# A Study of Doxycycline Release from pH-Responsive Chitosan-PLGA Coated Titanium Nanotubes

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THESIS

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**To my parents and teachers**

**Thank you for all your support and encouragement**

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## ABSTRACT

Nearly 35 million Americans are missing teeth in one or both jaw, 15 million of those people undergo replacement surgery, and 3 million get dental implants which is increasing by 500,000 yearly. The dental implants however are not always successful. Peri-implantitis is an inflammatory lesion of the tissues surrounding the implants that occurs in 12-43% of the patients. Implant failure can be caused due to bacterial infections, and/or biomechanical overload. Even poor oral hygiene, smoking, and diabetes increase the risk of implant failure, ultimately leading to peri-implantitis. In patients with peri-implantitis, *P. gingivalis* is found in large numbers as the causative bacteria of the inflammation. Peri-implantitis causes a drop in the pH around the implant leading to acidic conditions which results in the inflammation of the tissue ultimately causing implant failure. Current treatment methods for peri-implantitis include removal of bacterial plaque around the implant, surface decontamination of implants, and delivery of antibiotics by oral route. In more extreme cases, surgical procedures like the removal of the affected implant is performed. However, these solutions have limitations and may be invasive for the patient. Some procedures have to be repeated regularly, some are painful, and the antibiotics may not reach the site of infection leading to recurrence. In this research a novel method is developed to cure peri-implantitis. Titanium nanotubes are synthesized on the surface of pre-fabricated titanium dental implants by electrochemical anodization. The hollow nanotubes serve as a drug reservoir in which the antibiotic drug doxycycline is loaded. The drug loaded nanotubes are coated with layers of pH responsive polymers which degrade at low pH releasing the drug. Ultimately, the drug release study is performed at pH 6.0 and pH 7.4 with the help of UV-Visible spectrophotometer.

# 1. Introduction

## 1.1 Use of Titanium in medical implants

Titanium is suitable as an implant material for biomedical purposes. It was discovered at the University of Cambridge in the 1950s and used as a potential tissue replacement device [1]. Titanium and its alloys are used in medicine as implantable devices to replace hip and knee joints. It is used as anchorage devices serving as bone plates, dental implants and in cardiology as pacemaker devices. Many implant manufacturers use titanium due to its favored properties like corrosion and wear resistance, low toxicity, and biocompatibility. Titanium is used as a substitute for hard tissues at locations which undergo intensive mechanical movement and stresses, hence it is expected to have superior mechanical properties. The biomedical titanium alloys show a tensile strength of 500 to 1000 MPa. Their elastic modulus and fatigue strength is between 55 to 85 GPa and 265 to 816 MPa, respectively [2].

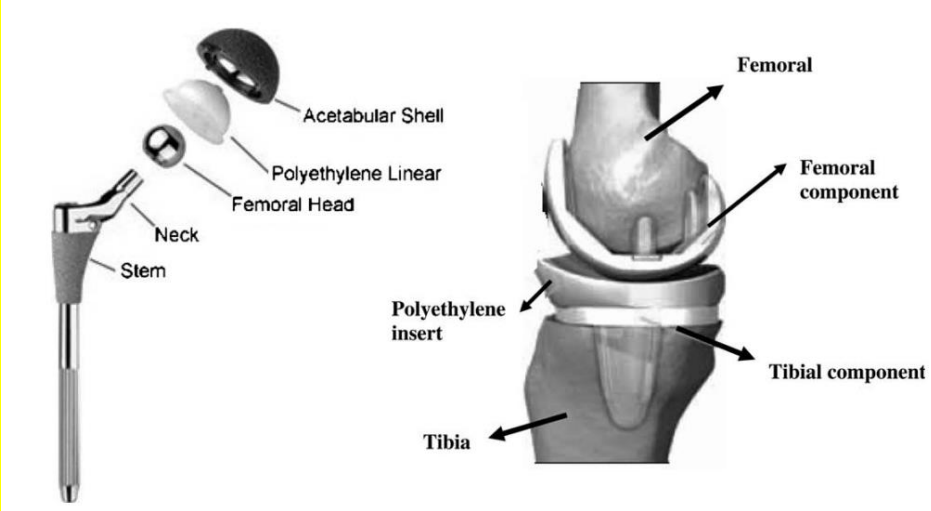


**Figure 1**- Titanium implants utilized in total-hip and total knee-reconstruction [3].

Titanium shows high biocompatibility [4]. Surface properties of titanium like surface chemistry and surface topography are important for the high biocompatibility and cell adhesion of titanium implants. The biocompatibility of titanium is due to the presence of a very stable passive layer of oxide ($TiO_2$) on its surface. The oxide layer repairs itself and regenerates even if it is damaged. The crystalline nature of the oxide layer has been found to be significant in protecting the metal substrate from corrosion [3].

### 1.2 Use of Titanium in dental implants

Branemark, a Swedish orthopedic surgeon was the first to use titanium dental implants for missing tooth. It is estimated that 30 million Americans are missing all their teeth in one or both jaws, and this number is growing at a rate of 500,000 every year. The different types of dental implants are osseointegrated implants, mini-implants, and zygomatic implants.

#### 1.2.1 Osseointegrated implants

Osseointegrated or endosseous dental implants are placed within the jaw bone. These implants can be made into different shapes like cylinders, screws, or blades (Figure 2).

**Figure 2**- Commercially available Titanium dental implants [4]

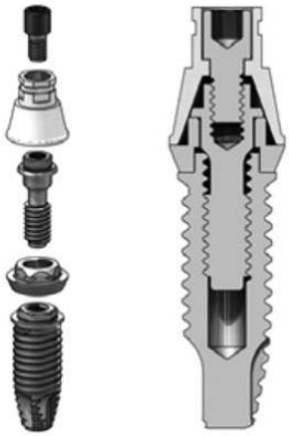

**Figure 3**- From bottom to top, a dental implant has several parts. (Left) These parts are: dental implant, prosthetic abutment and abutment screw; prosthetic cylinder and cylinder screw. (Right) Cross-section of the assembly on the left. [4]

The screw-shaped implants may have various connecting prosthetic parts (Figure 3). The implant is the main component which connects with the bone tissue and forms the bone-implant interface. The crown portion of the dental implants may be connected as a one-piece structure or a multi-piece structure. If it is a multi-piece structure, the second component may be the prosthetic abutment, which connects directly to the dental implant by means of an abutment screw. The third component of the implant system is the prosthetic cylinder, which is used to cast a crown in screw-retained dental implant crowns (Figure 3).

### 1.2.2 Mini-Implants

These are temporary implants used in orthodontic treatments which provide a secure anchoring. These are made up of Ti-6Al-4V alloys in place of commercial Ti, since the alloy has greater strength.

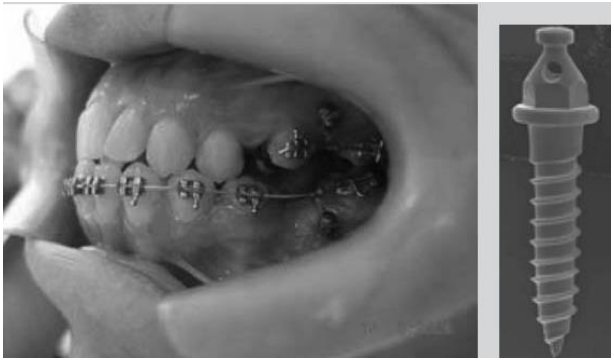

**Figure 4**- Mini-Implants used for the purpose of absolute anchorage to facilitate tooth movement. [4]

### 1.2.3 Zygomatic fixtures

Zygomatic implants are made of commercial Ti and are used in the treatment of atrophic maxilla or when there is a significant loss of the maxillary structure. They have a diameter and length of 4-5 mm and 30-53 mm respectively. They provide anchorage and support to the prostheses. It is placed in the posterior maxilla near the alveolar crest. (Figure 5).

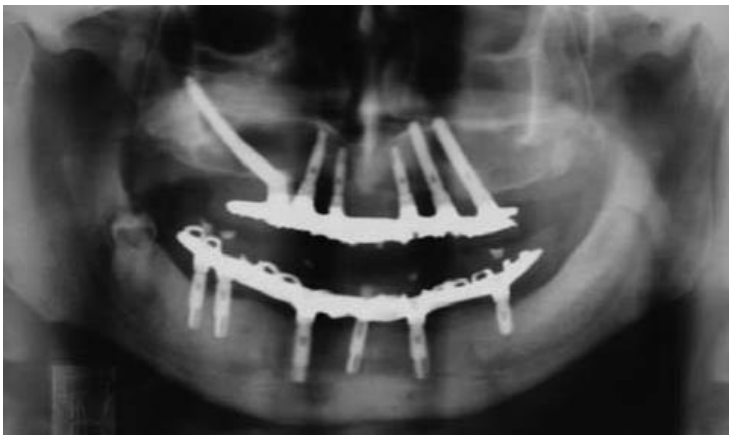

**Figure 5-** Panoramic radiograph taken of a patient presenting one left zygomatic Implant. Note extension of the implant at the level of the maxilla posteriorly in a high level. In addition, there is presence of a radiopaque image suggestive of bone loss around two-thirds of the zygomatic implant. [4]

### 1.3 Diseases associated with titanium dental implants

Due to their long term success and functional stability, titanium dental implants are increasingly being used for oral rehabilitation in patients with partial or complete loss of teeth. However, this has also led to the increase in the inflammatory conditions around a them

Diseases caused around a titanium dental implant are collectively termed as peri-implant diseases. Peri-implant mucositis and peri-implantitis are the major peri-implant diseases. Peri-implant mucositis is the swelling of the tissue around the implant. It can be cured with proper diagnosis and management; however, if left untreated it may lead to peri-implantitis (Figure 6).

