

Controlled Localised Drug Delivery Using Titania Nanotubes

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AMOGH KUMAR BARANWAL

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CERTIFICATE

This is to certify that the project titled **Achieving Sustained Drug Delivery using Titania Nanotubes**, submitted by **Mr Amogh Kumar Baranwal**, to the Indian Institute of Technology, Madras, for the award of the degree of **Bachelor of Technology in Engineering Design** and **Master of Technology in Automotive Engineering**, is a *bona fide* record of the research work done by him under our supervision. The contents of this project, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Dr Tuhin Subhra Santra
Project Adviser
Assistant Professor
Department of Engineering Design
Indian Institute of Technology Madras
Chennai 600 036

Place: Chennai
Date: May 3, 2019

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ABSTRACT

KEYWORDS: Nanotubes, Drug delivery.

One of the major reasons of implant failure in patients is poor integration with the host and subsequent infections in the regions surrounding the implant. Many techniques have been tried to tackle this, such as functionalizing the implants for better biocompatibility, taking antibiotics or other drugs orally, or intravenous injection. One of the recent trends in functionalizing Ti implants has been surface modification to form nanotubes of them. These nanotubes have shown qualities highly desirable in implants, such as biocompatibility, promotion of hydroxyapatite formation and the ability to load micro-particles such as drugs, proteins, etc. onto them.

These nanotubes can then be used to control the drug release rate in the body, giving a much more sustained drug release profile in comparison to dip coating a drug on the implant's surface. Furthermore, a polymer coating can also be applied on top of the nanotubes loaded with drugs, which enables further control over the drug release kinetics and is an important step towards achieving zero order drug release. The drug loading and release kinetics is also dependent on the physical features of the nanotubes such as length, diameter, aspect ratio and the intertubular distance. Thus, it becomes important to optimize the physical morphology of the nanotubes before employing them to control the drug release kinetics.

This project involved two major phases. The first phase was the fabrication of the nanotubes and optimization of the anodization parameters to obtain nanotubes of desired morphology. Physical features of nanotubes were observed after anodization under acidic electrolytes, organic electrolytes containing HF, and organic electrolytes containing fluoride salts. Similar studies were also carried out for varying potentials and anodization time for all the electrolytes. Trends were observed with increase in length of the nanotubes with increasing time of anodization. Anodization at higher voltages led to breakdown of the nanotubes to give an oxide layer on the surface. Finally, EG + HF electrolyte was chosen for preparing the final sample due to its regional consistency in the physical morphology and sufficient length of the nanotubes for drug loading studies.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	i
ABSTRACT	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABBREVIATIONS	vii
INTRODUCTION.....	1
1.1 BIOMATERIALS	1
1.2 TITANIUM ALLOYS AS BIOMATERIALS.....	3
1.3 SURFACE NANO-FUNCTIONALIZATION OF BIOMATERIALS	4
1.4 CONTROLLED DRUG DELIVERY THROUGH SURFACE FUNCTIONALIZATION	6
LITERATURE REVIEW OF TITANIA NANOTUBES (TNTs).....	8
2.1 SYNTHESIS OF TITANIA NANOTUBES	8
2.1.1 Chemistry of the Anodization Process	11
2.1.2 Growth Mechanism of the TNTs	12
2.1.3 Nanotube interspacing and self-ordering.....	13
2.2 FACTORS AFFECTING GROWTH OF TNTs	13
2.2.1 Electrolyte pH and fluoride ion concentration.....	13
2.2.2 Anodization potential.....	14
2.2.3 Anodization time	14
2.3 LOCALIZED DRUG DELIVERY THROUGH TNTs	14
2.4 PROSPECTIVE CLINICAL APPLICATIONS OF TITANIA NANOTUBES	15
2.4.1 Bacterial infection.....	16
2.4.2 Bone-implant integration	16

2.5 AIM AND OBJECTIVES	16
EXPERIMENTAL TECHNIQUES	18
3.1 PREPARATION OF TITANIUM SAMPLES.....	18
3.2 FORMATION OF TITANIA NANOTUBES.....	18
3.3 DRUG LOADING INTO NANOTUBES.....	19
3.3.1 Loading of the Drug.....	19
3.3.2 Polymer Loading	19
3.3.3 Drug Release studies.....	20
3.4 CHARACTERIZATION TECHNIQUES	20
3.4.1 Contact Angle Measurements	20
3.4.2 Scanning Electron Microscopy	20
3.5 CELL CULTURE ON TNT SAMPLES	21
3.5.1 MTT assay for cell cytocompatibility.....	21
3.5.2 Qualitative cell viability assay	21
RESULTS AND DISCUSSION	23
4.1 ANODIZATION OF TITANIUM ALLOY SAMPLES	23
4.1.1 Effect of anodization time on TNTs fabricated in acidic ($H_2SO_4 + HF$) electrolytes.....	23
4.1.2 Effect of anodization voltage on TNTs fabricated in acidic ($H_2SO_4 + HF$) electrolytes.....	23
4.1.3 Effect of anodization time on TNTs fabricated in HF + EG electrolytes	26
4.1.4 Effect of anodization voltage on TNTs fabricated in HF + EG electrolytes	26
4.1.5 TNTs fabricated in organic electrolytes containing fluoride salt	29
4.1.6 Fabrication of double layered TNTs in organic electrolytes	31
4.2 CONTACT ANGLE MEASUREMENTS	33
4.3 CELL CULTURE STUDIES	34
4.3.1 Fluorescence imaging of cultures cells.....	34
4.3.2 MTT Assay for cell cytocompatibility	38
4.4 DRUG LOADING AND RELEASE STUDIES FROM THE TNTs	39
4.4.1 SEM Imaging of TNTs loaded with Drug	39
4.4.2 Drug Release Studies	42
CONCLUSIONS	45
REFERENCES	46

LIST OF TABLES

Table 1: Mechanical properties of Ti alloys commonly used as implants	3
Table 2: Surface modification techniques employed for Titanium and its alloys (Liu, Chu and Ding, 2004).....	5

LIST OF FIGURES

Figure 1: Requirements of orthopaedic implant materials (Long and Rack, 1998).....	1
Figure 2: The FBR Reaction (Ratner, 2015).....	2
Figure 3: Titanium as a Biomaterial (Source: TitaniumTools.com)	3

ABBREVIATIONS

TNTs	Titania Nanotubes
PLGA	Poly (lactic-co-glycolic acid)
DI	De-Ionized
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
SEM	Scanning Electron Microscopy
DMSO	Dimethyl sulfoxide
EG	Ethylene Glycol
PBS	Phosphate Buffered Saline

NOTATIONS

Symbols	Descriptions
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CHAPTER 1

INTRODUCTION

1.1 BIOMATERIALS

Biomaterials are used to support, replace or enhance a tissue or muscle's function in the body (Uludağ, 2014). Biomaterials actively participate in biological functions of the body, sustaining or improving their performance, or inducing desired regenerative traits which would have otherwise not been possible. These materials may be synthetic or natural as per requirements. Biomaterials are made from metallic, ceramic, polymer or composite components based on their desired function. Biomaterials are used in different tissues and organs of the body such as hip joint replacements, artery stents and heart valves among many others (Daniel *et al*, 2017). However, to achieve successful integration into the body, these biomaterials must mimic the tissue or muscle being replaced or enhanced. This reduces the risk of rejection by the body. Thus, biocompatibility, meaning the ability of an implant to perform its function without any inappropriate host responses, becomes a crucial requirement for biomedical implants.

REQUIREMENTS OF IMPLANTS

<u>COMPATIBILITY</u>	<u>MECHANICAL PROPERTIES</u>	<u>MANUFACTURING</u>
<ul style="list-style-type: none">• Tissue reactions• Changes in properties<ul style="list-style-type: none">• Mechanical• Physical• Chemical• Degradation leads to<ul style="list-style-type: none">• Local deleterious changes• Harmful systemic effects	<ul style="list-style-type: none">• Elasticity• Yield stress• Ductility• Toughness• Time dependent deformation• Creep• Ultimate strength• fatigue strength• Hardness• Wear resistance	<ul style="list-style-type: none">• Fabrication methods• Consistency and conformity to all requirements• Quality of raw materials• Superior techniques to obtain excellent surface finish or texture• Capability of material to get safe and efficient sterilization• Cost of product

Figure 1: Requirements of orthopaedic implant materials (Long and Rack, 1998).

Biocompatibility is affected by various factors such as toxicity of the biomaterial, resistance to degradation in vivo, design of the implant and types of reactions that occur at the biological interface formed by the implant's surface and the biological environment (Ratner, 2015). Biocompatibility can be determined by in vitro or in vivo tests, by studying the interaction of the biomaterial with the cells and fluids. Upon in vivo implantation, a mild inflammatory reaction occurs which results in formation of a thin fibrous capsule that covers the implant surface with giant cells and macrophages. These giant cells and macrophages persist for the implant's lifetime; however, they cause no adverse effects on the biological environment beyond a period of three weeks. This composite reaction of the body to implants is called the FBR and is considered a characteristic of biocompatible materials.

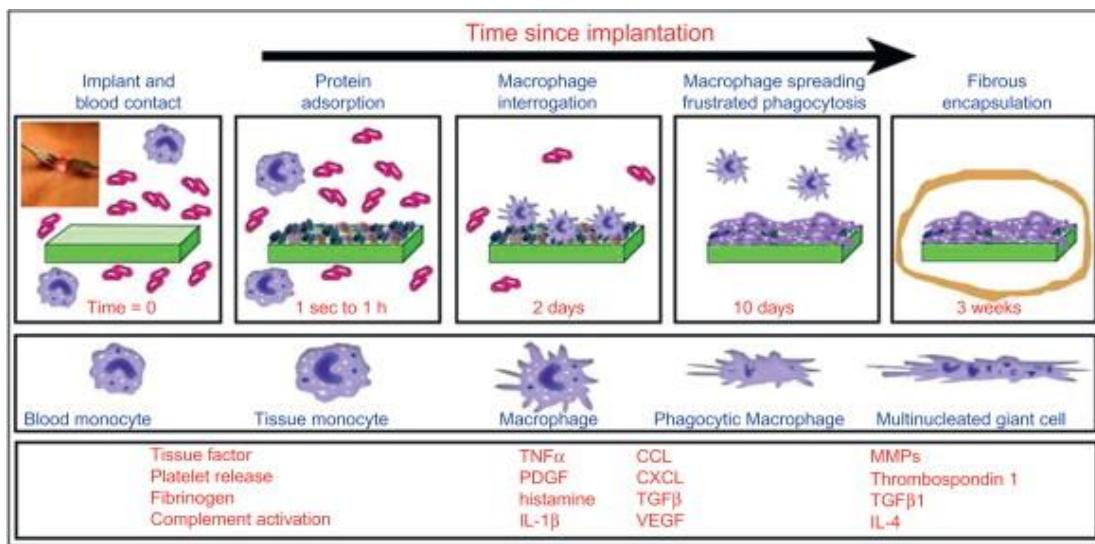


Figure 2: The FBR Reaction (Ratner, 2015).

Living cells interact with implants in many different ways (Moroni and Elisseeff, 2008; Schmalz and Galler, 2017). The biomaterial may promote cell adhesion, proliferation, differentiation or spreading, all of which are desirable traits for regenerative medicine. However, biomaterials may also cause apoptosis of cells or frustrated phagocytosis. This is harmful for the biological environment as it is followed by release of lysosomal content or proinflammatory factors such as cytokines into adjacent tissues. Thus, biocompatibility is a very important criterion for the successful implantation of biomedical devices.

