.1 Vancomycin Release from PLLA/TCP Composites

The calibration curve for vancomycin was given in Appendix A. The weight of the PLLA/TCP composites and the entrapped amount of vancomycin in the PLLA/TCP composites were calculated as 9.1% (w/w) of the composite was vancomycin (Table 4.1).

Table 4.1 The weight and the entrapped amount of vancomycin in the composites

Sample	Weight (mg)	Entrapped Amount of Vancomycin (mg)
VC	29.32	2.67
CVC	29.84	2.71

The release period was 5 weeks for CVC and a day for VC. The polymer coating was extended the release period for CVC. CVC released 1.57 mg vancomycin in the first day. In other words, CVC released 57.9 % of its entrapped amount of vancomycin in the first day. At the end of 5 weeks, CVC released 2.54 mg vancomycin totally. Finally; in terms of percentage, CVC released 93.8 % of its entrapped amount of vancomycin (Fig 4.6).

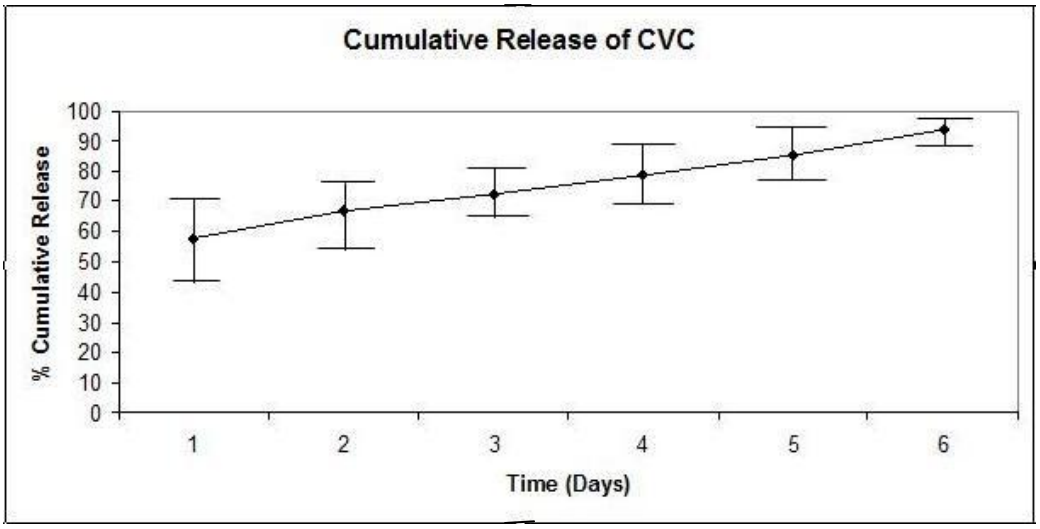


Fig 4.6 Cumulative release of CVC

VC released 2.67 mg vancomycin. This result was higher than the entrapment amount of vancomycin in the PLLA/TCP composites. With respect to the absorbance values, there would be certainly some inaccuracy in the measured values due to the interference of vancomycin peak at 280 nm with the polymer (PLLA) dissolved into the aqueous solution. The value found for VC was higher than the total drug content therefore; the polymer could be contributing to the measured absorbance value. In the preparation of the calibration curve, no polymer was included into the aqueous solution. The important point was, almost all of the vancomycin entrapped in VC was released in about the day.

4.2.2 High Performance Liquid Chromatography (HPLC) Assay

During the HPLC assay, the eluted vancomycin concentrations were below the detection rate and could not observed. Consequently, HPLC assay method was not successful.

4.3 In vitro Studies

4.3.1 Cell Culture Studies

In the third day, 5000 more cells were counted for CVC while, 5000 less cells were counted for VC according to control group with 35000 mean cell count. In the seventh day, 10000 more cells were counted in the control group than third day control group. The number of cells counted with CVC was the same with the control group whereas; the mean cell count was diminished 5000 cells for the VC. MSC adhesion was increased for all types of PLLA/TCP composites in the seventh day than the third day (Fig 4.7).