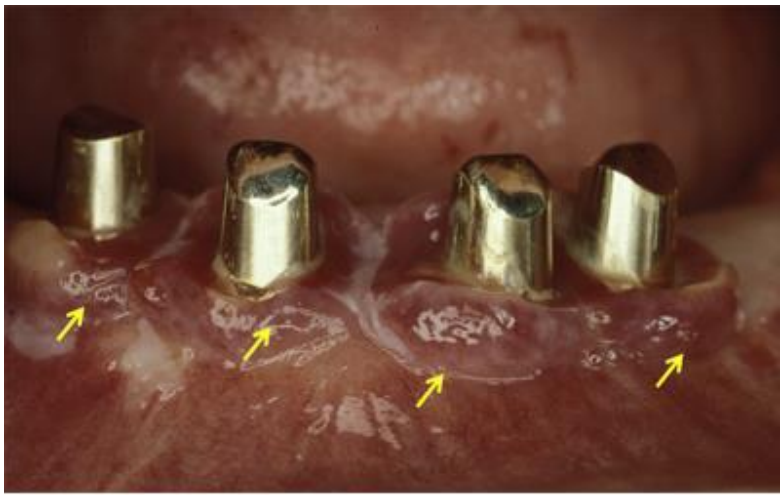

**Figure 6-** Frontal intra-oral view of four dental implants. Note erythematous and edematous peri-implant tissues (arrows). This image is suggestive of peri-implant mucositis, once there is no radiographic image suggestive of bone loss [5]

Peri-implantitis causes swelling of the peri-implant mucosa with bone loss. There also occurs a purulent discharge on probing and loss of bone density of at least 2.5 mm (Figure 7).

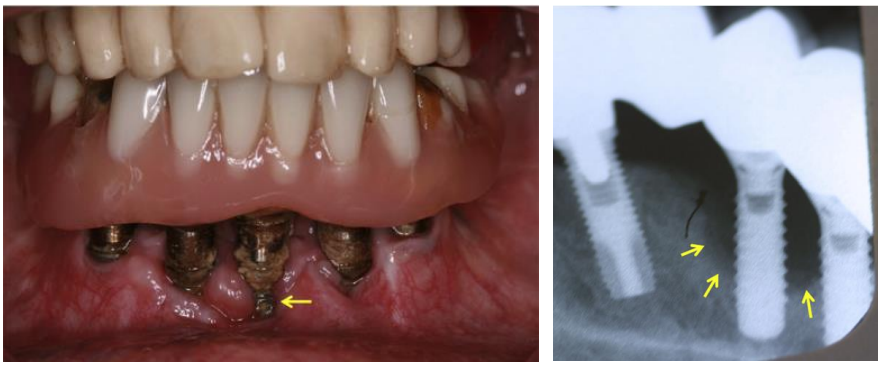

**Figure 7-** (Left) Frontal intra-oral view of an implant supported full-arch prosthesis over dental implants presenting clinical evidence of bone loss and exposure of the implant threads (arrow). (Right) Periapical radiographic image of dental implant showing image suggestive of bone loss to the 8$^{th}$ thread of the implant on the mesial and 9$^{th}$ thread on the distal (arrows). [5]

### 1.4 Causes of peri-implant diseases

Some of the well-known causes of peri-implant diseases are smoking, poor oral hygiene, and history of periodontitis [5]

#### 1.4.1 Poor oral hygiene

Due to the shape and the surface features of the implants, it might get difficult for the patients to clean or brush around the implant and maintain proper oral hygiene which may lead to plaque formation around the implant. The plaque accumulation may cause peri-implant pockets [5]

### 1.4.2 Previous History of Periodontal Diseases

Peri-implantitis is common among patients who have had a history of periodontitis or periodontal diseases and is increased significantly in these patients as compared to healthy patients [6]

### 1.4.3 Occlusal Overloading

Occlusal overloading or irregular excessive pressure on the implants is another cause of implant failure which may cause implant loosening. Occlusal overloading along with plaque accumulation, interferes with the bone-implant interface, which if not diagnosed properly, may result in implant failure (Figure 8) [5]

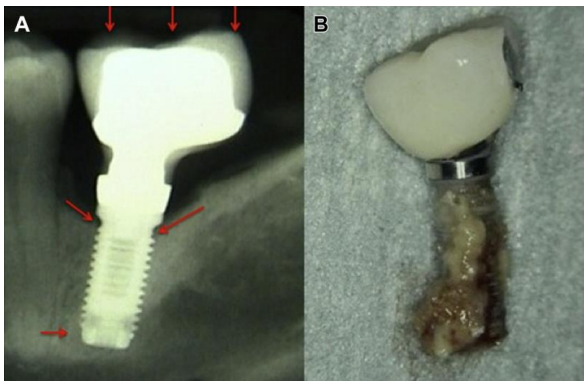


**Figure 8-** Radiographic periapical image suggestive of vertical bone loss on the mesial and distal aspects of the dental implant. B) Dental implant failed 8 months after loading. Note, presence of a distal extension of the implant that is not supported by the dental implant, which could have been the possible cause of biomechanical overload and dental implant failure [5]

### 1.4.4 Smoking

Tobacco smoking is a noteworthy cause of periodontitis and periimplantitis diseases. Nornicotine which is a metabolite of nicotine, increases the expression of RAGE in smokers, which causes secretion of cytokines leading to bone loss. [5].

### 1.4.5 Diabetes

Peri-implant diseases are common among patients with diabetes as compared to healthy individuals. This is due to the long-term hyperglycemia hinders tissue repair and immune responses. The chronic hyperglycemia increases the production of inflammatory cytokines like interleukines and matrix metalloproteinases inhibit osseointegration and long-term success of the implants. [5]

### 1.5 Treatment of peri-implantitis-

For the treatment of peri-implantitis debridement is the most commonly used technique, however, surgical and non-surgical techniques are also used for peri-implantitis treatment. A brief description of some of the treatment techniques is given below.

#### 1.5.1 Local Debridement

The cleaning of the implant is done by polishing. The polishing material is made up of a softer material than which is used for the implant. Generally, interdental brushes are used for polishing. Unlike metal instruments, these do not damage the surface of the implant. By using ultrasonic scalers with non-metallic tips implant surface damage can be prevented.

However, debridement does not always suffice the need of the surface decontamination with peri-implant pockets ≥5 mm and exposed implant threads [7]

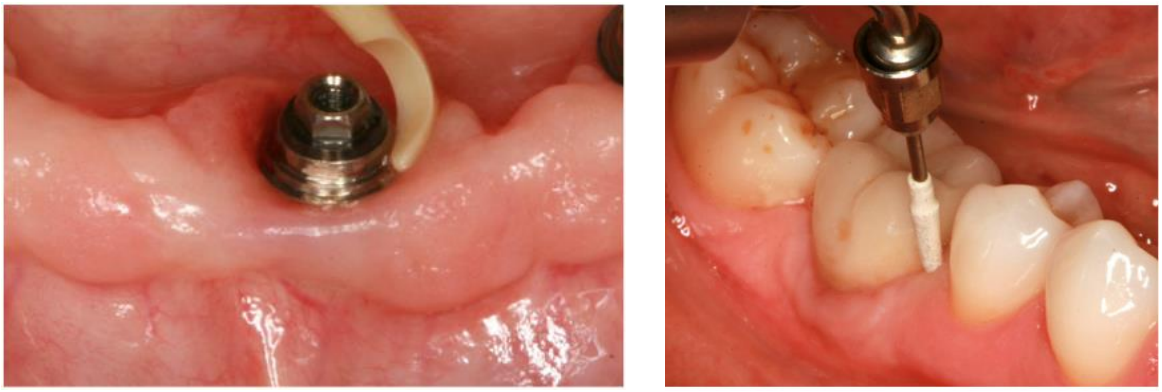

**Figure 9-** Debridement of peri-implant biofilm using a plastic curette and a polyetherketone coated ultrasonic tip. [9]

### 1.5.2 Implant Surface Decontamination

Some treatment methods involve decontaminating the implants. The most common methods of implant surface cleaning are air-powder abrasive technique with citric acid application, gauze soaked in citric acid, and gauze soaked in chlorhexidine and saline. Other methods involve mechanical or ultrasonic cleaning with soft plastic or curettes. Yet other methods include irrigation with 1% delmopinol, irrigation with tetracycline, photosensitization with Toluidine blue followed by soft laser irradiation. [8]

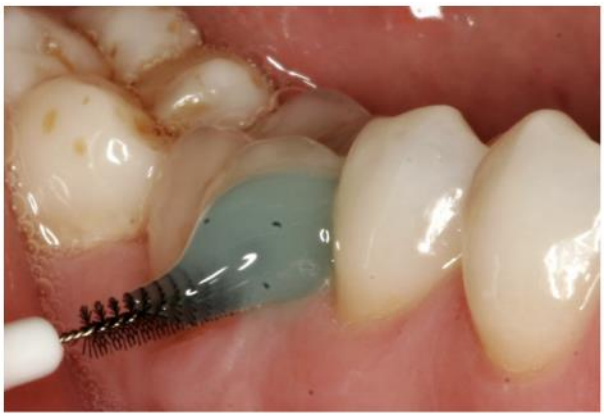

**Figure 10-** Peri-implant cleaning procedure with an interdental brush and chlorhexidine [9]

### 1.5.3 Treatments involving surgical methods

Treatment of peri-implantitis by surgical interventions depends on the access and exposure of the affected area. Access flap surgery, apically positioned flaps surgery, and regenerative surgical techniques are the major surgical treatment methods.

In the access flap surgery, the objective is to maintain and conserve the soft tissue and only to decontaminate the affected implant. This is done by making intracrevicular incisions around the implant, raising the mucoperiostal flaps buccally and palatally, followed by cleaning of the implants. Finally, the flaps are placed in position and sutured. But this technique can only be used when there very less bone loss.