1.2 TITANIUM ALLOYS AS BIOMATERIALS

The biological, physical and mechanical properties of Titanium and its alloys provide highly desirable traits in implants, such as high damage tolerance. They boast of highly sought-after properties such as lightweight, high resistance to corrosion, bio inertness and good biocompatibility, and high strength (Matković, Slokar and Matković, 2006). They have also been shown to integrate relatively easily with bone and other tissue, called osseointegration. Due to these reasons, Titanium alloys are the most commonly used biomaterials for hard tissue replacement, such as dental and orthopaedic implants (Niinomi, 2002). They also show high chemical stability due to the spontaneous formation of thin oxide layer on their surface. This oxide layer is also believed to be the main reason for good biocompatibility of Titanium alloys (Zhang *et al*, 2001).

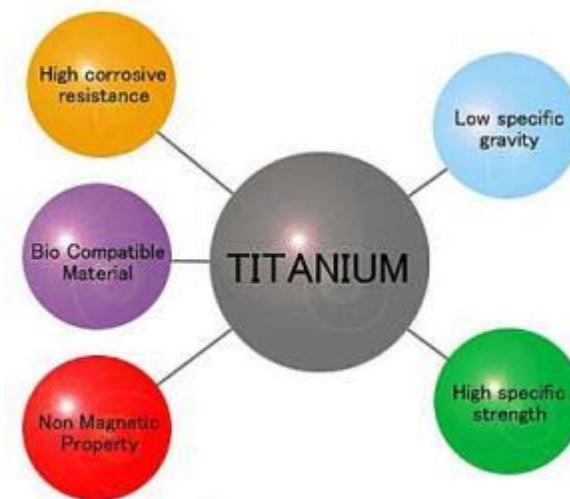


Figure 3: Titanium as a Biomaterial (Source: TitaniumTools.com)

Table 1: Mechanical properties of Ti alloys commonly used as implants

Alloy Designation	Microstructure	Elastic Modulus in GPa	Yield Strength in MPa	Ultimate Tensile Strength in MPa
Cp Ti grade I	α	102	170	240
Cp Ti grade II	α	102	275	345
Cp Ti grade III	α	102	380	450
Cp Ti grade IV	α	104	483	550
Ti-6Al-4V	α/β	110	850-900	960-970
Ti-6Al-4V ELI	α/β	113	795	860
Ti-6Al-7Nb	α/β	105	921	1024
Ti-5Al-2.5Fe	α/β	110	914	1033

Ti-12Mo-6Zr-2Fe	Metastable β	74-85	1000-1060	1060-1100
Ti-15Mo-5Zr-3Al	Metastable β Aged $\alpha + \beta$	75 88-113	870-968 1087-1284	882-975 1099-1312
Ti-Zr		N/A	N/A	900
Ti-0/20Zr-0/20Sn-4/8Nb-2/4Ta+ (Pd, N, O)	α/β	N/A	726-990	750-1200
Ti-15Zr-4Nb-2Ta-0.2Pd	α/β	94-99	693-806	715-919
Ti-13Nb-13Zr	α'/β	79	900	1030
Ti-15Mo-3Nb-0.3O (21SRx)	Metastable β + silicides	82	1020	1020
Ti-35Nb-7Zr-5Ta (TNZT)	Metastable β	55	530	590
Ti-29Nb-13Ta-4.6Zr	β	80	864	911
Ti-35Nb-7Zr-5Ta 0.4O (TNZTO)	Metastable β	66	976	1010

Titanium oxide surfaces have been shown to promote cell adhesion and chemical adsorption of proteins (Ahmed *et al*, 2012). Chemical composition and thickness play an important role in protein adsorption, which in turn is crucial for implant-tissue integration in the body. Protein adsorption is a major step in reorganization of tissue around the implant to accommodate it (Feng *et al*, 2002).

1.3 SURFACE NANO-FUNCTIONALIZATION OF BIOMATERIALS

Majority of the interactions between a biomaterial and the surrounding physiological environment are dependent on the surface properties of the biomaterial. Due to this, surface modification can play an important role in modifying the behaviour of the biomaterial in its environment and improve adaptability and performance. A biomaterial's resistance to surface corrosion or wear can be improved by changing its microstructure or composition. Surface modification allows for improving properties such as cyto-compatibility and osseointegration without affecting the mechanical strength or robustness of the biomaterial.

Titanium implants, used as bone implants, have been shown to be separated from bones after implantation by a thin mineral layer, which prevents true integration between the implant and bone. Thus, in order to overcome this limitation and promote adhesion between titanium and bones, many surface modification techniques have been employed for improving osseointegration and bioactivity of titanium.

Table 2: Surface modification techniques employed for Titanium and its alloys (Liu, Chu and Ding, 2004)

Surface modification methods	Modified Layer	Objective
Mechanical Methods: Machining Grinding Polishing Blasting	Rough or smooth surface formed by subtraction process	Produce specific surface topographies: clean and roughen surface; improve adhesion in bonding.
Chemical Methods: Chemical treatment Acidic treatment	<10 nm of surface oxide layer	Removes oxide scale and contamination
Anodic oxidation	~10 nm to 40 µm of TiO ₂ layer, adsorption and incorporated of electrolyte anions	Produces specific surface topographies; improved corrosion resistance; improves biocompatibility, bioactivity or bone conductivity
Chemical Vapour Deposition	~10 nm of TiN, TiC, TiCN, diamond and diamond like carbon thin film	Improve wear resistance, corrosion resistance and blood compatibility
Biochemical methods	Modification through silanized titania, photochemistry, self-assembled monolayers, protein-resistance,etc.	Induce specific cell and tissue response by means of surface-immobilized peptides, proteins or growth factors
Physical methods: Thermal spray Flame spray	~30 to ~200 µm of coatings,such as Titanium HA, Calcium Silicate, Al ₂ O ₃ , ZrO ₂ , TiO ₂	Improve wear resistance, corrosion resistance and biological compability

Plasma spray HVOF, DGUN		
PVD Evaporation Ion planting Sputtering	~1 µm of TiN, TiC, TiCN, diamond and diamond like carbon thin film	Improve wear resistance, corrosion resistance and blood compatibility

1.4 CONTROLLED DRUG DELIVERY THROUGH SURFACE FUNCTIONALIZATION

After successful implantation, it is desired that the body undergoes rapid healing and there are no external factors causing infections or other adverse effects due to surgery or implantation. The most common method of achieving this is through sustained release of drugs which reduce inflammation or act as anti-bacterial agents to prevent or cure infections, directly into the site of implantation (Liu *et al*, 2016). The drugs can also promote osseointegration, thus reducing the healing time and ensuring good integration between the bone and implant (Wu *et al*, 2015). This form of local drug delivery is performed by loading drugs onto the implant before the implantation process. However, very small amounts of drugs can be directly loaded (through physisorption) onto the natural surfaces of implants made of biomaterials such as Titanium and its alloys. These direct loading techniques also suffer from poor sustained drug release as physisorption allows for almost all the loaded drug to diffuse into the physiological environment very rapidly (Raval, Parikh and Engineer, 2010; Rajgor, Patel and Bhaskar, 2011). Thus, surface modification methods have been proposed to ensure sufficient drug loading onto the surface of Titanium implants followed by sustained release inside the body (Liu, Chu and Ding, 2010).

Nanotubes formed on surfaces of implants can serve as agents for loading and subsequent localized drug delivery. These nanotubes must have a good area to volume ratio to allow drug loading, and their dimensions must be controllable during fabrication. Titania Nanotubes (TNTs) are an excellent example of such nanotubes for enabling sustained localized drug delivery (Gulati *et al*, 2012). Thus, TNTs of appropriate dimensions fabricated on the surface

of titanium alloy implants can facilitate sustained and controlled drug release for better osseointegration and faster healing.

CHAPTER 2

LITERATURE REVIEW OF TITANIA NANOTUBES (TNTs)

The first self-organized nanotubes formed on Titanium substrate were reported by Zwilling *et al.* (Zwilling, Aucouturier and Darque-Ceretti, 1999). Though the overall uniformity was poor with non-homogeneity in wall thickness, the authors established that fluoride ions were the major factor for formation of these self-assembled nanotubes. They used an electrolyte containing chromic acid and hydrofluoric acid for anodization.

2.1 SYNTHESIS OF TITANIA NANOTUBES

The surface of the substrate with TNTs consists of two layers:

1. Barrier layer: The Titanium oxide formed in the initial stage of anodization is called the barrier layer. As the thickness of this layer increases, it increases resistivity of the substrate by restricting the flow of electrons and ions.
2. Porous/Tubular layer: The tubular or nanoporous structures develop in a direction perpendicular to the barrier layer and can be altered to be self-organized. In a electrolyte containing fluoride ions, the Titanium oxide formed breaks down to give TiF_6^{2-} complexes, which are stable in water. This breakdown rate is stronger at the base of the tubes, due to the presence of a stronger electric field in that region. The electric field can also weaken the bonds between Ti and O in Titanium oxide, thus creating another pathway for dissolution of the oxide layer.