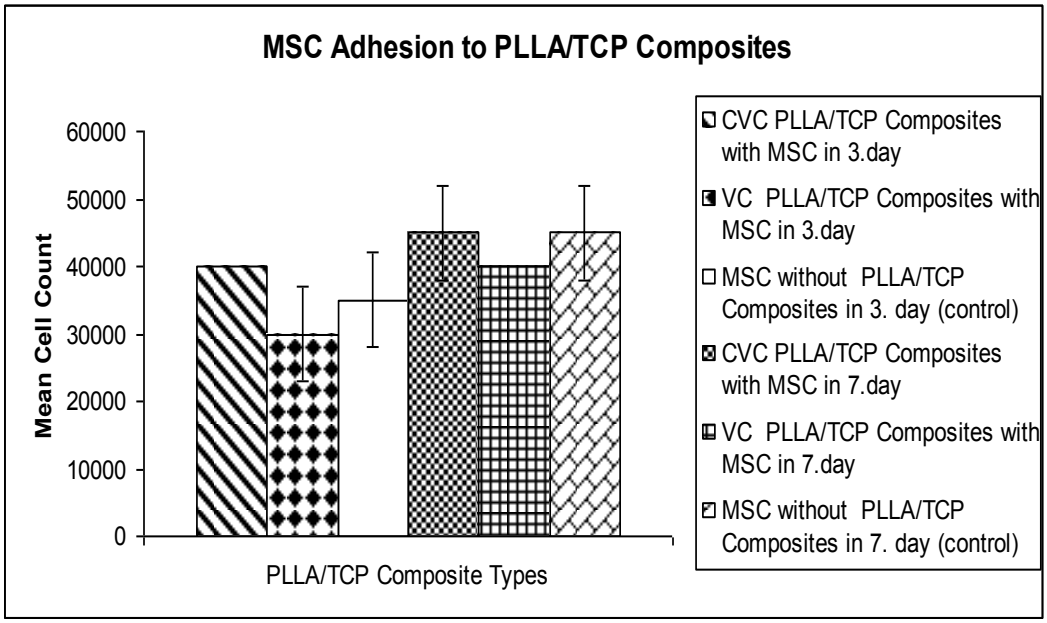


Fig 4.7 MSC adhesion to PLLA/TCP composites

In the same assay with Saos-2 cells, the mean cell count for the control group was 65000 cells higher in the seventh day than the third day with 50000 mean cell count. In the third day, mean cell count was equal for the CVC and control group but, 5000 more cells were counted for VC. In the seventh day, mean cell counted

for the CVC and VC were 70000 and it was 45000 cells lower than the control. Saos-2 cells adhesion increased for all types of PLLA/TCP composites in the seventh day than the third day (Fig 4.8).