The apically positioned flap surgery is done so that the patient can self-perform cleaning of the implants and to maintain oral hygiene. This surgery is also done to minimize the pockets around the affected implants. In this technique the margin of the affected tissue is removed and the implant surface is thoroughly cleaned. The implant remains exposed to the oral cavity after the flaps are sutured. [9]

Regenerative surgery is performed when there is considerable bone loss around the affected implant. The objective is to obtain re-osseointegration, using reconstructive and regenerative materials. The surgery is performed by making intracrevicular incisions and elevating the lingual and buccal periosteal flaps. The affected area is decontaminated and grafting is done using autologous bone or bone substitute graft and then covered with a resorbable material and the flaps are sutured. If the bone loss has advanced to a stage where the implant cannot be saved, explantation (removal of the implant) is performed. [9]

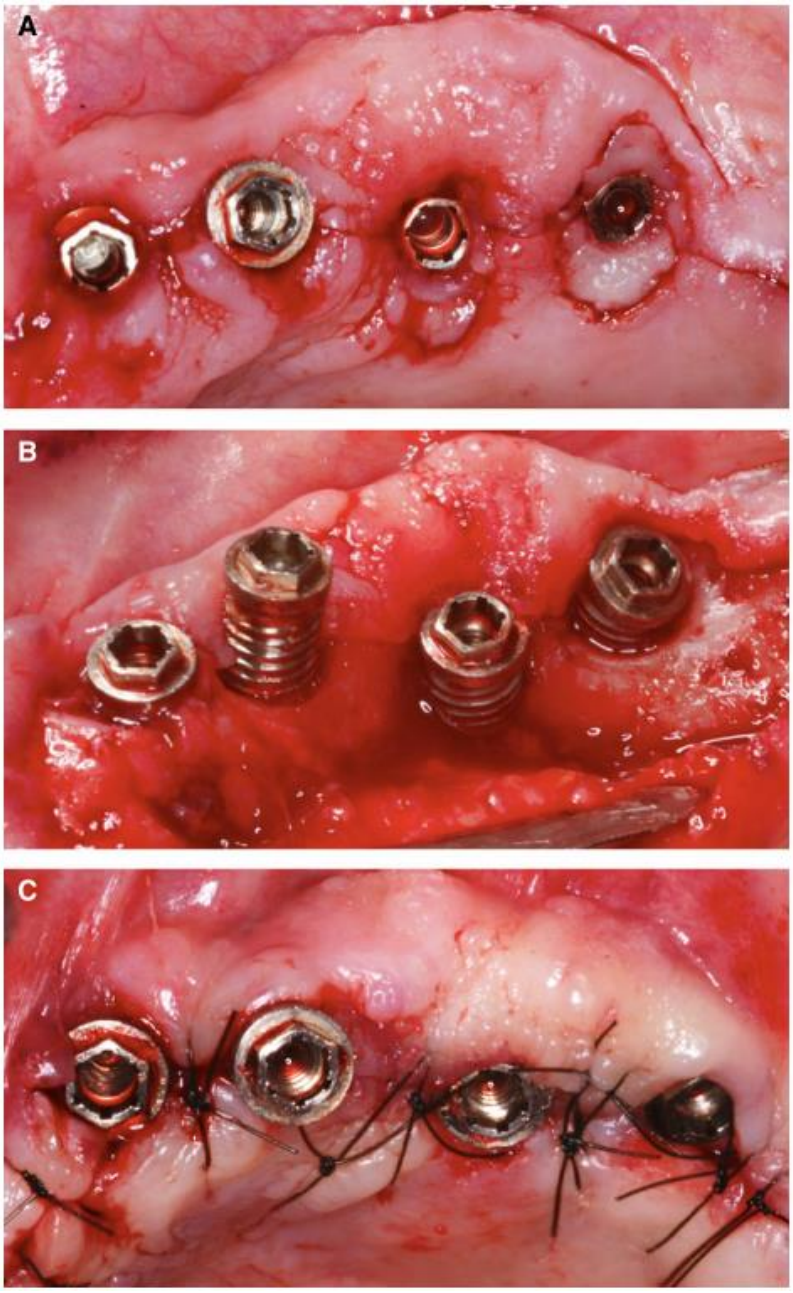


**Figure 11-** Surgical therapy- Resective therapy. Incision (top), mucoperiosteal flaps (middle), suturing (bottom) [9]

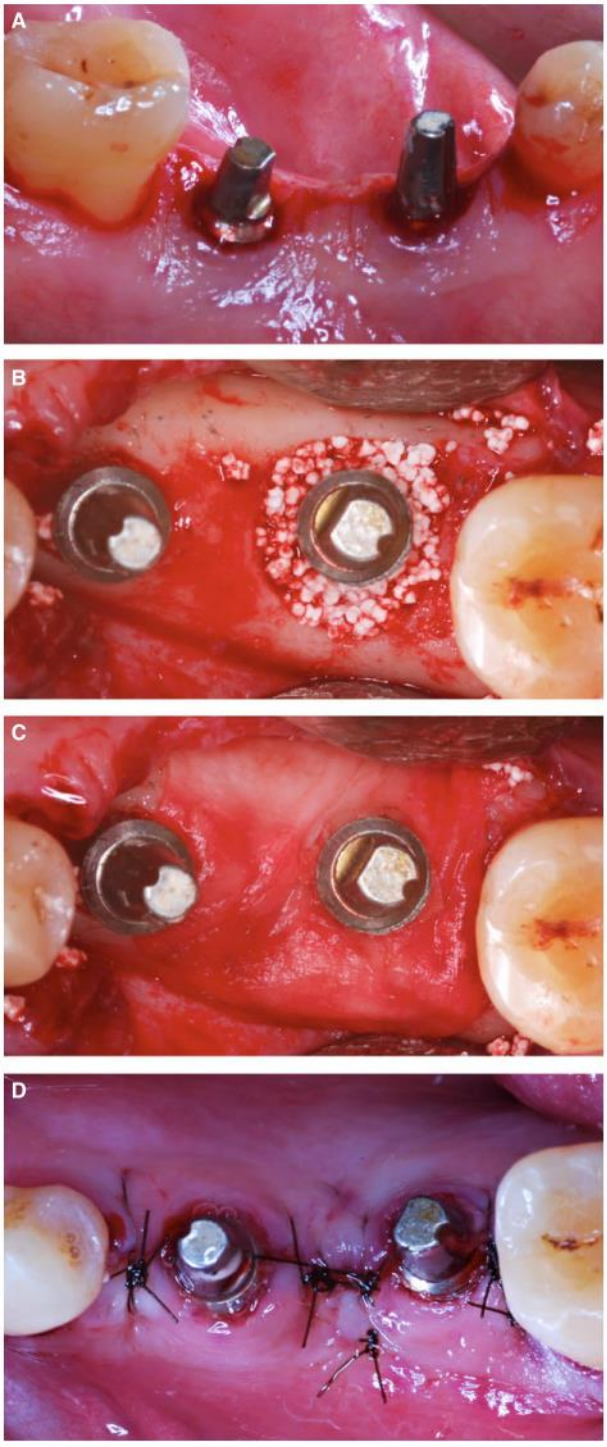


**Figure 12-** Surgical therapy- Access flap therapy [9]

### 1.5.4 Non-surgical Treatments

Non-surgical treatment of peri-implantitis generally involves local debridement, implant decontamination, and systemic antibiotic therapy. Some new methods have recently been developed for the non-surgical treatment of peri-implantitis. These include irradiating the affected area with lasers. Erbium-doped yttrium-aluminium-garnet (Er:YAG) laser irradiated on the surface of the affected implant can effectively debride the implant surface and has a high bactericidal effect without any morphologic changes to the implant surface. Two clinical trials [10] and a case study carried out in 2013 utilized diode laser for treating peri-implantitis.

The lasers used were 660 nm and 810 nm with 100mW power for 10 seconds and 1.96 W power for 6 minutes. The studies showed significant reduction in bleeding and inflammation of the affected sites concluding that photodynamic laser therapy can serve as a substitute treatment method for peri-implantitis. Other type include use of $CO_2$ laser. $CO_2$ lasers use photonic energy in the wavelength range of 9300-10600 nm.[12]

$CO_2$ lasers clean the surface of the implants by removing contaminated and inflamed tissues without affecting the morphology of the implant surface. [13] $CO_2$ lasers also kill the microorganisms on the implant surface without changing the surface topography.

Romanos et. al.[14] did clinical human studies using $CO_2$ laser on implant surfaces. In their study they used 2, 3, and 4 W laser irradiation for 1 minute followed by autologous grafting. Their study indicated the reduction in the pocket depth and sulcus bleeding.

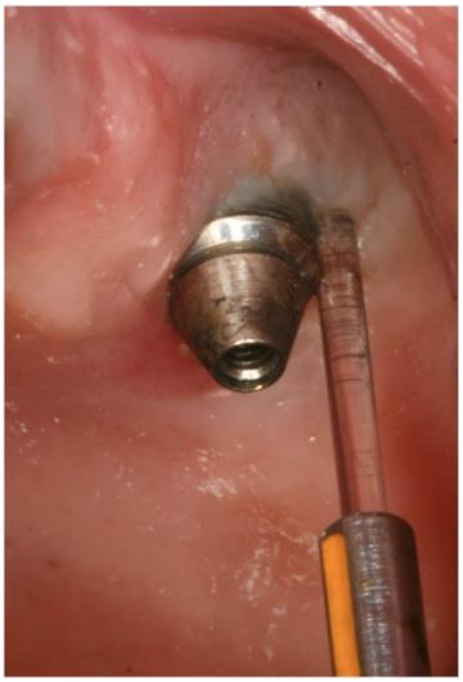

**Figure 13-** Debridement of peri-implant by Er:YAG laser [9]

### 1.6 Use of antibiotics in treatment of peri-implantitis

Antibiotics have been used in the prevention and/or treatment of implant failure. These are prescribed for prophylaxis during the post-operative period for 10 days to avoid early implant failure.[15] Oral implant surgical procedures have a bacterial infection rate of 10-15%, but this can be reduced to 1% with the proper use of prophylactic antibiotics.[16] But long term prophylactic treatments are being challenged due to the rise of antibiotic resistance bacteria, hence it is being replaced by single dose prophylaxis.[17,18]

Bacteria are the major causative agents for the start and progression of peri-implantitis. Gram-negative, anaerobic bacteria like *Porphyromonas gingivalis, Tannerella forsythia,* and *Aggregatibacter actinomycetemcomitans* are predominantly found associated in chronic peri-implantitis.[19]

Antibiotics are delivered either systemically through the oral route or by direct application around the dental implant. The treatment duration should be short so as to prevent any microbiological relapse, however it should have characteristics like rapid action, bacteriostatic and bactericidal activity, resistance to spawn mutants, and should be effective conditions of low pH [20]

Some of the widely used antibiotics for peri-implantitis therapy are- amoxicillin, penicillin, and antibiotics of the tetracycline group. Amoxicillin, a derivative of penicillin, is a broad-spectrum antibiotic which shows good absorption an bioavailability.