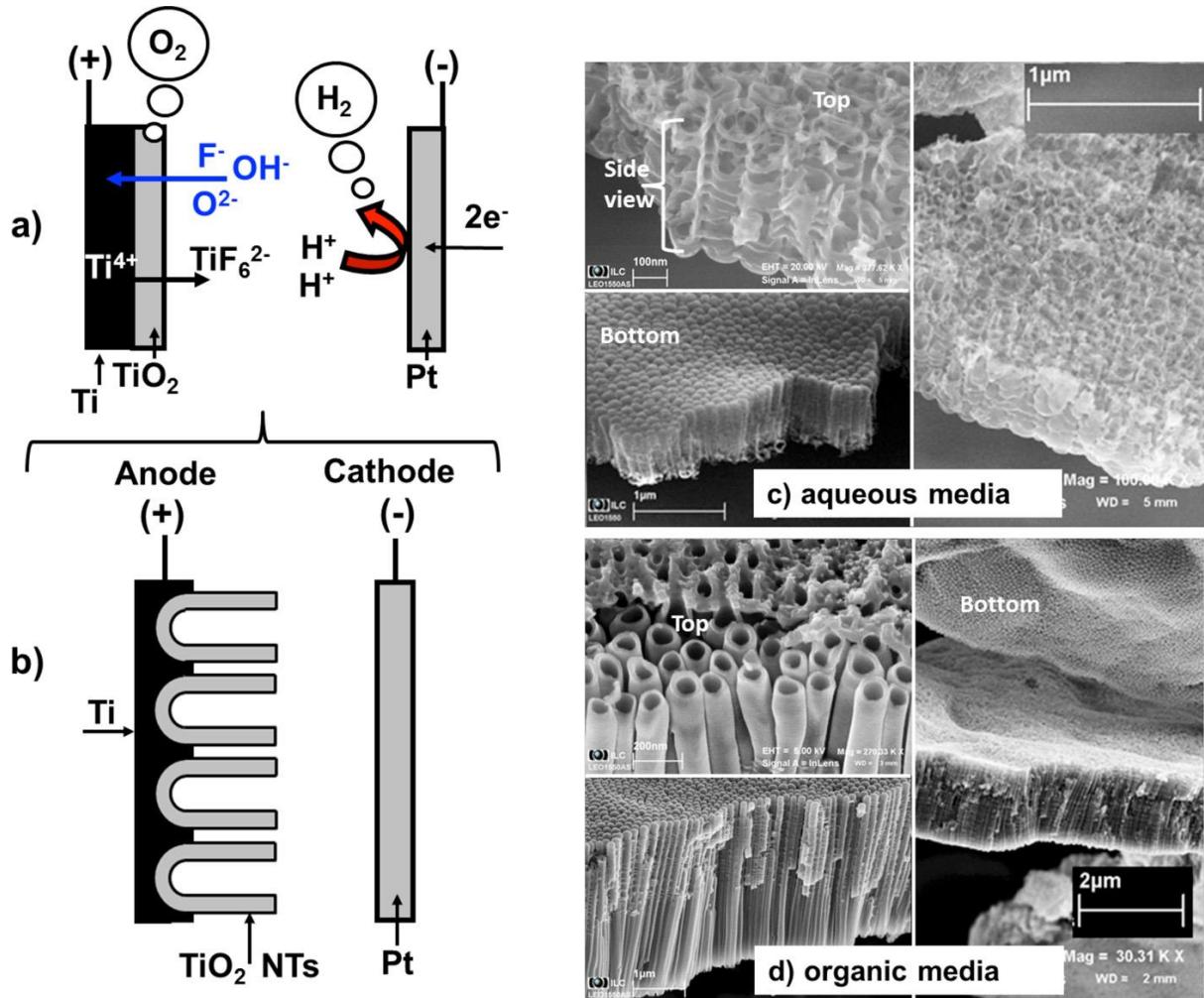


Figure 4: (a) & (b) The chemical process by which the barrier layer and porous/tubular layer consisting of the nanotubes are formed. **(c)** Samples of nanotubes fabricated in aqueous media. **(d)** Samples of nanotubes fabricated in organic media (Regonini *et al.*, 2013).

The TNTs are broadly classified into 3 generations based on their length and electrolyte used for fabrication:

1. First Generation: Hydrofluoric acid in low concentration together with another acid used as electrolyte. TNTs formed were only upto 500 μm long (Raja, Misra and Paramguru, 2005).
2. Second Generation: Water-based electrolytes containing fluoride salts used for anodization. TNTs upto 5 μm long were obtained (Macak, Sirotna and Schmuki, 2005; Macak *et al.*, 2006; Jaroenworaluck *et al.*, 2007).

3. Third Generation: Organic solvents such as ethylene glycol or glycerol containing fluoride salts and small volume of water used as electrolytes. TNTs upto 100-1000 μm in length were obtained.
4. Fourth Generation: Once again, organic solvents containing fluoride ions and small amounts of water are used as electrolytes. However, the anodization consists of multiple steps or techniques for optimizing the self-arrangement or length of the nanotubes formed. Double-layered or hexagonal nanotubes come under the fourth generation.

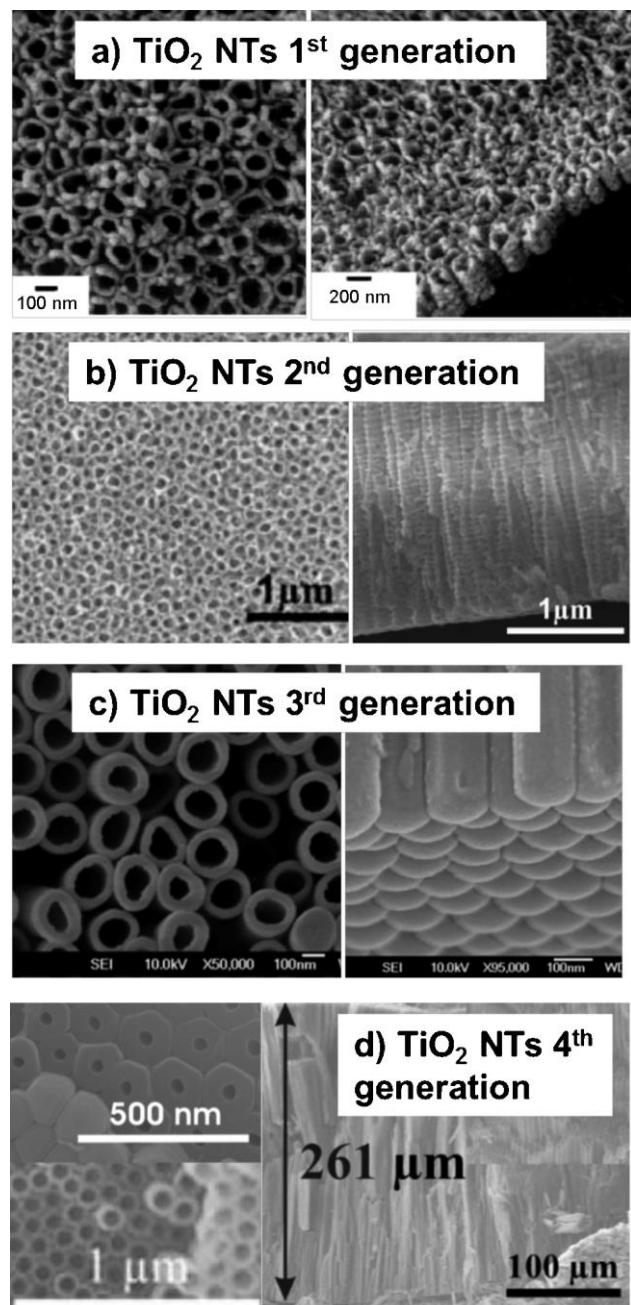
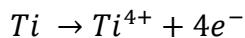


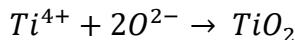
Figure 5: Examples of typical structure and morphology of different generations of Titania Nanotubes (Regonini *et al*, 2013).

2.1.1 Chemistry of the Anodization Process

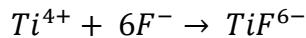
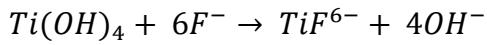
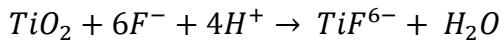
In an aqueous media, the outer layer of the anodic metal has more hydroxyl ions in the form of $\text{Ti}(\text{OH})_4$ and the inner layer has more Titanium oxide molecules. This TiO_2 exists as $\text{TiO}_2\text{-H}_2\text{O}$ hydrated molecules. There is a concentration gradient of these hydrated molecules across the anode. It is believed that on the outer layer, the Ti metal undergoes oxidation to give Ti^{4+} ions and 4 electrons per molecule.



Oxide is produced when these Ti^{2+} ions interact with hydroxyl and oxygen ions to give TiO_2 .



The fluoride ions in the electrolyte react with the hydroxyl molecules, the oxide molecules and the metal Ti too to form titanium hexafluoride complexes.



Studies have shown that the competition between the oxide formation and the etching of the oxide by the fluoride ions is the main driving force behind the formation of nanotubes.

The next major question is what drives the morphology to take the nanotubular form instead of just forming nanopores. It has been observed that nanopore formation is prominent in highly aqueous electrolytes and nanotube formation is prominent in organic electrolytes containing low volume of water (Taveira *et al*, 2005; Regonini *et al*, 2012). Thus, water content plays an important role in determining the morphology of the TNTs (Raja, Gandhi and Misra, 2007; Wei *et al*, 2010).

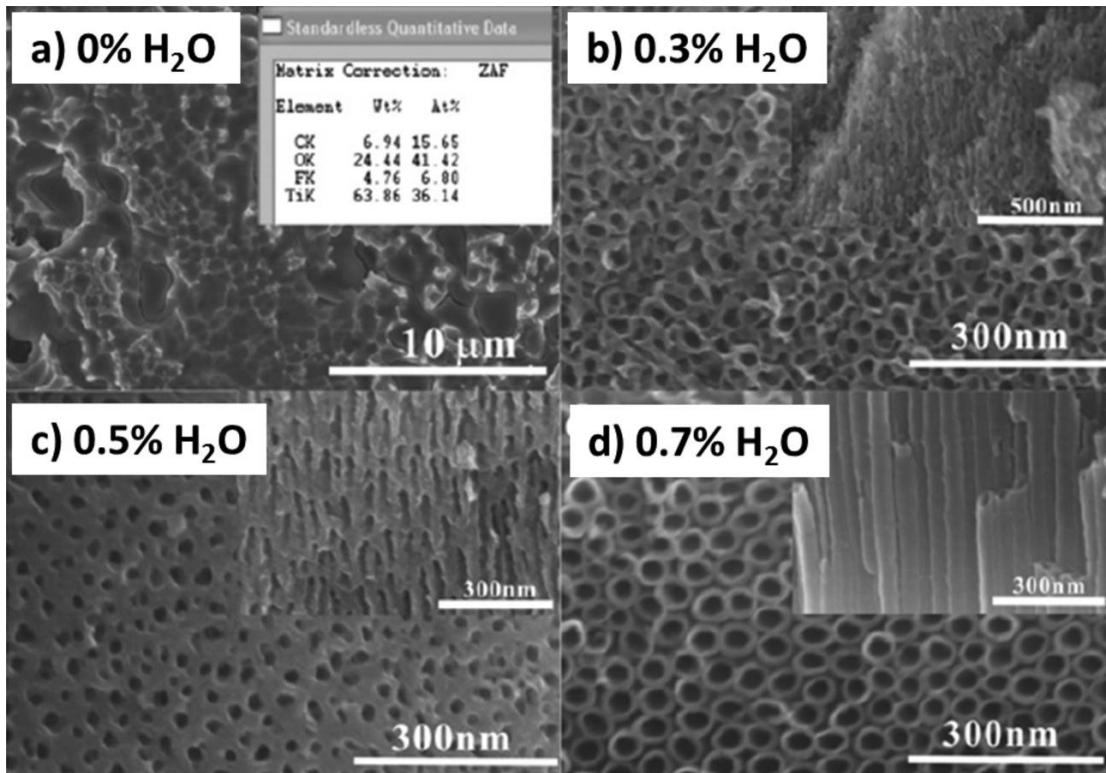


Figure 6: Images of nanopores and nanotubes formation for different volume percentages of water added to aqueous electrolytes. (a) shows patchy formation on the surface in the absence of water. (b) and (c) show formation of porous layers and slight nanotube formation. (d) shows formation of ordered nanotubes due to presence of 0.7% water in the organic electrolyte (Wei *et al*, 2010).

2.1.2 Growth Mechanism of the TNTs

As the anodizing process begins with an applied voltage, the current sharply drops to a low value. This happens due to the initial increase in thickness of the oxide layer, which is highly resistant and can grow upto 50 nm thick at 20-25V (Macak *et al*, 2006). In order to maintain the current flow from this point onwards, the fluoride or hydroxyl ions have to penetrate through this thick oxide layer and reach the metal. Due to the aggressive nature of penetration of the fluoride ions, pores are formed on the oxide layer. However, the current still keeps reducing at this stage, as the oxide formation process is still dominant in comparison to the oxide etching by the ions.

The current eventually reaches a minimum and then slowly rises again. This occurs due to the nucleation of several pores on the oxide layer, which provides lot of alternate pathways for the ions to flow into the metal. At this point, the transition from pores to nanotubes begins.