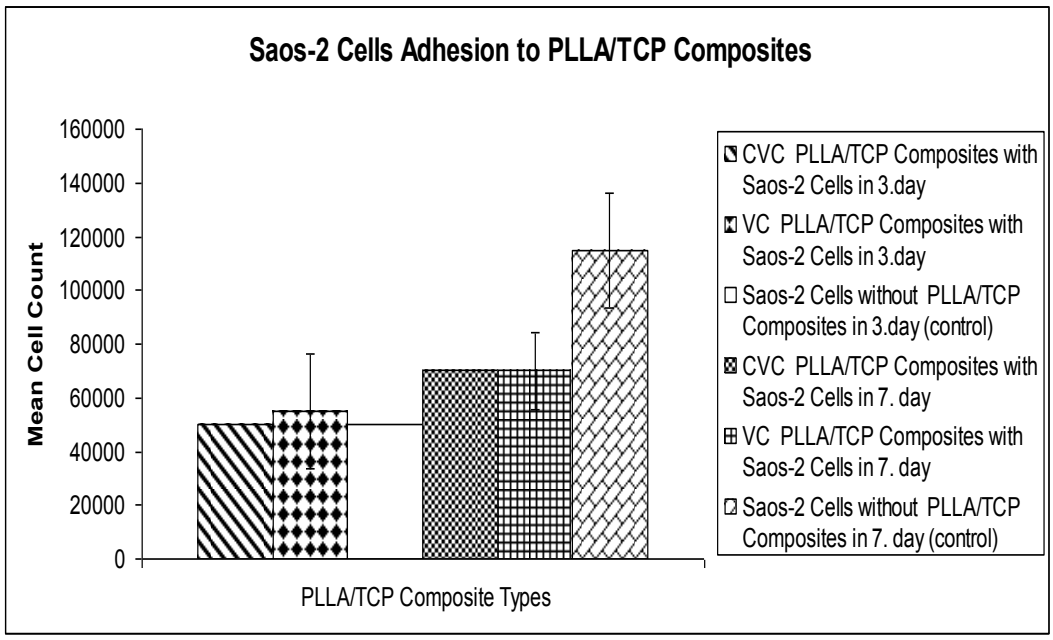


Fig 4.8 Saos-2 Cells adhesion to PLLA/TCP Composites

The MTT cell proliferation assay showed that, MSC proliferated more with CVC in 3. day than 3. day control (p=0.03). MSC proliferation was higher for the 7. day control than the 3. day (p=0.02). In the 7. day, again MSC proliferated more with CVC than the control (p=0.03) (Fig 4.9). While working with the Saos-2 cells, more cells proliferated with VC than CVC in the 3. day. Saos-2 cells proliferation was higher for the 7. day control than the 3. day (p=0.02). In the 7. day, Saos-2 cell proliferated more with the VC than the control group (Fig 4.10).