The microbial enzymes cleave the beta-lactam ring present in the molecular structure of amoxicillin. Penicillin is a narrow-spectrum antibiotic having bactericidal action against most *Streptococcus* species and oral anaerobes.[21]

Tetracyclines are considered as useful accompaniments in the treatment of periodontal diseases due to 3 advantages- as antibiotics tetracyclines suppress periodontitis causing gram-negative organisms, tetracyclines concentrate in the gingival crevicular fluid of the periodontal pockets in higher concentrations, and these antibiotics bind to the surface of the tooth and are slowly released for some time even after the drug intake is stopped.

Tetracyclines also have non-anti-microbial property like altering the host response, promote fibroblast and connective tissue attachment to the tooth surface, and the ability to inhibit collagenolytic enzymes which inhibits connective tissue degradation and bone resorption.[22] Tetracycline-HCl (100 mg/ml) is used to decontaminate root surfaces.[23]

Doxycycline is important in treatment of peri-implantitis because of its high availability in the gingival crevice about 7-10 times higher than other antibiotics. Also its action is not just limited to antimicrobial activity but it also shows anticollagenase and anti-inflammatory property. Other than these it promotes reattachment and prevents bone resorption. A study conducted in 2017, showed that the minimum concentration of doxycycline released from drug loaded titanium nanotubes was 1.29 ug/ml over a period of 28 days successfully suppressed the bacterial growth. [24,25]

### 1.6.1 Anti-collagenase effect of Tetracyclines

Different tetracyclines have different anti-collagenase activity. Minocycline was found to inhibit collagenase extracted from polymorphonuclear cells at a concentration of 2 to 25 µg/ml, whereas human fibroblasts' collagenolytic activity was resistant till 250 µg/ml concentration.

The concentration of tetracycline required to inhibit 50% ($IC_{50}$) of the activity of activated collagenase from human polymorphonuclear cells was found to be much lower than the collagenase extracted from human fibroblast cells. The anti-collagenase activity of minocycline or doxycycline is more effective than tetracycline. The $IC_{50}$ of these antibiotics is reported to be 15 and 190 µM, respectively as compared to 350 µM for tetracycline.

The higher inhibitory activity of doxycycline is due to its ability to bind $Zn^{2+}$ more tightly than other tetracyclines.[22] Doxycycline ($M_w$- 1025.89) is a yellow crystalline powder, readily soluble in water. It is active against many gram positive and gram negative. Use of doxycycline for the treatment in periodontitis results in significant gain in gingival attachment and decrease in pocket depths. Doxycycline inhibits PMN collagenase in vitro at $IC_{50}$= 26 µM.[25] Collagenases breakdown connective tissue macromolecules like matrix metalloproteinases (MMPs).[26]

Doxycycline inhibits the matrix metalloproteinase activity by depriving them of the divalent cations ($Ca^{2+}$ and $Zn^{2+}$) binding sites. Doxycycline chelates these cations which reduces their protein degrading activity. [26] This activity is observed even at submicrobial doses which led to the development of submicrobial doxycycline doses (SDD) [26]

The systemic use of sub-microbial dose of doxycycline (20mg taken 2 times a day for 6 – 9 months), in conjunction to non-surgical periodontal treatment had significantly greater clinical benefits than those with non-surgical periodontal treatment alone in the treatment of periodontitis. [27]

Doxycycline has shown to improve wound healing, to increase osteogenic mediators, and to reduce collagenase activity. Recently, the benefits of doxycycline as an osteogenic agent were observed in *in vivo* peri-radicular surgeries, in the treatment of infra-bony defects and in the downregulation of osteoclastogenesis *in vitro*.[28]

## 2. Surface modifications of titanium dental implants

Titanium implants are improved to enhance the surface properties of the implants but keeping the desirable mechanical properties intact. Surface modifications are carried out either by mechanical, chemical, or physical methods. Some advantages of surface modifications are, increase in corrosion and wear resistance, increase in surface energy, higher wettability, improved cell proliferation, and improved osseointegration process. [29]

Surface modification treatments can either be additive or subtractive. In the additive methods other materials are added to the surface of the implant either by coating or impregnation. Meanwhile, in the subtractive methods the superficial layer is removed to roughen the surface. Some of the additive surface treatments are plasma spraying and HA/CaP coating. The common subtractive methods of surface modification are sandblasting, acid etching, and anodization. Apart from these methods some of the other surface modification techniques are mechanical methods like grinding or machining, chemical methods like treatments with acid or alkali, and physical methods like ion implantation, laser treatment, and sputtering. Plasma spray coating and acid etching are the preferred surface modification methods since these provide higher porosity and greater bone-implant contact (BIC). The following sections give a brief description of the commonly used surface treatment methods.[29]

### 2.1 Plasma Spray Coating

In this method rough implant surfaces are produced by projecting titanium powder into a plasma torch. The titanium particles are then forced with high velocity and pressure on the implant surface where they get condensed and fuse together. This forms a thick film on the implant surface of about 30 µm. To get a uniform surface the thickness should be around 40-50 µm. This leads to an increase in the surface area of the implant which subsequently increases the tensile strength at the interface of the bone and the implant because of changes in the three-dimensional topography [11]. In a study carried on minipigs, rapid bone-implant interface formed on the titanium plasma spray coated surface than with smooth surface.

However, this method also has disadvantage in that the particles of titanium sometimes separate from the implant surface due to fretting or corrosion [21,22,23]. The metallic debris is also found in the liver, spleen, macrophages and lymph nodes [21, 25].

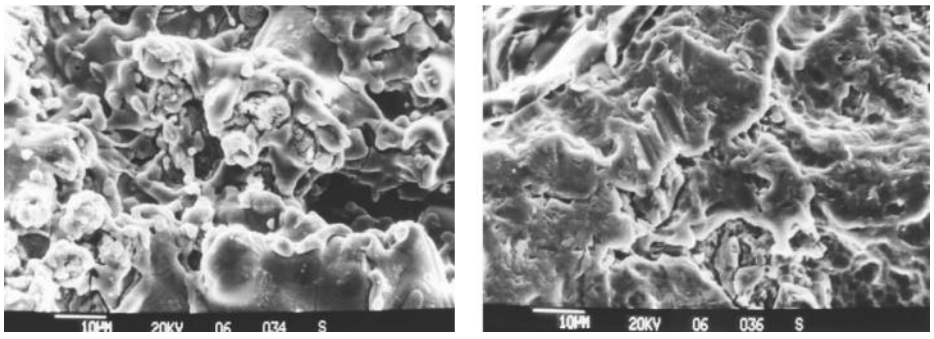


**Figure 14-** FESEM image of surface morphology of plasma sprayed titanium dental implant [43]

## 2.2 Sandblasting

Sandblasting is a technique used for roughening the surface of the titanium dental implants. In this method ceramic particles are forced on the surface using pressurized air. The roughness of the surface is directly related to the size of the particles blasted. Alumina ($Al_2O_3$), titanium oxide ($TiO_2$), and calcium phosphate particles are used as blasting materials since these are, biocompatible and do not interfere with the osseointegration process. However, the disadvantage of this method is that since the blasting material remains embedded into the implant surface, they may get released in the surrounding tissue and hinder the osseointegration process [27].

Titanium dental implants are blasted using titanium oxide particles. The particles used for sandblasting are of 25 um. A study showed enhanced bone-implant interface with $TiO_2$ blasted surfaces than with machined surfaces [28,29,30,31].

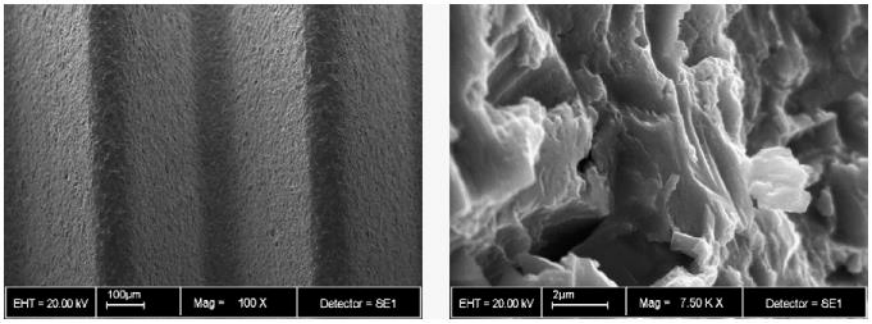


**Figure 15-** FESEM image of titanium oxide blasted dental implant surface [44]

## 2.3 Acid Etching

Acids such as HCl, $H_2SO_4$, HF and $HNO_3$ are used for the etching of the surface of titanium dental implants. These acids form micro pits on the surface with the size of 0.5 to 2 um in diameter [38,39]. This is usually done by immersing the implants in a highly concentrated mixture of HCl and $H_2SO_4$ which produces a micropatterned, rough surface. This surface also enhances higher osseointegration which helps maintain the implants for a longer duration of about 3 years [40,41]. Dual acid-etched surfaces promote osteoconductivity by attaching the fibrin and osteogenic cells which promotes the bone growth on the implant surface subsequently enhancing bone development due to the specific surface structure [42,43,44,45].

## 2.4 Electrochemical Anodization

Anodizing of titanium in acids by use of high voltage produces micro- or nano- porous structures and thickens the oxide layer. The oxide layer dissolves due to the convection lines formed by the current passing through the electrolyte during anodization [54–57,58]. This process depends on electrolyte temperature, current density, composition and concentration of acids, which makes the process rather complex. Anodized surfaces show enhanced properties as compared to machined surfaces [59,60].

A comparison between the anodized titanium implants and regular titanium implants of similar shapes showed considerable increase in the clinical success rate [61]. Two mechanisms for the success in the higher osseointegration have been proposed: biochemical bonding and mechanical interlocking through bone growth in pores [55,62,63,64].

# 3. Control of drug delivery and release by use of polymers

Polymers are important in the development of drug delivery systems as they provide controlled and/or sustained release of therapeutics, and also in the delivery of drugs. The polymers used for the drug delivery can be bioactive or biodegradable. Bioactive polymers combined with therapeutics can provide their own therapeutic benefits and the biodegradable ones can improve release kinetics. Conventionally, the polymers used for drug delivery are derived from cellulose and categorized as solvent-activated, biodegradable, or stimuli responsive systems.[31] A brief description of each these systems is discussed further.

## 3.1 Solvent-based Systems

In these systems dehydrated hydrophilic polymers and the drug are wrapped together. In systems which do not have a plasticizing aqueous solvent the diffusivity and the glass transition temperature, $T_g$ is low. When the system is exposed to moisture the polymer absorbs water and swells, releasing the drug. The drug release happens because the system alters from a glassy state to a rubbery state, relaxing the polymer.