Finally, the current reaches a maximum value when there is equilibrium between the etching away of the oxide layer at the oxide/electrolyte interface and formation of new oxide at the oxide/metal interface. The fluoride ions have a greater etch rate at the base of the nanopores/nanotubes formed, due to the electric field being stronger in that region. Thus, the tubes get deeper as time progresses.

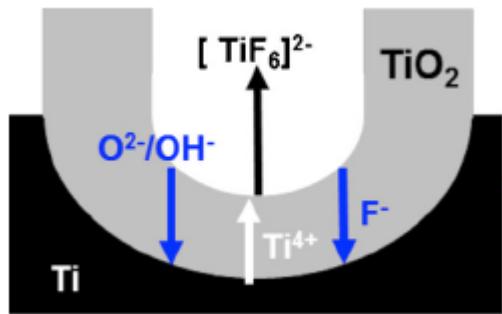


Figure 7: The movement of various ions during the anodization process (Regonini *et al*, 2013).

2.1.3 Nanotube interspacing and self-ordering

The transition from pores to nanotubes and the initial nanotubes growth process play a major role in determining the interspacing between nanotubes and their self-ordering. Ono *et al*, observed highly self-ordered nanotube arrays were formed under high current density and applied potential across the electrodes (Ono *et al*, 2004; Ono, Saito and Asoh, 2004). Yoriya *et al*. found out that the self-ordering was also dependant on the conductivity of the electrolyte.

2.2 FACTORS AFFECTING GROWTH OF TNTs

The balance between oxide formation and oxide etching by fluoride ions is very crucial to the growth of TNTs. There are several factors discussed in this section that affect the growth parameters of anodized TNTs.

2.2.1 Electrolyte pH and fluoride ion concentration

Higher oxide etch rate is observed in acidic electrolytes, which might be the primary reason for 1st generation TNTs, which used hydrofluoric acid for anodization, to be less than 500 nm long (Macak, Tsuchiya and Schmuki, 2005). The F⁻ concentration in the electrolyte must be

carefully balanced to avoid over etching and ensure enough growth of the nanotubes (Kaczmarek, Klekiel and Krasicka-Cydzik, 2010).

2.2.2 Anodization potential

A linear relation has been observed between the diameter of the TNTs formed and the potential applied (up to 60V) across the electrodes. Similarly, a linear relationship has also been observed between the thickness of the oxide layer formed at the base of the nanopores or nanotubes, and the voltage applied.

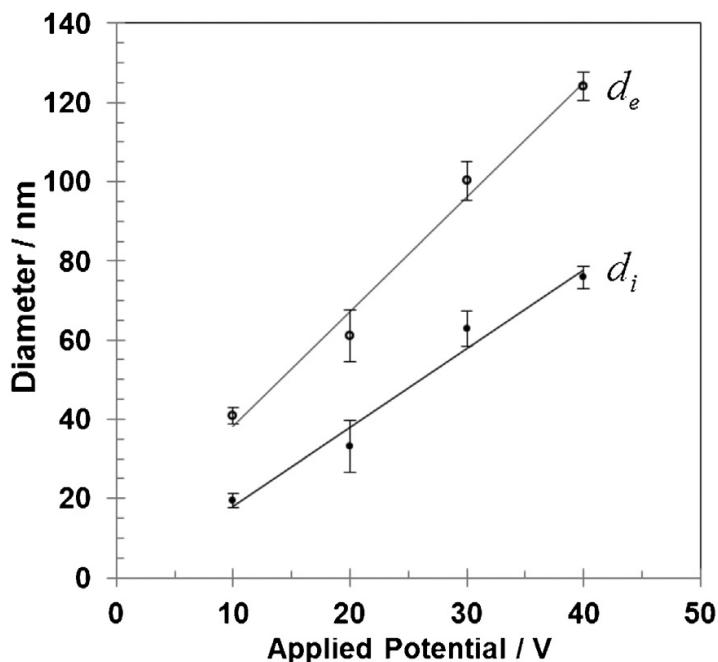


Figure 8: Graph showing linear relation between applied voltage and diameter of the nanotubes formed by anodization (Regonini *et al*, 2012).

2.2.3 Anodization time

In aqueous media, the fluoride etching is too aggressive to allow for formation of long nanotubes. Hence it is important to optimize the anodization time to obtain TNTs of desired lengths. Longer anodization times allow for longer length of nanotubes. Organic electrolytes enable formation of nanotubes up to 1000 μm in length with anodization times to several hours (Maggie Paulose *et al*, 2007).

2.3 LOCALIZED DRUG DELIVERY THROUGH TNTs

There has been lot of research on loading and release of drugs via titanium nanotubes for applications in dental implants, bone tissue engineering and orthopaedic implants. Different types of drugs such as antibacterial, anti-cancer, anti-inflammatory, antifungal or proteins can be loaded into these nanotubes for enabling sustained and controlled release. The drug loaded onto the TNTs is released via simple diffusion into the surrounding medium, if it is reasonably soluble in the medium and does not precipitate. Aw et al. (Sinn Aw, Kurian and Losic, 2014) discussed the influence of various nanotube parameters such as length, diameter, hydrophilicity and aspect ratio of the nanotubes, on the drug release kinetics of drugs loaded on TNTs. Caliskan et al. (Çalışkan *et al*, 2014) reported sustained release of the drug gentamicin from titanium nanotubes by controlling the aspect ratio of the TNTs.

Simovic et al.(Simovic, Losic and Vasilev, 2010) researched the possibility of further controlling the drug release kinetics by coating a polymer coating on top of drug-loaded TNTs to take a step closer towards achieving zero order release. Biodegradable polymers such as PLGA and chitosan, known to have impressive biocompatibility and osseointegration properties, were used as polymer coatings for drug release studies by Gulati et al. (Gulati *et al*, 2013). Thus, dip coating of such polymers on top of drug loaded TNTs can be a crucial development towards achieving zero order release from these surface modified implants.

2.4 PROSPECTIVE CLINICAL APPLICATIONS OF TITANIA NANOTUBES

Titanium implants are majorly used in orthopaedic implants as bone or joint replacements. One of the major problems with bone implants is bone infections, which can require multiple revision surgeries. Infections caused by implants and surgeries can also cause severe pain to patients and might even lead to death (Rodan and Martin, 2000). This is where surface modifications to implants such as TNTs provide a direct yet simplistic solution for these setbacks. Localized drug delivery can be used to treat inflammatory reactions, bone infections, whilst also being able to improve bone-implant integration, promoting faster regeneration of tissue around the implant and reducing chances of rejection by the host.

2.4.1 Bacterial infection

Most of the current failures of bone implants occur due to infections in the bone after the surgery. In practice, this is countered by administering antibiotics to the patients after implantation, in order to fight any bacterial infections that may develop post-operation. Researchers have studied the effects of loading various drugs such as penicillin (Yao and Webster, 2009) or silver nanoparticles (Zhao *et al*, 2011) into the TNTs with in-vitro antibacterial studies. Loading drugs onto the TNTs has shown to effectively reduce bacterial adhesion to a great extent, while also showing potential for supporting proliferation and adhesion of osteoblasts (Popat *et al*, 2007). Thus, this drug loading technique shows great future promise for bone implants.

2.4.2 Bone-implant integration

Another important reason for failure of implants in the host body is unsuccessful integration with the surrounding tissue or bone. This can often lead to immunorejection of implant by the body, or sustained inflammation, and might require revision surgeries or even complete implant replacement in some cases. However, with certain surface modifications, implants can be made bioinert or even biocompatible which enables successful integration with the host body. In case of bone implants, this involves promotion of adhesion and proliferation of bone cells such as osteoclasts and osteoblasts. Many surface roughening techniques applied to titanium implants on the nanoscale level such as machining, chemical etching and anodization have been shown to improve integration with surrounding bone and tissue (Rungsiyakull *et al*, 2010). However, nanotubes and nanopores have shown to promote osseointegration much more than other nanoscale roughness morphologies. This is because of the high rate of formation of hydroxyapatite on these nanotubes in comparison to other types of surfaces (Pittrof, Bauer and Schmuki, 2011). This hydroxyapatite then promotes cell adhesion and proliferation to an exceptional extent. This can be further improved by using a biodegradable polymer such as chitosan or PLGA, which also improve cell adhesion and proliferation to a great extent (Xin *et al*, 2009). Thus, TNTs with polymer coatings can enhance the osseointegration capability of titanium implants.

2.5 AIM AND OBJECTIVES

The broad objective of this project was to fabricate Titania Nanotubes in different kinds of electrolytes and use them as a means for obtaining sustained drug release in-vitro.

The first aim of this project was to study the influence of different anodization parameters on Titanium Alloys such as anodization time, voltage and adjust them accordingly to obtain TNTs of dimensions suitable for drug loading applications. SEM imaging was used to qualitatively judge the features of the TNTs formed in various experiments, and a plan was charted out to obtain optimum features.

After obtaining TNTs of the desired parameters, Ciprofloxacin drug was loaded onto them for studying the drug release in PBS. Polymer (PLGA) layers of different thicknesses were coated onto samples to compare the amount by which they would control the drug release kinetics and observe trends regarding the same. Finally, osseointegration of these prepared samples was tested by testing for adhesion and proliferation of MG-63 bone cancer cells on them. Fluorescence imaging and MTT assay was done for checking the cell growth and proliferation on these samples.

CHAPTER 3

EXPERIMENTAL TECHNIQUES

3.1 PREPARATION OF TITANIUM SAMPLES

95% pure CP-Ti sheets of 1mm thickness were ultrasonically cleaned with acetone before anodization. Few of the 1mm thickness sheets were etched with Kroll's reagent (91ml H₂O + 6.5ml HNO₃ + 2.5ml HF) to obtain a pure titanium surface free of impurities. The samples were cut into pieces having 30mm x 10mm cross-sectional area.

3.2 FORMATION OF TITANIA NANOTUBES

Anodization was performed by using a 30mm x 10mm x 1mm Platinum sample as the cathode and a prepared Titanium test sample as the anode. The distance between the electrodes was 2 cm. The electrolyte was continuously stirred using a magnetic stirrer at 500 RPM. Anodization was carried out at different voltages between 10-60V using DC power supply and the anodization time was varied between 0.5-8h to optimize the parameters of the TNTs formed. First, anodization was carried out to form 1st generation TNTs using an electrolyte containing 50ml of 0.08M HF and 50ml of 1M H₂SO₄. This was followed by experiments for fabrication of 2nd generation TNTs, using a combination of 50ml Ethylene Glycol and 50 ml of 0.08M HF as the electrolyte. Finally, 3rd generation TNTs were fabricated using organic electrolytes containing fluoride salts and less than 10% by volume of water.

3.3 DRUG LOADING INTO NANOTUBES

3.3.1 Loading of the Drug

Ciprofloxacin is an antibiotic used to treat a variety of bacterial infections. It belongs to the fluoroquinolone class of antibiotics (Fisher *et al*, 1989). It is commonly used to treat bone infections, joint infections and skin infections. One of the major applications of loading ciprofloxacin onto titanium implants could be to treat bone infections such as osteomyelitis. A 0.5mg/ml solution of ciprofloxacin in DI water was prepared. 100 µl of this solution was loaded onto 2cm x 2cm area samples of fabricated TNTs. Thus, 50 µg of the drug was loaded onto a 2cm x 2cm area of the TNTs substrate.