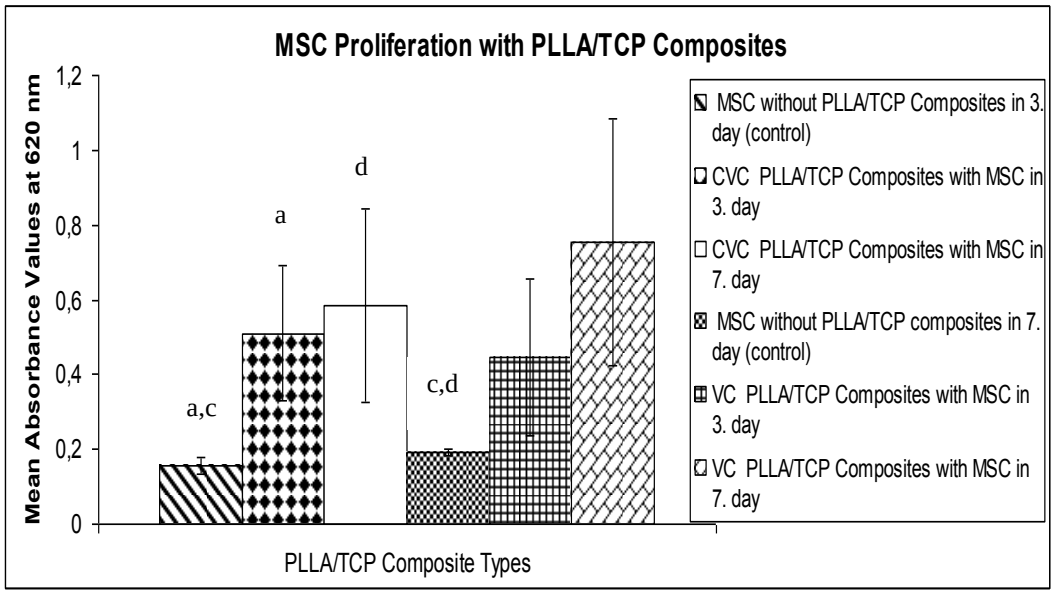


Fig 4.9 MSC proliferation with PLLA/TCP composites

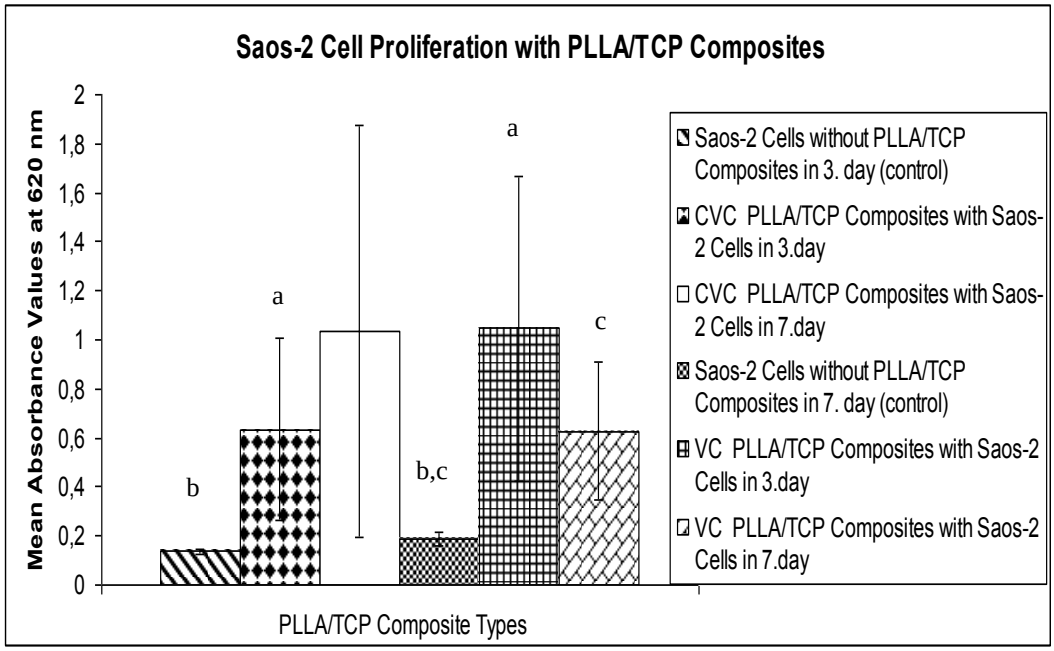


Fig 4.10 Saos-2 Cell proliferation with PLLA/TCP composites

According to the mineralization studies, calcium was identified in the composites, which did not interact with the cells. When they interacted with the cells, the cells differentiated and calcium was identified. There was no significant difference between the control groups and the composites with the cells (Fig 4.11, 4.12).