### 3.2 Biodegradable Systems

Biodegradable polymer are important for drug delivery systems. Degradation of the polymer occurs when the covalent bonds in it are cleaved by chemical reactions. Degradation can be a bulk or a surface phenomenon. In surface degradation, the volume of the polymer remains unchanged due to the slow degradation, whereas, in bulk degradation the physical size of the polymer does not change until it is completely degraded. The polymers need to have hydrolytic or proteolytic labile bonds in their structure to be chemically degradable

### 3.3 Stimuli Responsive Systems

Stimuli responsive polymers are linear or cross-linked polymers. These are also called as smart polymers. These polymers undergo physical or chemical change when under the influence of an external stimuli. Temperature and pH are the common stimuli used to bring about structural or behavioral changes in the polymer, but other stimuli, like electromagnetic radiation, redox potential, ultrasound, and biochemical agents can be used. Physical stimuli (temperature, ultrasound, light and electromagnetic fields) change the energy level of the polymer system. Chemical stimuli, like, pH, redox potential, chemical agents respond to the stimuli by altering the molecular interactions between polymer or between polymer chains.

### 3.3.1 Stimuli Responsive Systems based on pH

The difference in the pH values of specific organs such as the gastrointestinal tract, has been exploited for the control of the delivery of drugs and also to trigger the release of the drug.[32] Inflamed tissues have lower pH than the normal tissues, generally producing acidic conditions in the extracellular environment [31] Due to anaerobic fermentation and inflammation, the pH around the bacterial infections drops very low in the range of 5.5 to 6.5. In such cases, ionizable pH responsive polymers incorporated with antibiotics are utilized as therapeutic delivery vehicles.[32]

Khanal et.al. in their study [33] used chitosan coated PLGA nanoparticles for the controlled delivery of diclofenac sodium. The 390-420 nm sized nanoparticles were synthesized by double emulsion solvent evaporation technique in which diclofenac sodium as a model drug and chitosan as a pH responsive polymer was used. The drug was released from the system at a pH of 5.5. Other examples of pH responsive polymers are poly(*N*,*N*-dimethylaminoethyl methacrylate) (PDMAEMA), poly(amido amine)s, poly(L-lysine) (PLL), modified chitosans[34] poly(acrylic acid) (PAA), polyethylenimine (PEI), poly(L-histidine), aminoalkyl methacrylate copolymer (Eudragit E), polyvinylacetal diethylaminoacetate, hydroxypropylmethylcellulose phthalate, hydroxypropyl methacrylamide.[35]

# 4.Project Objectives

The current treatments for peri-implantitis involve decontamination by physical and chemical means. Antibiotics, applied at the local site or delivered systemically, are used in chemical purification. Locally delivered antibiotics could be used in small volume or concentration in comparison to antibiotics delivered systemically. Local delivery of antibiotics also reduces the risk of side-effects like vomiting, nausea, hypersensitivity, and gastrointestinal distress.

The objective of this project is to improve oral and dental health related to implants. This objective will be attained by synthesizing a nanoporous surface on the titanium dental implants and loading the nanopores with antibiotics like doxycycline. The drug loaded nanotubes would be coated with a pH responsive polymer which would degrade under the acidic conditions found during a peri-implantitis infection. The degradation of the polymer would result in the release of the drug around the infected area thus reducing the healing time and increasing the success rate. This would also lead to greater osseointegration of the implant with the surrounding tissue.

The benefit of this strategy is that it can improve the state of the currently established titanium dental implants without any major changes to the surgical procedure or the implant design.

We propose to study the drug release from the nanotubes under the diseased pH and normal pH. The final objective is to study the viability of cells by MTT assay. This would be achieved by growing the cells on titanium surface, titanium nanotube surface and titanium nanotubes coated with pH responsive polymers.

Following are the specific aims of the project-

1. To synthesize titanium nanotubes on the surface of titanium dental implants by electrochemical anodization
2. To load doxycycline in the titanium nanotubes on the dental implants
3. To coat the drug loaded titanium nanotubes with pH responsive Chitosan-PLGA polymer
4. To study the release of doxycycline from the nanotubes at pH of 6.0 and 7.4
5. To check the cell viability by MTT assay

# 5. Materials and Methods

## 5.1 Synthesis of nanotubes on titanium dental implants by electrochemical anodization

The nanotubes were synthesized on commercially available, prefabricated titanium dental implants (Biohorizons Implant Systems, Inc., Birmingham, AL) by electrochemical anodization method. The implants were first sonicated in acetone for 15 minutes to remove the impurities on their surface and then rinsed under deionized water. To prepare the nanotubes on the implant surface, anodization was carried out in an ammonium fluoride, $NH_4F$ (Sigma-Aldrich, St. Louis, MO, USA) electrolyte containing 0.2 wt% $NH_4F$, 10 vol% $H_2O$, and ethylene glycol. The anodization was carried out in a beaker containing the electrolyte in which the Ti dental implant served as the working electrode and a graphite rod was used as a cathode. The two electrodes were connected to a DC power source (Keithley 2400 SourceMeter) which supplied 60 V for 2 hours. The anodization was carried under mild stirring at room temperature. The anodization setup is shown in the figure.

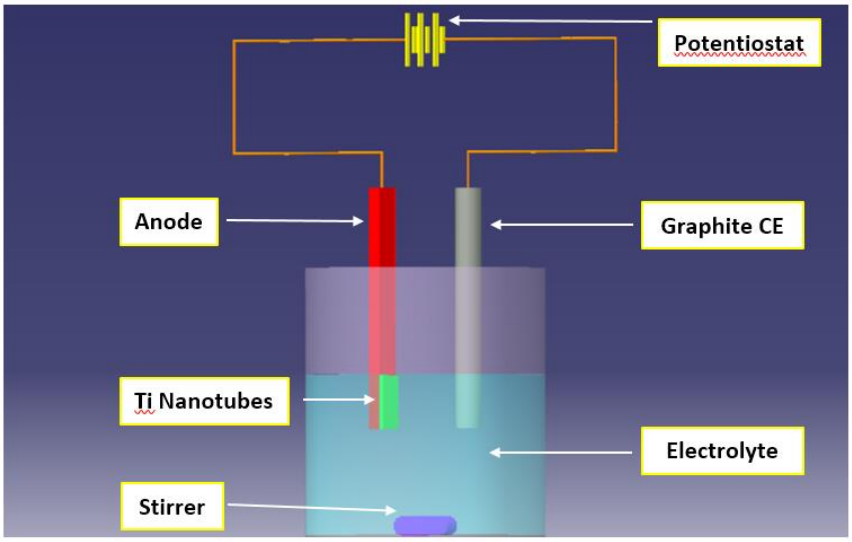


**Figure 16-** Schematic of the electrochemical anodization apparatus

### 5.2 Drug loading in the titanium nanotubes

A solution of doxycycline (Sigma-Aldrich, St. Louis, MO, USA) with a concentration of 50 mg/ml was prepared in deionized water to use as a model drug. The drug loading in the titanium dental implants was carried as per the procedure of Choi et. al.[36] In brief, the dental implants were kept immersed in the drug solution for 1 hour and then kept in a desiccator attached to vacuum until the samples were completely dry. To achieve maximum volume of drug in the nanotubes the drug loading procedure was repeated 3 times. To check the presence of the drug in the nanotubes, the samples were analyzed under an EDS.

### 5.3 Polymer coating on drug loaded titanium nanotubes

Polymer coating on the drug loaded titanium nanotubes was done as per the procedure described by Gulati et. al.[37] The polymer solution was prepared by dissolving 1% chitosan in acetic acid. 5% Doxycycline (Sigma Aldrich) was added to the chitosan solution for maximum drug entrapment. Another solution of PLGA (50:50 LA:GA; $M_n$ 35,000-45,000 Da) was prepared by dissolving 2% PLGA in dichloromethane (Sigma Aldrich) and 5% triethyl citrate (Sigma Aldrich) was used as a plasticizer. The coating was done by dipping the dental implants in the polymer solution. The implants were first dipped in the chitosan solution and then in the PLGA solution. The dipping procedure was repeated 5 times with alternate chitosan and PLGA coatings. After each dipping the implants were kept in the incubator at 40°C till they were completely dried.

### 5.4 Drug release study at different pH

The implant samples were separated into two groups. The samples in the first group was used to study the drug release at pH-6.0 and the samples from the second group at pH 7.4. The sample size of the release study at the designated pH values was n=3. A phosphate buffer solution of known pH value was used for the release of the drug. The release study was performed for a period of about 14 days since, this is the initial wound healing period after an implant is placed. The release was done in 2 ml Eppendorf tubes and filled with 1 ml phosphate buffer. After designated time points the complete buffer solution from the tubes was removed and replaced with fresh buffer. The samples withdrawn were then analyzed using an UV spectrophotometer at 353nm and the release curve was plotted.

### 5.5 MTT cell viability assay

The viability of the cells after incubation was checked by MTT assay. Prior to performing the assay 3 samples were prepared- first sample was the titanium dental implant, titanium dental implant with nanotubes synthesized on its surface served as another sample, and the third sample was titanium dental implant with nanotubes and coated with Chitosan-PLGA polymer. MTT assay was done according to the protocol of Agrawal et.al.[38]

The assay was performed by seeding $1x10^4$, 3T3-J2 fibroblast cells cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) combined with 10% Fetal Bovine Serum (FBS, Gibco), and 1% penicillin-streptomycin (PenStrep, Gibco) in a tissue culture flask in a $CO_2$ incubator at 37°C till a confluent growth was achieved. MTT solution (Sigma) was prepared by diluting MTT in PBS (5 mg/ml). Then the cells were seeded in a poly-L-lysine precoated 24 well culture plate by keeping the concentration of the cells same as initial and incubated for 24 hours. After incubation, 10 vol% MTT was added to each well and plate was kept for incubation for 4 hours in dark. Finally equal volume (as of the seeded media) of DMSO was added to each well and the plate was incubated for 20 minutes in dark. The absorption was checked on a microplate reader (Synergy™ H1, BioTek) at 570 nm since the cell viability is directly related to the absorption.