3.3.2 Polymer Loading

Poly Lactic-Co-Glycolic Acid (PLGA) is a copolymer of poly lactic acid and poly glycolic acid and is one of the best biodegradable polymers currently used for implant functionalization to improve biocompatibility. PLGA is also highly sought after for its ability to act as a controller for sustained drug delivery into the body as degradation of PLGA promotes its ability to act as a barrier for burst release of drugs. PLGA undergoes degradation via cleavage of its backbone ester linkages into oligomers and monomers (Ramchandani and Robinson, 1998; Amann *et al*, 2010). For drug loading, PLGA was dissolved in 1.5%, 0.75% and 0.375% weight/volume percentage solutions of chloroform. Chloroform being a volatile solvent, led to quick loading of a PLGA layer on top of the TNTs substrates. 50 µl of the solutions prepared were loaded on top of the respective 2cm x 2cm samples.

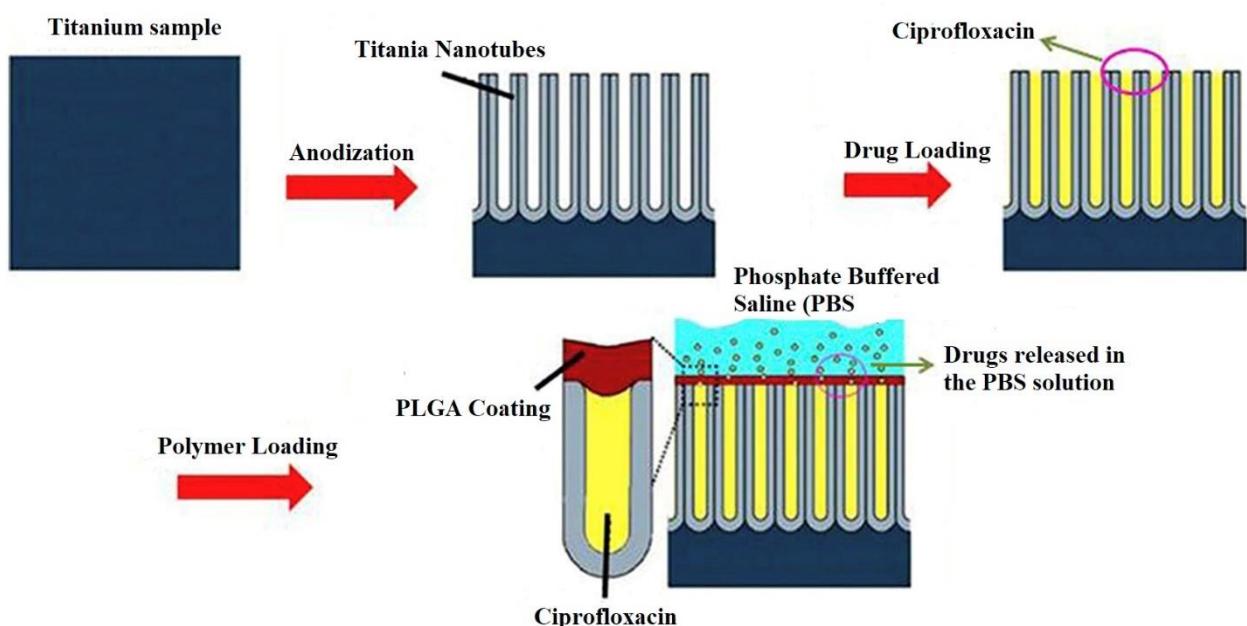


Figure 9: Schematic of the drug and polymer loading and release process into the TNTs substrate.

3.3.3 Drug Release studies

Drug release was studied by immersing the loaded TNTs samples in 50 ml PBS solutions. The amount of drug released was estimated by measuring the absorbance of the sample at 270 nm, which is the wavelength for peak absorbance of ciprofloxacin. Measurements were taken at many intervals starting from the first minute. Five readings were taken on the first day to observe the initial burst release of the drug. This was followed by readings taken every 24 hours over a period of 7 days. A standard graph for the absorbance vs concentration of ciprofloxacin in PBS was prepared and used to measure the drug concentration in the test samples after obtaining absorbance. Finally, the drug release data was represented in a graph portraying the duration vs drug release percentage. The drug release percentage was calculated by dividing the amount of drug released in sample reading by the total amount of drug loaded on the 2cm x 2cm sample, multiplied by 100 (Nagiah *et al*, 2013).

3.4 CHARACTERIZATION TECHNIQUES

3.4.1 Contact Angle Measurements

Contact angle gives a quantitative measure of the wetting of a solid by a liquid. Its value is defined by the angle a liquid drop makes with a solid at the liquid-solid-air three-way interface. If water is taken as the liquid, a low contact angle means that the surface has good wettability and is hydrophilic, whereas a high value of the contact angle means the surface is hydrophobic. The surface properties of the solid such as contact angle varies with the surface texture and roughness. Even nanoscale changes on the surface can have significant impact on the wettability of a solid. Water contact angle measurements for the TNTs samples were taken using the sessile drop method. 8 µl droplets were dropped on the samples using a syringe and 3 readings were used for each sample to obtain the average contact angle.

3.4.2 Scanning Electron Microscopy

The Quanta 200 FEG scanning electron microscope from the Sophisticated Analytical Instrument Facility, IIT Madras, was used to analyze the fabricated TNTs samples and obtain nanoscale images of the morphology.

3.5 CELL CULTURE ON TNT SAMPLES

3.5.1 MTT assay for cell cytocompatibility

MG-63 osteosarcoma cells were cultured on the fabricated TNT samples and cell viability was quantitatively determined using an MTT assay (Van Meerloo, Kaspers and Cloos, 2011).

3.5.1.1 Principle of the assay

Only live cells on the substrate can reduce yellow tetrazolium salt present in the media to insoluble purple formazan salt. The mitochondria of the cells perform this reduction. Following this, the cells are dissolved in DMSO solution and spectrometry is performed at 570 nm to detect the live cells.

3.5.1.2 Procedure

1. Added 0.5mg/ml MTT solution in a 1:10 ratio to the cell culture media volume.
2. Incubated for 4h in a CO₂ incubator at 37 °C.
3. Discarded the medium and add 100 µl of DMSO.
4. Took absorbance readings of the sample at 570 nm.
5. Used the following formula for cell viability calculation:

$$\% \text{ cell viability} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control sample}} * 100$$

3.5.2 Qualitative cell viability assay

MG-63 cells were cultured on the TNT samples and incubated for 8 hours in CO₂ incubator at 37 °C to allow for cell adhesion and proliferation. Fluorescence imaging was used to detect live and dead cells after incubation. Calcein AM dye was used to stain live cells and imaging was done with a 490 nm excitation filter and 520 nm emission filter. Propidium iodide was used to stain the dead cells, having an excitation wavelength of 493 nm and emission wavelength of 636 nm.

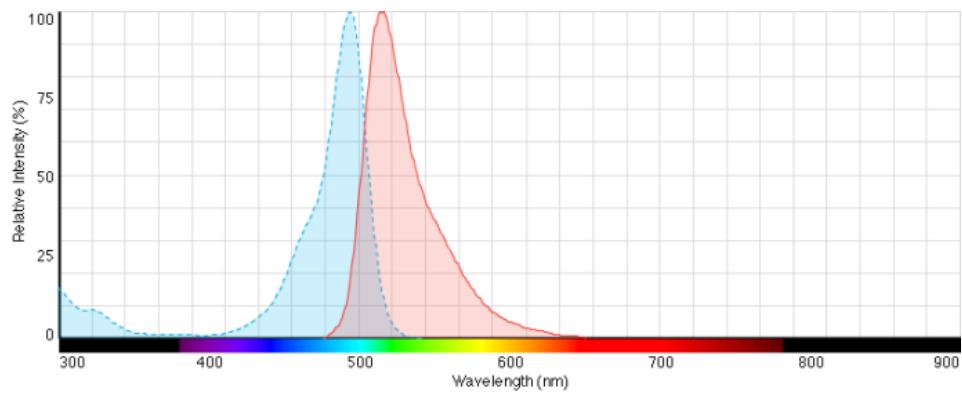


Figure 10: Excitation (blue) and emission (red) intensity for fluorescence imaging of Calcein AM stained cells. Image taken from ThermoFisher Scientific.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 ANODIZATION OF TITANIUM ALLOY SAMPLES

4.1.1 Effect of anodization time on TNTs fabricated in acidic ($H_2SO_4 + HF$) electrolytes

Titanium alloy samples were anodized for different durations ranging from 30 minutes to 8 hours under low voltages to observe changes in the physical properties of the TNTs. For samples which were anodized for 2 hours or less, clear porous TNTs could be observed. For higher duration (4h – 8h) of anodization, clear TNTs around 400nm in length were observed with SEM. However, this was only observed when the voltage was 15V or lower. For samples with higher voltage, lot of patchiness and precipitate was observed on top of the anodized substrate, with no presence of TNTs. This suggests that the acidic electrolyte is too aggressive to allow formation of long TNTs having higher diameters. It is possible that the formed nanotubes were etched out by the fluoride ions, thus leaving behind only the barrier oxide layer. Thus, acidic electrolytes only work for shorter durations and at lower voltages to give short TNTs of lesser diameters.

4.1.2 Effect of anodization voltage on TNTs fabricated in acidic ($H_2SO_4 + HF$) electrolytes

Anodization was carried out for different potentials across the electrodes ranging from 10V to 110V. For potential of 15V and below, formation of well-developed nanopores and nanotubes was observed on the samples. The exact depth and morphology of these features was dependent on the duration of anodization as well as the voltage applied.

For voltages above 15V and anodization for longer than 2 hours, 100% precipitation was observed on the surface of the Titanium alloy samples after anodization. This suggests that for higher voltages in this electrolyte, the fluoride ions etch out the oxide layer faster than new

oxide is formed. Thus, the oxide nanotubes get thinner until they completely break off, leaving behind the precipitated and patchy surface on the substrate.

For potential of 40V across the electrodes and anodization for 1 hour, few flower-like patterns were observed on the sample surface instead of any nanotubes. Similarly, for various samples, anodization at 60V for 1 hour also lead to the formation of non-uniform, sparse flower-like structures instead of nanotubes. Finally, for 110V anodization for 1-hour duration resulted in a substrate completely devoid of nanotubes. Only precipitate and a thick oxide layer was observed on the surface.