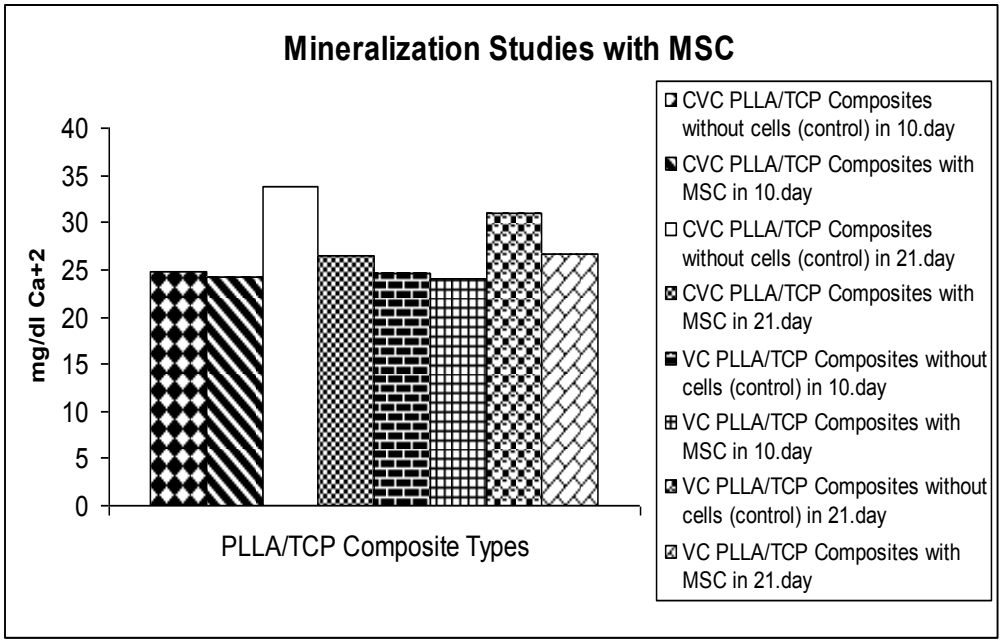


Fig 4.11 Mineralization studies with MSC

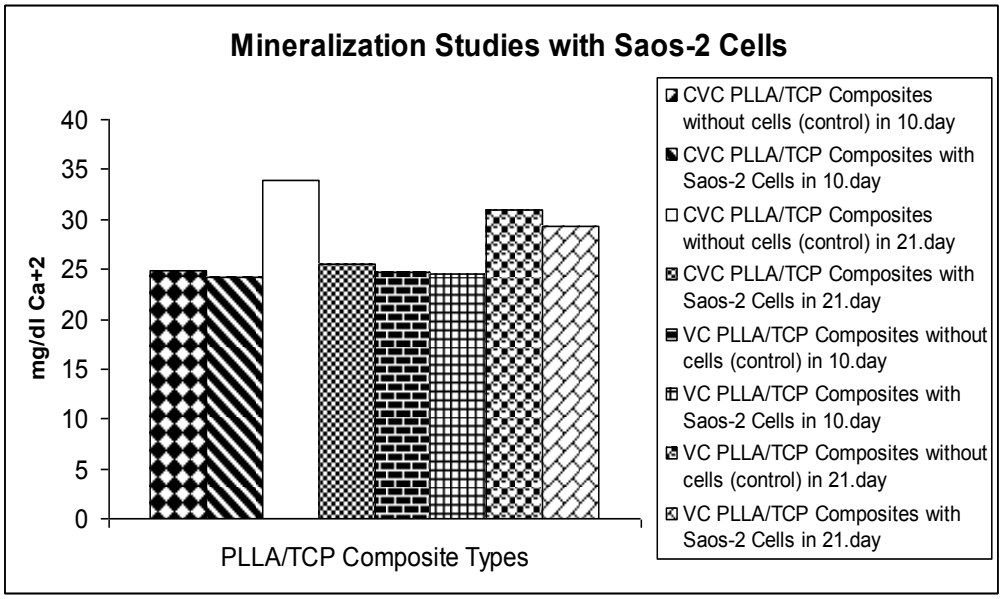


Fig 4.12 Mineralization studies with Saos-2 Cells

4.3.2 Microbiological Studies

4.3.2.1 Microbiological Studies Before In vivo Studies

In the study, the results were same for the first, second and the fourth days. The antibiotic test disc developed 18 mm inhibition zone diameter and MRSA was sensitive to the test disc.

CVC developed 11 mm zone diameter and MRSA was intermediate to CVC. On the other hand, VC developed 17 mm zone diameter and MRSA was sensitive to VC (Fig 4.13).