# 6. Results and Discussion

## 6.1 Synthesis of nanotubes on titanium dental implants by electrochemical anodization

After the anodization process, the samples were imaged by a JEOL JSM-6320F Field Emission Scanning Electron Microscope (FESEM). Images were taken at different magnifications to check for the synthesized nanotubes on the implant surfaces. Cross-sectional images were also taken to measure the length of the formed nanotubes. Analysis of the images by ImageJ software was done to measure the dimensions of the nanotubes.

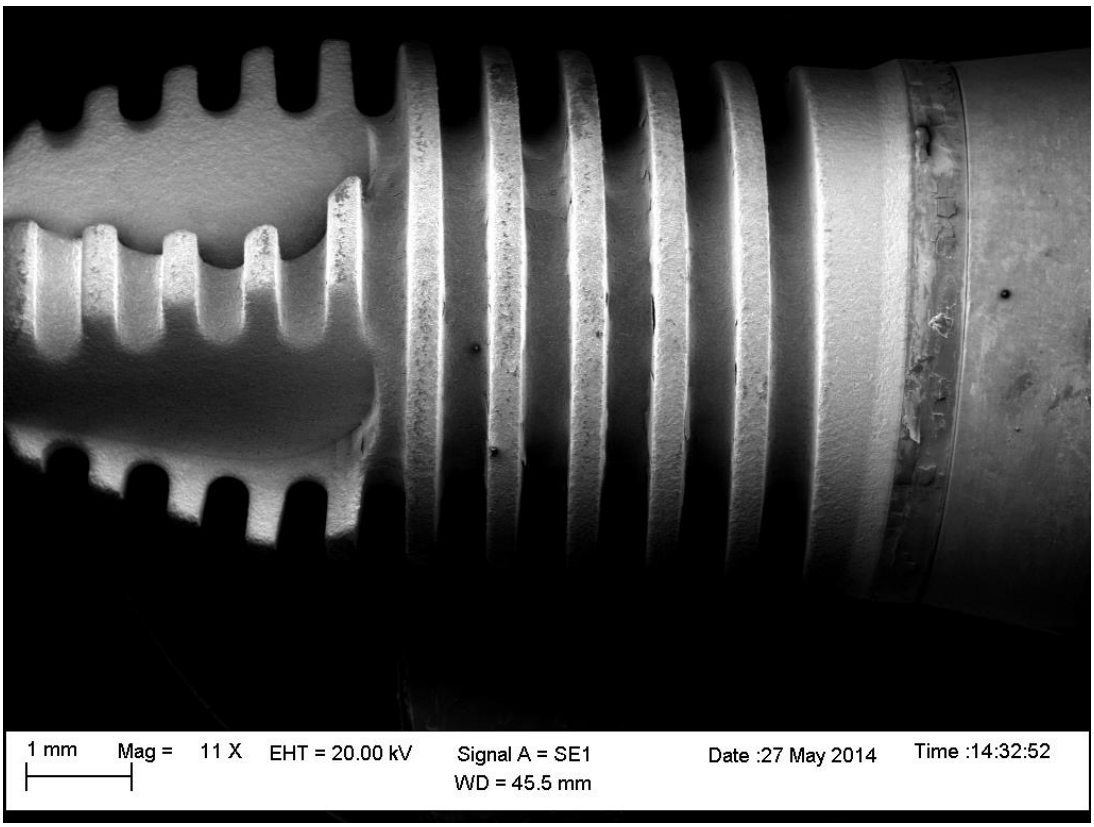


**Figure 17**- Low magnification FESEM image of a titanium dental implant

The implant samples were imaged from a top-view and also cross sectional view. The top view images showed well-aligned, circular nanotubes with diameter of 100±10 nm. (Figure 18)

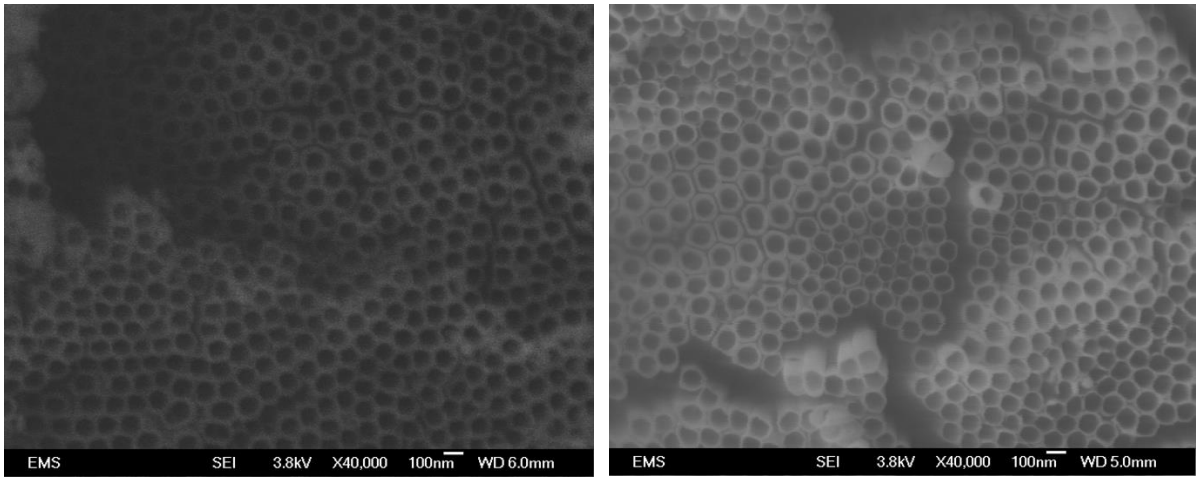


**Figure 18**- FESEM image showing the top view of the nanotubes formed on the dental implants (Scale- 100 nm)

To determine the length of the nanotubes cross-sectional FESEM images were also taken. The length of the nanotubes was found to be 10±2 µm.

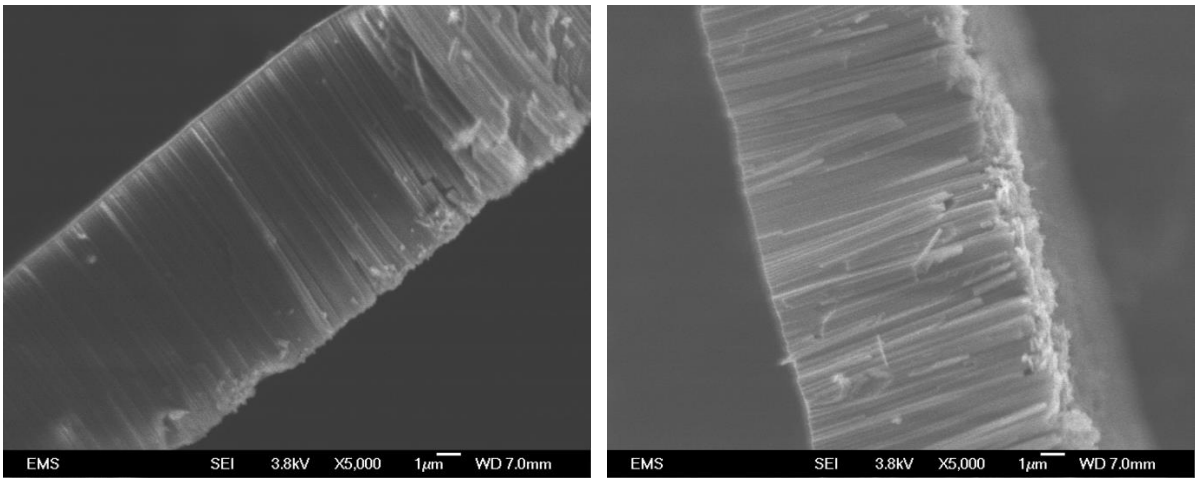


**Figure 19**- FESEM image showing the cross-section view of the nanotubes formed on the dental implants (Scale- 1 µm)

## 6.2 Doxycycline loading in the titanium nanotubes

To check for the presence of the drug in the nanotubes, the drug loaded samples were subjected to an EDS. The EDS spectrum showed a peak at 0.392 keV which is an indication of the presence of doxycycline in the nanotubes since doxycycline has nitrogen in its composition.


**Figure 20**- EDS spectrum of the drug loaded titanium nanotubes. The spectrum shows a N peak at 0.392 keV.

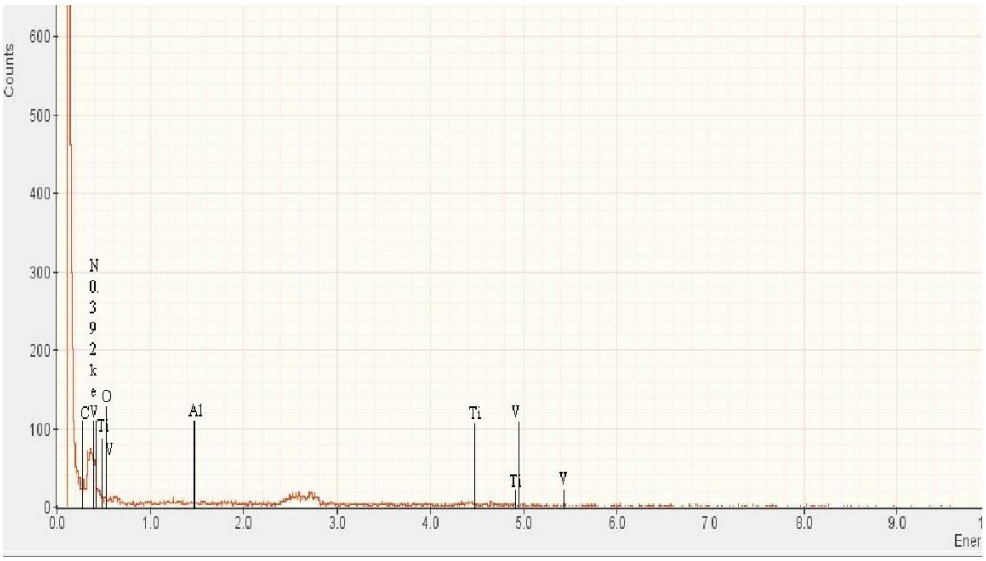


**Figure 21**- EDS spectrum showing peaks of N, C, Ti, O, V, and Al

### 6.3 Chitosan-PLGA coating on the nanotubes

The implant samples were imaged by an FESEM after the dip coating process. The samples which were dipped once showed a very thin layer of polymer coating, with the openings of the nanotubes. However, some nanotubes were covered by the polymer (shown in red circle in figure 22a below). The samples which were dipped five times showed a thick layer of polymer on their surface and the nanotubes appeared completely coated with the polymer. (Figure 22b)