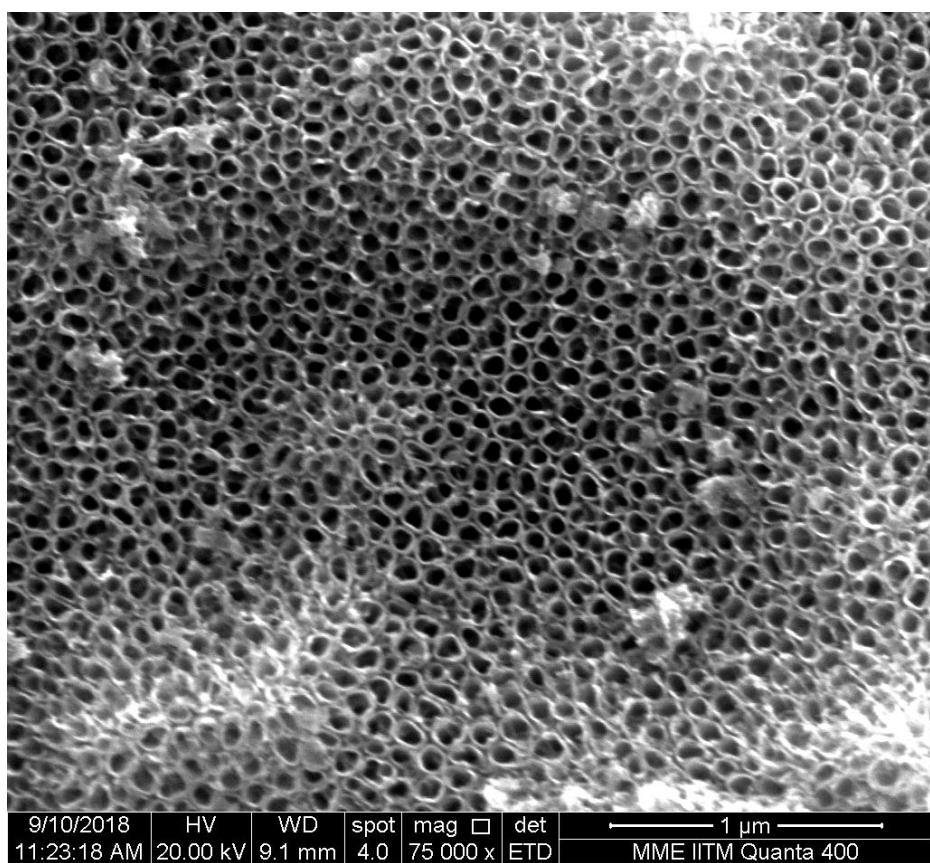


Figure 11: TNTs formed after anodization with the acidic electrolyte for 1 hour under 15V. Some precipitate showed on the surface.

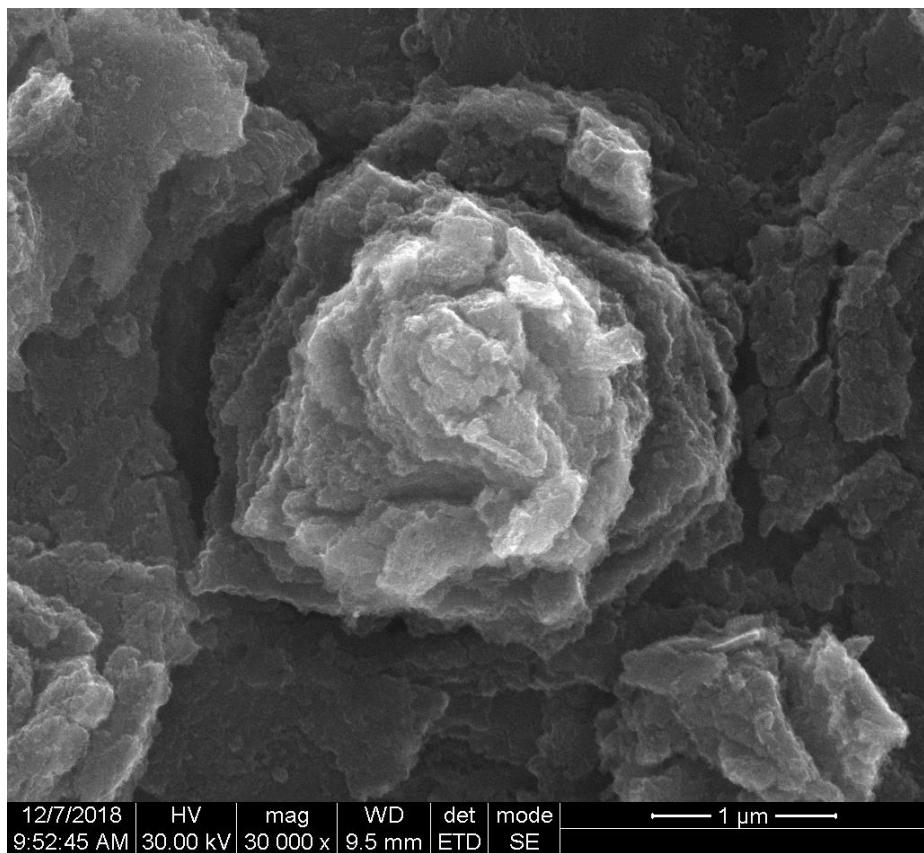


Figure 12: SEM image showing Flower-like morphology observed on the surface on anodization in acidic electrolyte at 60V for 1 hour.

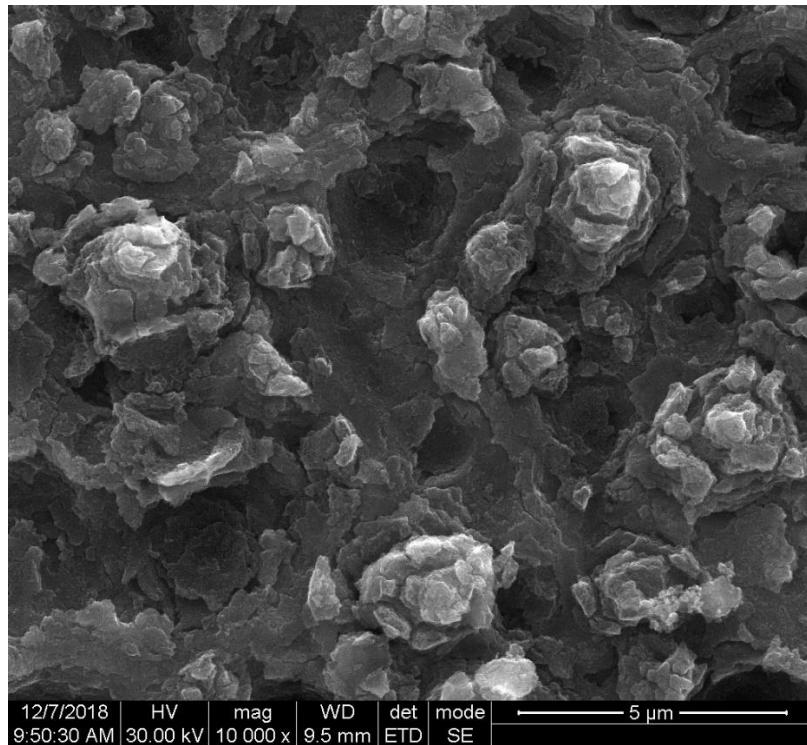


Figure 13: SEM image showing the non-uniform distribution of the flower-like structures over the sample's surface.

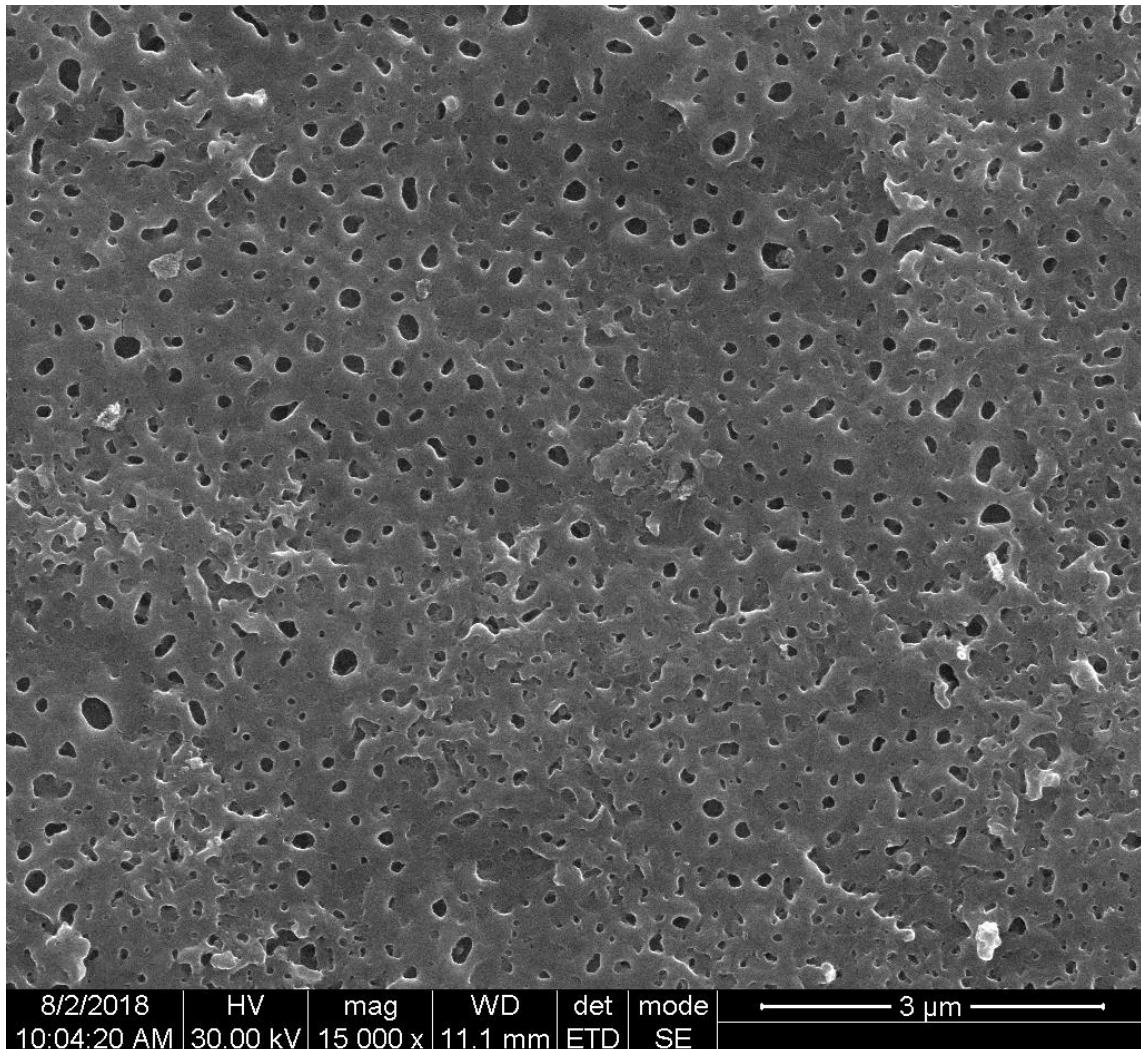


Figure 14: Sample after anodization using acidic electrolyte for 1h at 110V. No well-defined TNTs are visible.

4.1.3 Effect of anodization time on TNTs fabricated in HF + EG electrolytes

Increasing the anodization time for TNT samples anodized in electrolyte containing a combination of Ethylene Glycol and Hydrofluoric acid resulted in formation of long and slightly ordered nanotubes. The nanotubes also show less precipitate on top.

4.1.4 Effect of anodization voltage on TNTs fabricated in HF + EG electrolytes

Titania nanotubes were fabricated using this electrolyte with anodization voltage ranging from 10V to 30V. The main motivation behind this was to obtain nanotubes longer in length than the first-generation nanotubes fabricated using the acidic electrolyte.