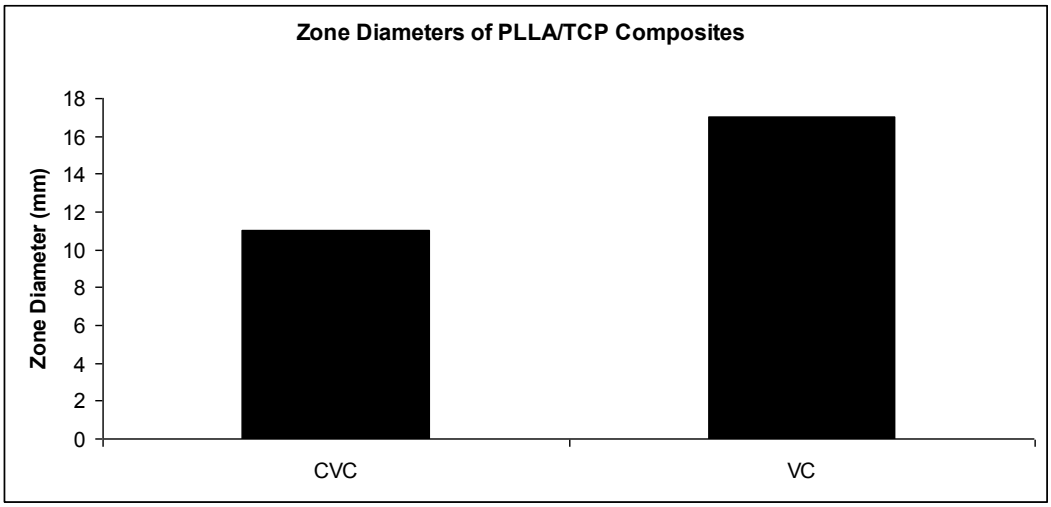


Fig 4.13 The zone diameters of PLLA/TCP composites

4.3.2.2 Microbiological Studies During In vivo Studies

Microbiological assessment was utilized to confirm osteomyelitis. In all the plates, presence of MRSA colonization was recorded (Fig 4.14).

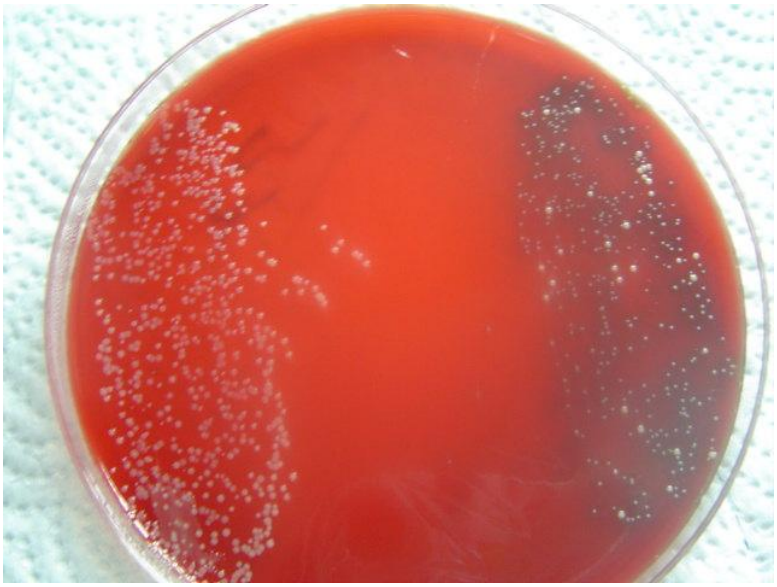


Fig 4.14 A blood agar plate photograph confirming presence of MRSA colonization

Before terminating the in vivo studies, operated tibiae were swabbed with sterile test stripes. For the VC, CVC and VUC without osteomyelitis groups, all the plates were free of bacteria and no growth was present (Fig 4.15).

For the first week, in VUC group, MRSA grew in four and Escherichia coli (E.coli) grew in two blood agar plates.

For the sixth week; in VUC group, MRSA grew in three and E.coli grew in three blood agar plates.

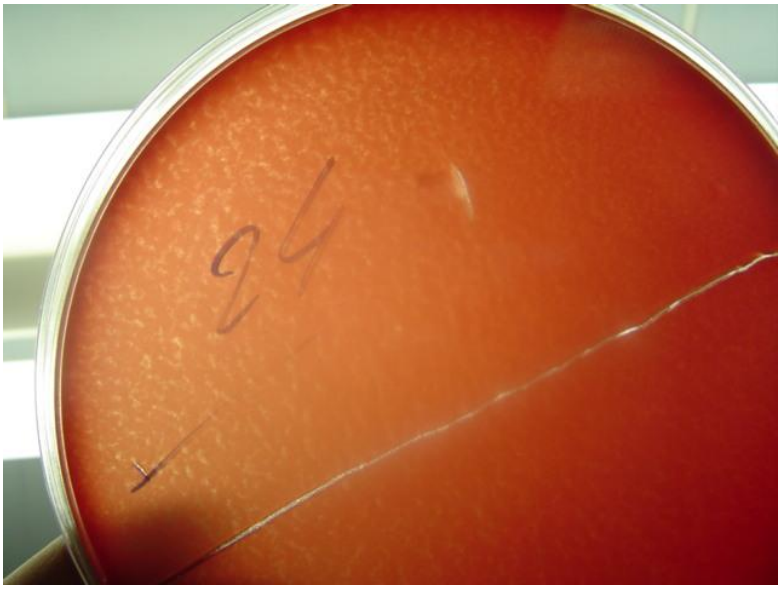


Fig 4.15 A clean blood agar plate confirming absence of bacteria

For microbiological evaluation, the criteria were (1) MRSA formation and (2) E.coli formation. Parameter 1 was graded as, (0) absent and (1) present. Parameter 2 was graded as, (0) absent and (2) present.