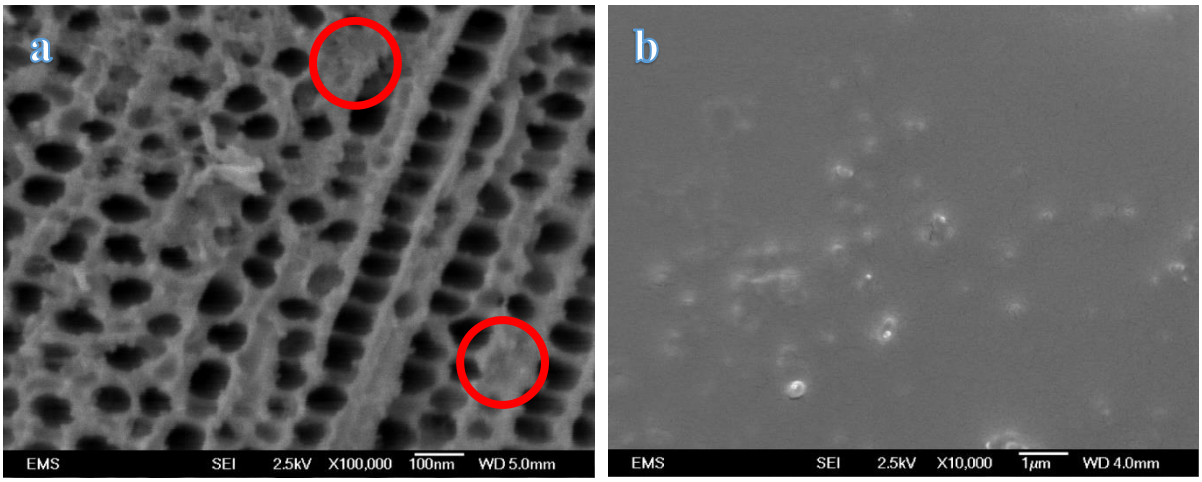


**Figure 22**- FESEM image of the dental implants after a one time (a) and five times (b) dip-coating into the Chitosan-PLGA solution. Red circles in (a) show the completely coated nanotubes

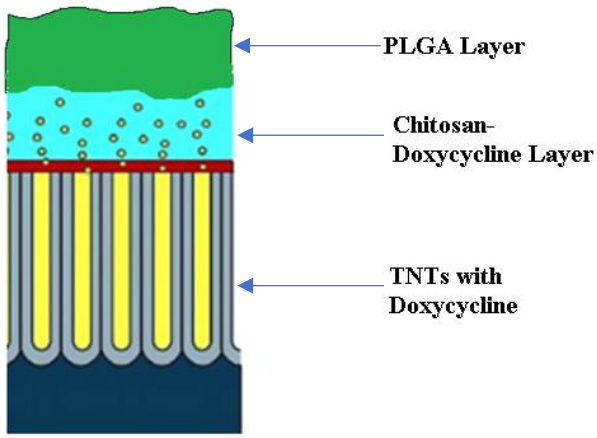


**Figure 23**- Schematic showing the cross-section of the Doxycycline filled and Chitosan-Doxycycline-PLGA coated Titanium nanotubes (Modified from Ref.37)

### 6.4 Drug release study at different pH

The drug release study was carried out at pH 6.0, and 7.4. The daily and cumulative drug release values were plotted on a graph. At all the pH values, doxycycline showed a burst release. The rapid release of doxycycline in the PBS solution can be attributed to the presence of the drug in the chitosan layer and also due to the hydrophilic nature of doxycycline. At pH 6.0 the cumulative release of the drug was 1647 µg/ml within a period of 9 days (figure 24) and at pH 7.4 the cumulative drug release value was 3574 µg/ml for a period of 15 days (figure 26).