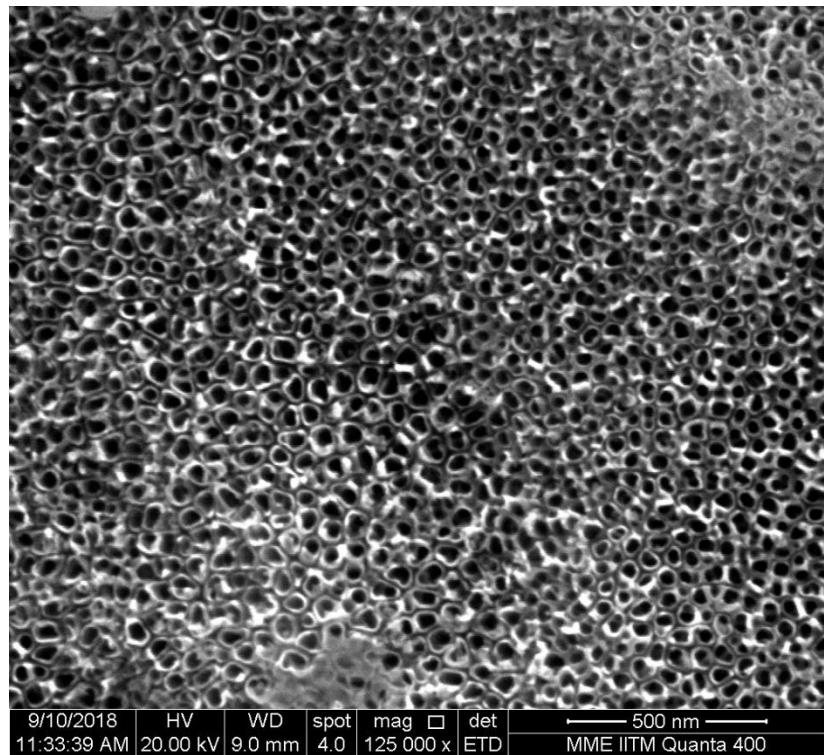


Figure 15: Clear porous TNTs formed on the surface after anodization with HF + EG electrolyte for 2 hours under 10V.

Nanotubes showing better arrangement in comparison to the ones fabricated by using the acidic electrolyte were obtained. Anodization for 8 hours resulted in formation of nanotubes 600-700 μm long, which provide enough volume on the substrate for loading microparticles such as drugs. Following this, anodization experiments were performed using organic electrolytes containing fluoride ions and small amount of water to obtain TNTs $> 1\mu\text{m}$ in length.

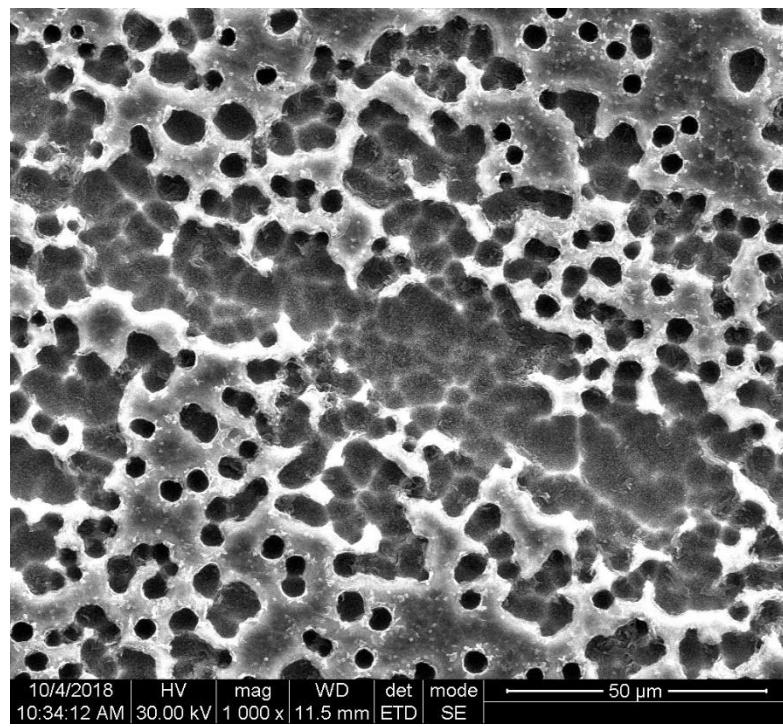


Figure 16: TNTs anodized with HF + EG for 8h under 30V showing precipitate spread over the surface.

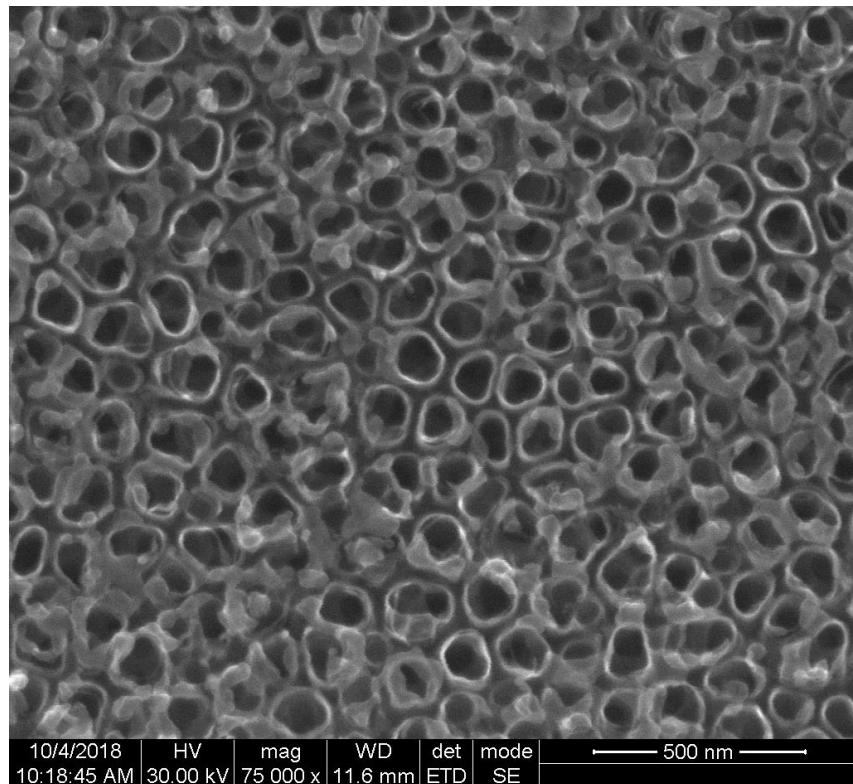


Figure 17: SEM image of TNTs fabricated using HF + EG electrolyte. Anodization time was 8h at 20V potential across the electrodes.

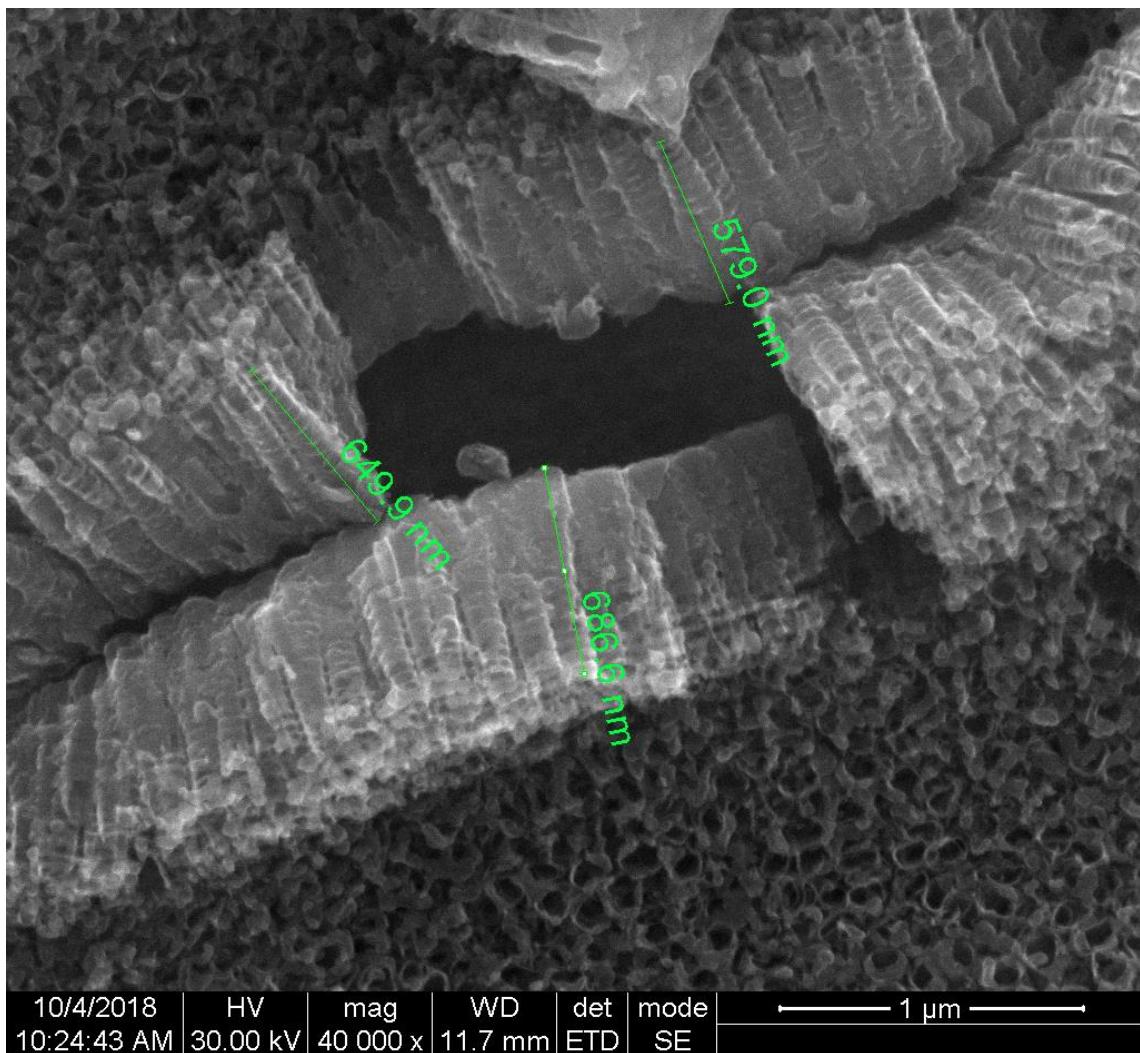


Figure 18: SEM image showing length (~650 nm) of TNTs fabricated using HF + EG electrolyte. Anodization time was 8h at 20V potential across the electrodes.

4.1.5 TNTs fabricated in organic electrolytes containing fluoride salt

Nanotubes fabricated by anodization in Ethylene Glycol containing less than 10% water by volume and 0.3% of NH₄F demonstrated lengths longer than 1μm. The nanotubes were highly self-ordered, but the surface morphology was not uniform over the entire substrate. Nanotubes upto 4μm in length were obtained by anodizing for just 2 hours. However, many of the tubes appear to have broken off into smaller tubes. There was no precipitate or oxide layer observed on the top, suggesting that the ion attack is less aggressive compared to previous cases. While these tubes could be very useful for drug loading applications because of the available loading volume, due to the non-uniformity in tube length, the previous samples would serve better for uniform drug release and loading efficiency across all samples.