The scores were added for each sample of each group, mean values and standard deviation were calculated (Table 4.2).

Table 4.2 The microbiological evaluation scores

	Group Names			
	Control	VC	CVC	VUC
Week1	0^{a}	0^{b}	0^{c}	1.3 ± 0.9^{d}
Week6	0^{e}	0^{f}	0^{g}	1.5 ± 1.1^{h}

a, b, c-d: p=0.001, e,f,g-h: p=0.001

There was no significant difference between control, VC and CVC groups whereas, between VUC and control, VUC and VC, VUC and CVC groups there were significant difference. VUC group had surviving bacteria while all other groups were free of the bacteria.

4.3.3 Radiography

After the evaluation of the graphies, intra observer and inter observer correlation coefficients were calculated by Pearson product correlation. The intra observer correlation coefficient was 0.98 and inter observer correlation coefficient was 0.95. According to the coefficients there was no significant difference in or between the examiners.

The scores were added for each composite of each group according to examiner and examination time. Mean values and standard deviation were calculated (Table 4.3).

Table 4.3 The radiological evaluation scores

	Groups			
	VC	CVC	VUC	VUC without Osteomyelitis
Week1	10.9 ± 1.0^{a}	6.1 ± 0.3	5.0 ± 0.2	6.3 ± 0.3^{b}
Week6	3.0 ± 0.3^{c}	6.0 ± 0.8	4.8 ± 0.3	9.2 ± 0.4^{d}

a-c: p=0.001; b-d: p=0.0024

CHAPTER 5

DISCUSSION

Osteomyelitis is a serious and difficult to treat infectious disease of bone, mainly caused by MRSA. The blood flow is poor for infection area and the systemically given antibiotics can not eradicate the pathogen which is protected by biofilm formation (48). In order to treat these infections, local controlled drug delivery systems can be used. These systems release the drugs at desired amounts to the target and eliminate the systemic toxicity and side effects of the drugs (49). The vancomycin loaded PLLA/TCP composites consist of three elements: an antibiotic to treat and control chronic implant related osteomyelitis, a biodegradable polymer for controlled antibiotic release and a reabsorbable ceramic for both antibiotic release and bone healing. Vancomycin, a glycopeptide antibiotic was chosen as an antibiotic that has bactericidal activity against MRSA, causing implant related chronic osteomyelitis. Vancomycin loaded PLLA/TCP composites were developed as coated and uncoated composites to understand the effect of PLLA coating for the elution of vancomycin from the composite. The composites had 0.005±0,002 cm^3/g normalized internal pore volume with the range between 0.0003-0.009 cm^3/g and. The PLLA coating changed the surface morphology of the composites as, CVC had smooth, network like surface while the VC had rough surface. Vancomycin was released from CVC for 35 days and a day for the VC. Adams et al. found that, vancomycin was released from poly methylmethacrylate for 28 days (50) and Liu et al. found, vancomycin was released from poly(D,L)-lactide-co-glycolide over 55 days (43). The PLLA coating prolonged the release period by eluting lower amounts of vancomycin while extended release was observed for the VC as, VC released all vancomycin entrapped in the composites in a day.

The amount of vancomycin released from the composites was analyzed spectrophotometrically while HPLC assay was not successful due to inappropriate storing conditions of the solutions. The solutions were stored at 4˚C instead of -80˚C for two months before the HPLC assay and vancomycin in the solutions decomposed.