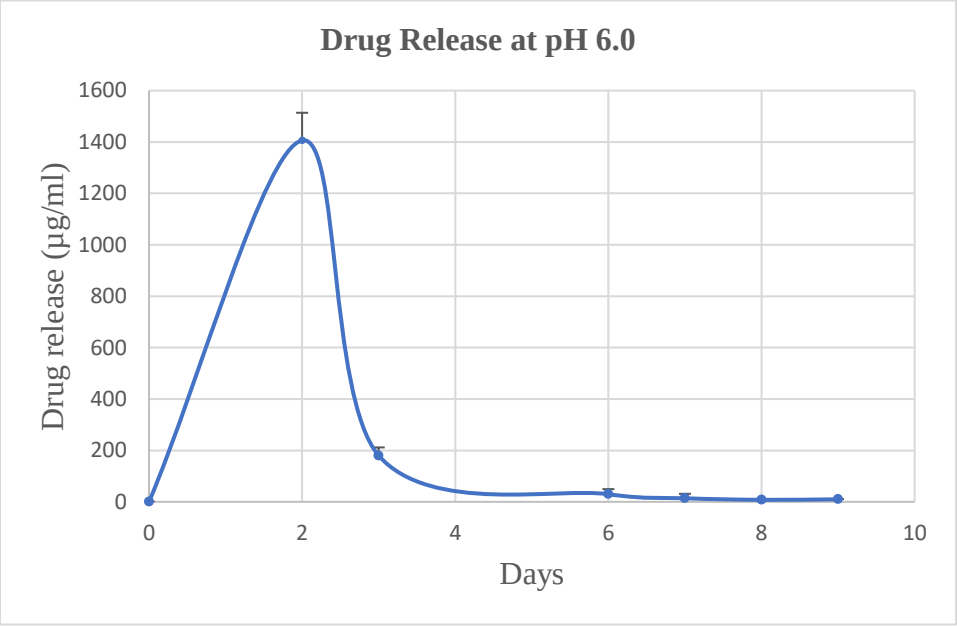


**Figure 24-** Graph showing drug release at pH 6.0

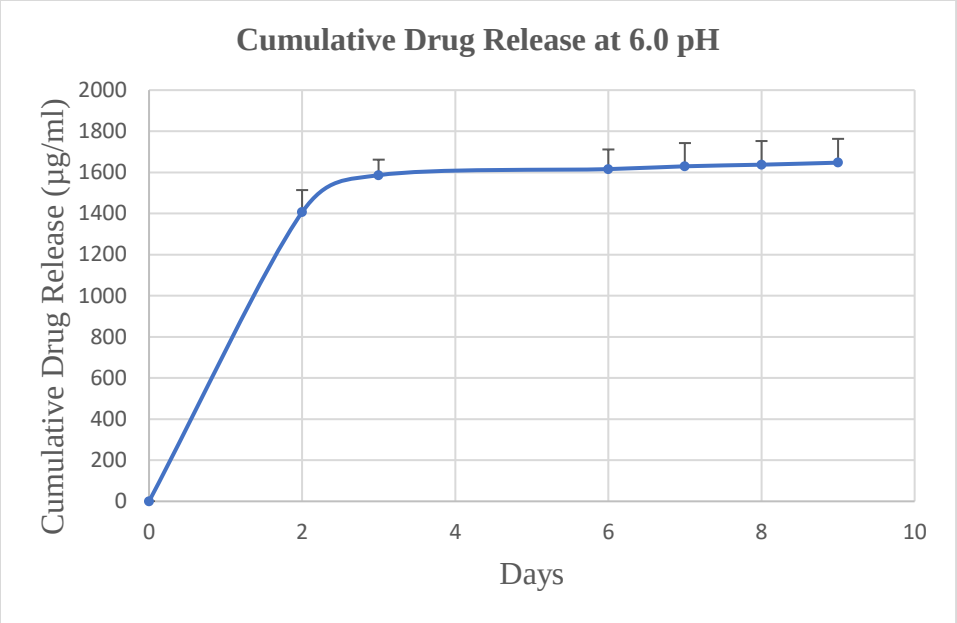


**Figure 25**- Graph showing cumulative drug released at 6.0 pH

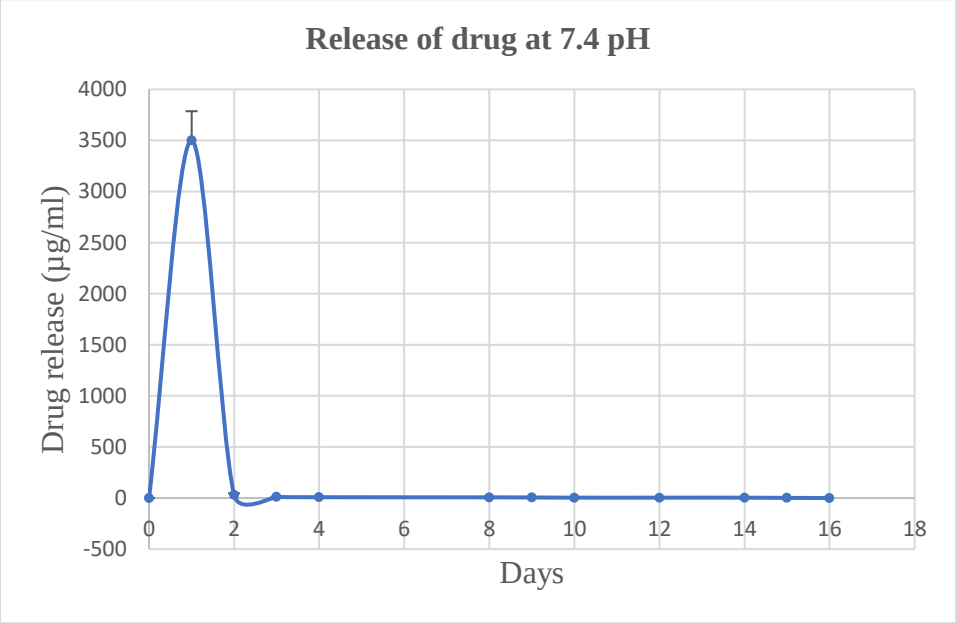


**Figure 26**- Graph of daily drug release at pH 7.4

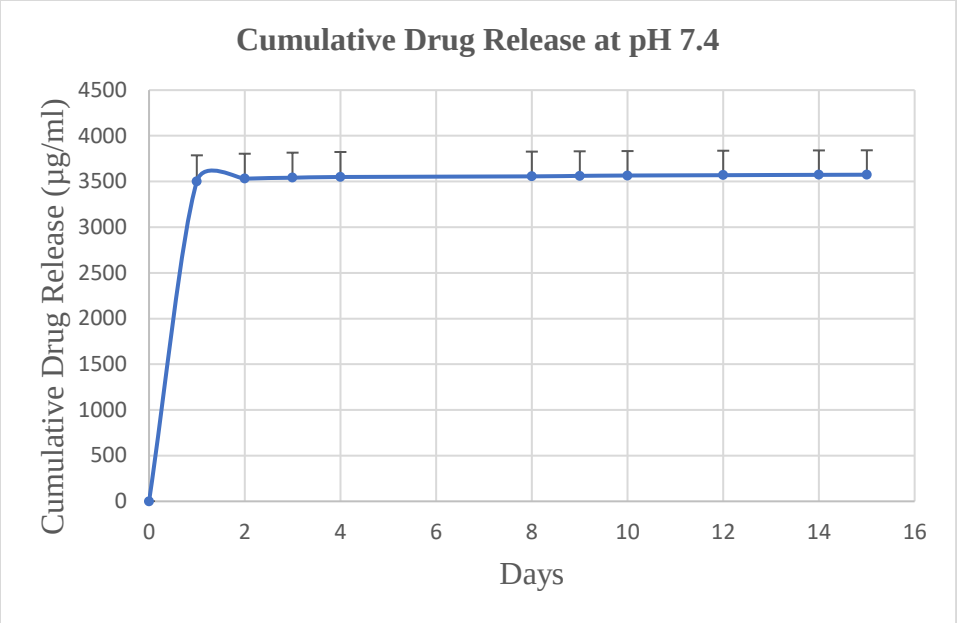


**Figure 27**- Graph of cumulative drug release at pH 7.4

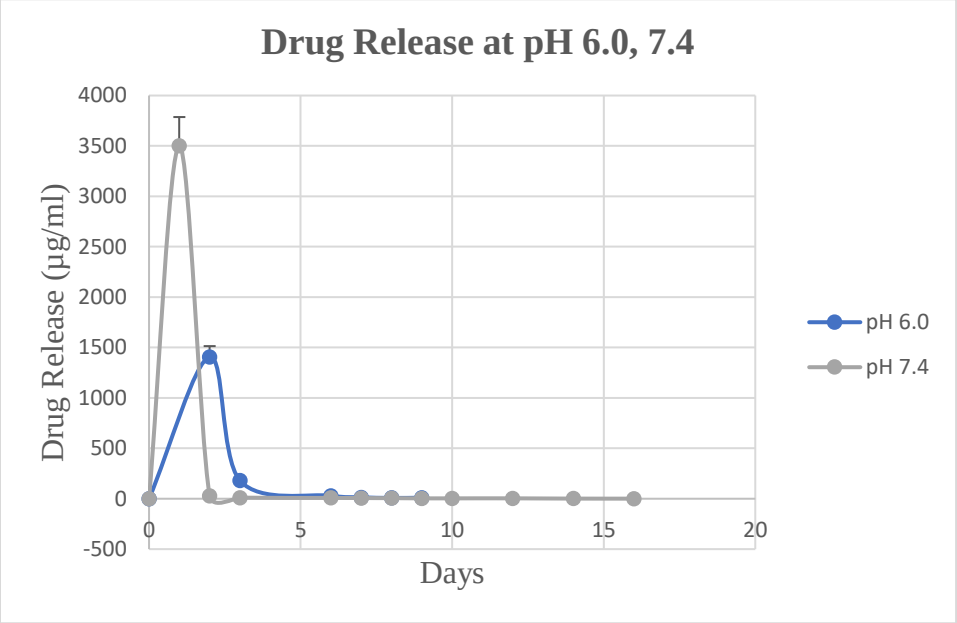


**Figure 28**- Combined graph showing the drug release at pH 6.0 and 7.4

### 6.5 MTT assay to check for the cell viability of the samples

The MTT assay results showed that the cells grown on the surface of titanium, Ti nanotubes, and Ti nanotubes coated with Chitosan-PLGA were viable after 24 hours of incubation. The MTT assay clearly shows an increase in cell viability as compared between pure titanium, Ti nanotubes, and Chitosan-PLGA coated Ti nanotubes. The highest cell viability was shown by the Chitosan-PLGA coated Ti nanotubes. This may be due to the properties of the polymers used. Chitosan is structurally similar to glycosaminoglycans, which are present in abundance in the extracellular matrix. Chitosan is also favors cell attachment, and increases the integration of the implant with the bone by preventing fibroblast growth. PLGA increases osseointegration, decreases fibroblast growth, and stabilizes the implant.[37]

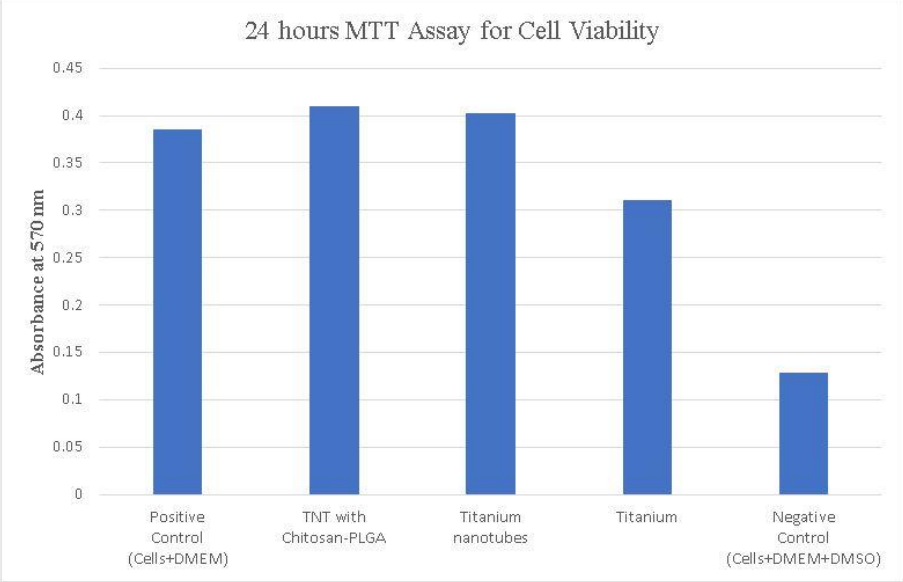


**Figure 29-** MTT assay absorbance results (Statistical analysis performed using one-way ANOVA. *P<0.05* was considered as statistically significant)

Cells viability in terms of % based on MTT absorbance results

| Sr. No. | Sample | % Cells viability |
|---|---|---|
| 1 | Titanium nanotubes coated with Chitosan-PLGA | 100% |
| 2 | Titanium nanotubes | 98.04% |
| 3 | Titanium | 75.85% |

# 7. Conclusion

The titanium nanotubes of 100 nm diameter and 10 μm length were successfully synthesized on the surface of the titanium dental implants. The dimensions of the nanotubes were confirmed by imaging under FESEM and image analysis done by ImageJ software.

Doxycycline was loaded in the titanium nanotubes which was verified by EDS spectrum which showed a N peak at 0.392 eV. The doxycycline loaded titanium nanotubes were coated with Chitosan-PLGA polymer and the drug release study was done at pH 6.0 and 7.4 in a UV-Vis spectrophotometer at 353 nm. Although there is an initial burst release of doxycycline from the nanotubes, the cumulative drug release for a period of 10 days would inhibit the bacterial growth. However, further study of polymer coating is needed to prevent the burst release of the drug.

The MTT assay results confirmed the high cell viability of the 3T3 cells where titanium nanotubes coated with Chitosan-PLGA showing highest values due to the intrinsic properties of the polymers. If the cell viability on titanium nanotubes coated with chitosan-PLGA is considered as 100% then the cell viability of titanium nanotubes and only titanium was found to be 98.04% and 75.85% respectively. This indicates that the cells are compatible with the polymer and hence can be used to coat on commercially available titanium dental implants with nanotubes.

# 8. Future Scope

The following work needs to be done in the future to enhance the performance of the implants-

1. The burst release of the drug needs to be controlled so that a sustained, long term release of doxycycline is achieved.
2. The drug release at pH 7.4 should be avoided so that it is not released under normal conditions.
3. The kinetics of the drug release is another area of study in the future.
4. Addition of growth factors like bone morphogenic protein (BMP) in the nanotubes would accelerate the healing process.
5. *In vivo* animal studies need to be carried out to determine the feasibility of the implants.

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# VITA

## Vikram Dube

### EDUCATION

**Master of Science in Bioengineering** **Aug. 2015 – May 2018**
*University of Illinois, Chicago*
GPA- 3.51

**Bachelor of Science in Applied Biotechnology** **Jun. 2006 – Jul. 2010**
*University of Pune, India*
GPA- 77% with distinction

### EXPERIENCE

**Research Assistant** **Jan. 2016** – **May 2018**
*In-situ Nanomedicine Laboratory, University of Illinois, Chicago*

- Optimized a protocol to synthesize 100 nm titanium nanotubes on titanium dental implants using a patented technology
- Loaded doxycycline drug in the titanium nanotubes
- Coated the drug loaded titanium nanotubes with pH responsive polymers for controlled drug release
- Studied the drug release from the nanotubes under pH of 6.0 and 7.4 by Ultraviolet-Visible spectroscopy
- Achieved a sustained release of doxycycline from nanotubes for 10 days
- Achieved 100% cell viability of 3T3 cells on titanium nanotubes coated with Chitosan-PLGA

**Teaching Assistant** **Feb. 2017 – May 2017**
*University of Illinois, Chicago*

- BioE 410: Regulatory affairs for medical devices- Supervised the daily class activities and evaluated students' exams

**Project Executive** **Jul. 2012 – Aug. 2013**
*Powerdeal Energy Systems, Nashik, India*

- Supervised to set up the initial infrastructure for the new biotechnology lab
- Identified and evaluated new projects for implementation and reported their technical and financial feasibility
- Interfaced with management and reported on the project progress periodically
- Supervised and planned interdepartmental project focused on production and characterization of Zeolites