Although the MRSA was intermediate to CVC and sensitive to VC before the in vivo studies but; during the in vivo studies, microbiological analyses showed that osteomyelitis could be treated with both VC and CVC. The reason was the duration difference between the studies. The microbiological studies before in vivo studies ended after 4 days while the in vivo studies took 6 weeks to end. As a result, the hypotheses approved that osteomyelitis was treated with PLLA/TCP composite and vancomycin was released from the CVC for 35 days and inhibited MRSA proliferation. In VUC, E.coli was colonized in some cultures instead of MRSA as a result of contamination. For VUC implanted without developing osteomyelitis, all the cultures were free from bacteria so there was no significant immune response to the composite material.

Radiography results showed that, there was a significant difference ($p=0.001$) between VC implanted for 1 week and VC implanted for 6 weeks. The radiological evaluation score was decreased for 6 weeks which signaling the treatment of the infection. Also, there was a significant difference ($p=0.0024$) between VUC implanted for 1 week without developing osteomyelitis and VUC implanted for 6 weeks without developing osteomyelitis. The radiological evaluation score increased by the sixth week due to the composite degradation. The acidic degradation products of PLLA decreased the pH value and inflammatory response was induced in vivo (51). The decrease in the pH also developed bone resorption (52).

It was assumed that, the composite with larger surface area would promote more cell attachment and spreading (53). The surface area of CVC was larger than VC

as the coating increased the surface area and CVC promoted more cell attachment than VC for both MSC and Saos-2 type cells. For spreading, CVC were capable of more cells spreading than VC for MSC but, more cells spreading were promoted by VC for Saos-2 cells. For mineralization, there was no significant difference between VC and CVC, cells differentiated and calcium was identified. There was not a negative response of the composite for mineralization. It was certain that VC and CVC promoted cell adhesion, proliferation and mineralization so; the hypothesis was proven concerning the biocompatibility of the composites in vitro.

CHAPTER 6

CONCLUSION

The PLLA/TCP composite is an in vitro biocompatible material that can treat the chronic implant related osteomyelitis with the loaded vancomycin.

Vancomycin can release for 35 days if the composite is coated with PLLA and inhibits MRSA proliferation. The only disadvantage is the extended release of the uncoated vancomycin loaded PLLA/TCP composite that releases all entrapment amount of vancomycin in a day. Additional work can be done to overcome the condition.

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APPENDIX A

CALIBRATION CURVE OF VANCOMYCIN

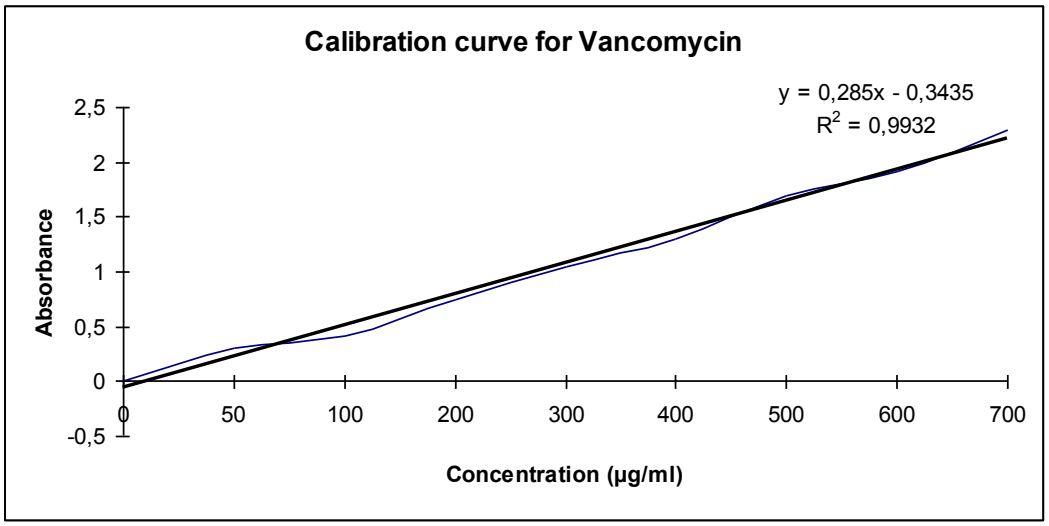

Fig A.1 The calibration curve for vancomycin at 280 nm