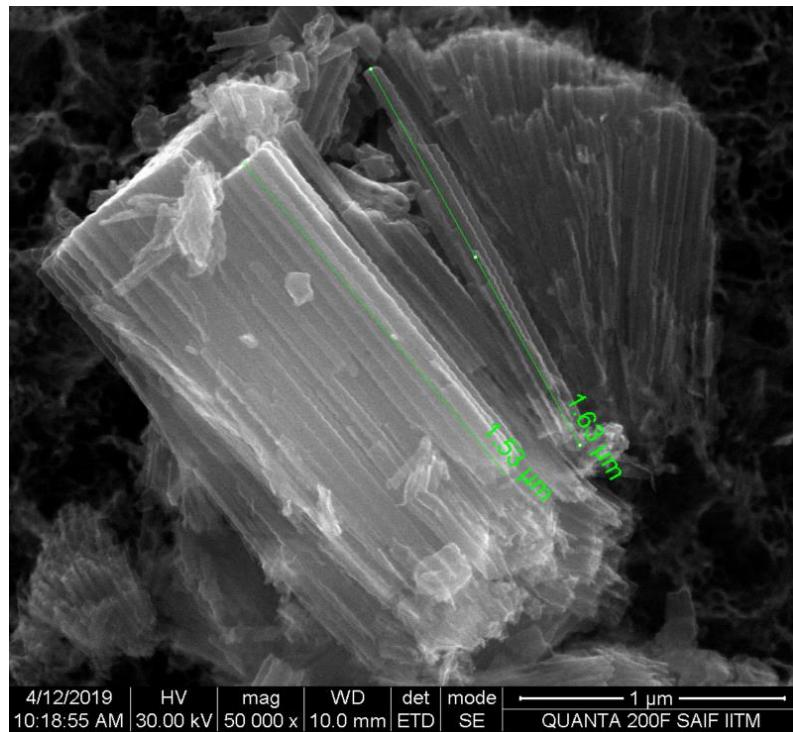


Figure 19: SEM image showing length of the nanotubes (~1.6μm) anodized in organic electrolyte containing fluoride salt for 1 hour.

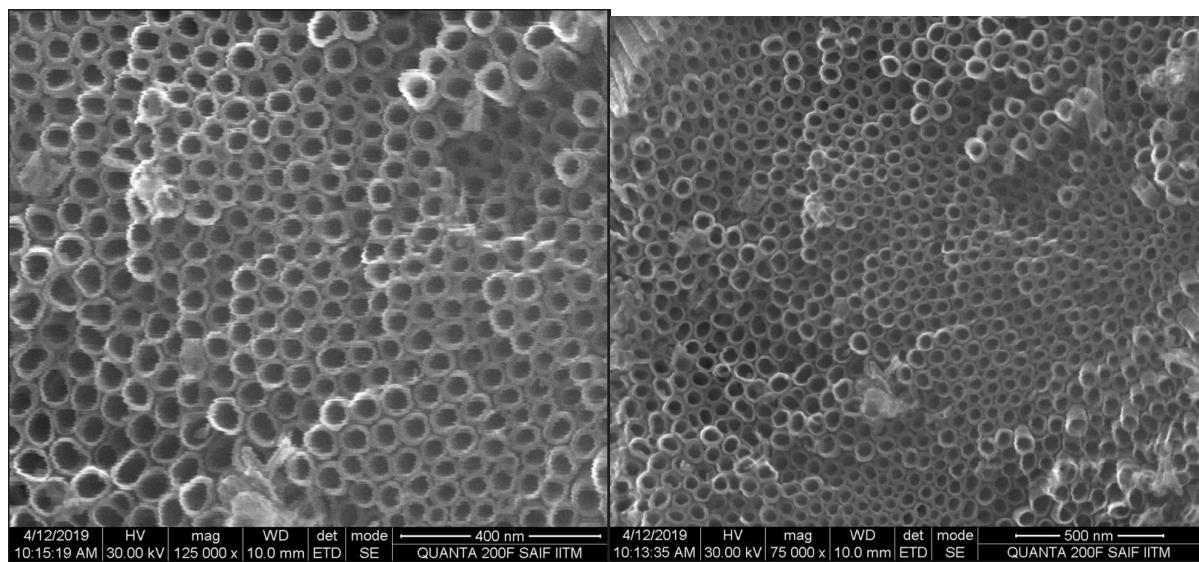


Figure 20: SEM images showing the orderly arrangement of nanotubes in one region of the sample anodized with organic electrolyte containing fluoride salt.

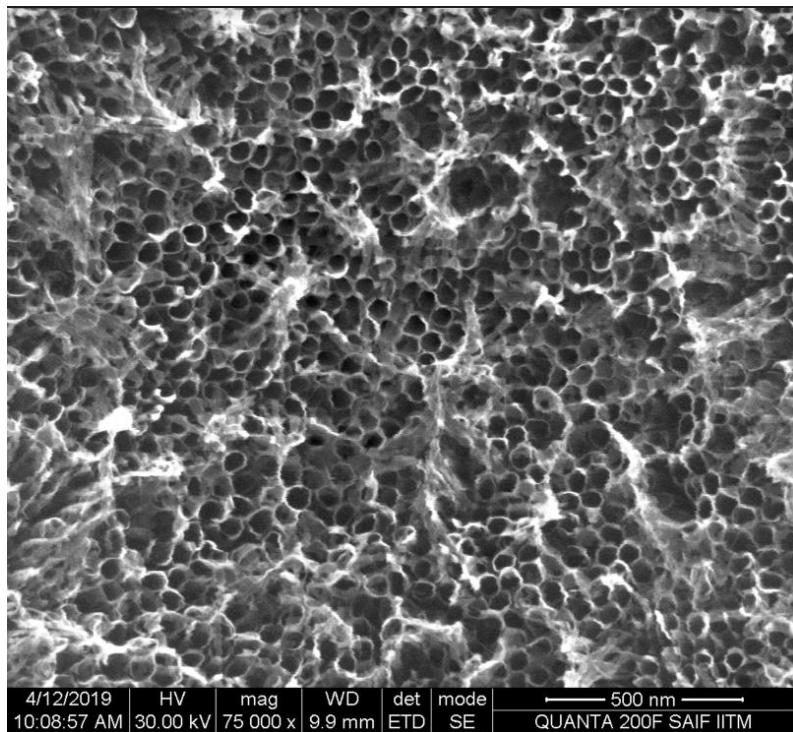


Figure 21: SEM image on another region of the same sample as above showing highly non-uniform surface with many broken nanotubes.

4.1.6 Fabrication of double layered TNTs in organic electrolytes

In order to deliver a combination of drugs, double layered TNTs could be fabricated using organic electrolytes. This was done by anodization at 20V for 80-90 minutes, followed by increase in voltage up to 60V and anodization for further 30 minutes. The bottom layer of the TNTs consisted of thicker tubes and were up to $2\mu\text{m}$ long, whereas the upper layer consisted of thin nanotubes which were up to 700nm long. Such a substrate could allow for multiple stage sustained drug release and be more efficient than single layer nanotubes in drug delivery.

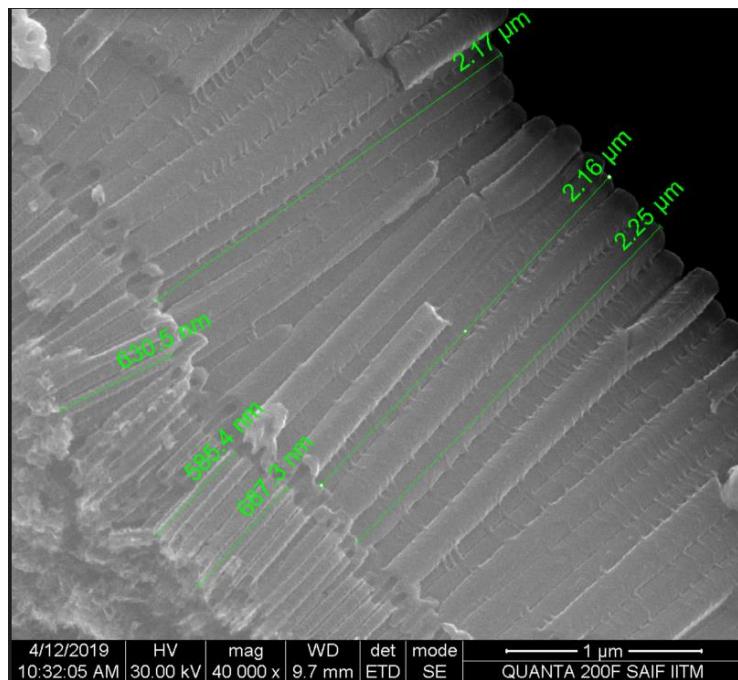


Figure 22: SEM image showing the lengths of the double layer TNTs fabricated. Top layer has thin nanotubes roughly 630 nm long and bottom layer has roughly 2μm long thicker nanotubes.

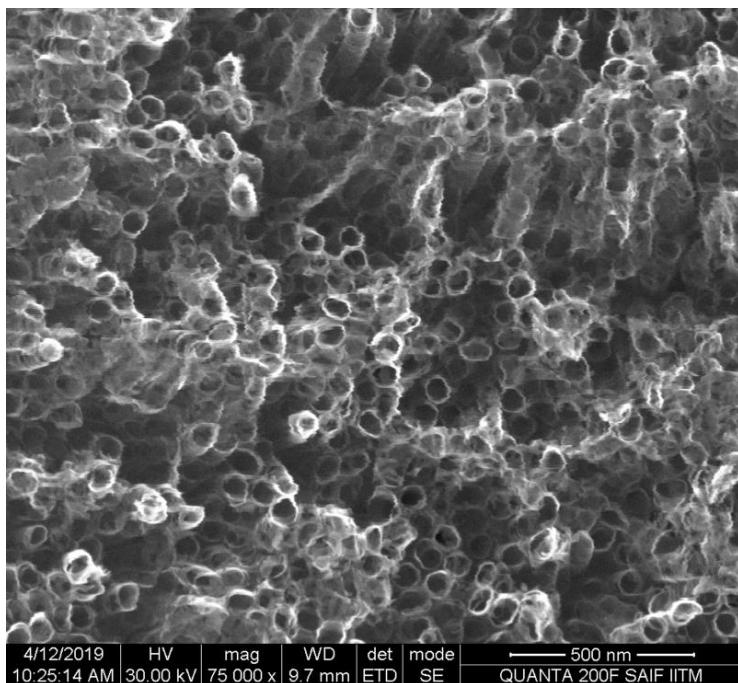


Figure 23: SEM image showing uneven and highly non-uniform surface morphology of the double layer TNTs. Most thin tubes seem to have broken off.

However, these samples also suffered from breakage of the nanotubes formed, especially on the upper layer. The tubes on the upper layer were broken off in several places on the substrate and their lengths were non-uniform. Thus, while being a future prospect, due to the current cons, the TNTs fabricated using HF + EG electrolyte combination were used for drug release studies. They offered better reproducibility, consistent morphology across the entire surface, self-ordering and sufficiently long nanotubes for drug loading applications.

4.2 CONTACT ANGLE MEASUREMENTS

Wettability of the anodized samples was measured using the contact angle measurement device. The unanodized substrate was shown to have a contact angle of 73deg. The samples showed super hydrophilicity after anodization in some cases. There was atleast a decrease of 50deg in the value of the contact angle of the substrate before and after anodization. Substrates with better wettability have shown to be able to promote cell and protein adhesion in host body. Therefore, the fabricated TNTs become hydrophilic and promote cell adhesion even further. The sample anodized with EG + HF electrolyte showed an average contact angle of 14.9deg across the anodized region. Similarly the sample anodized with EG containing fluoride salt showed a mean contact angle of 25deg.

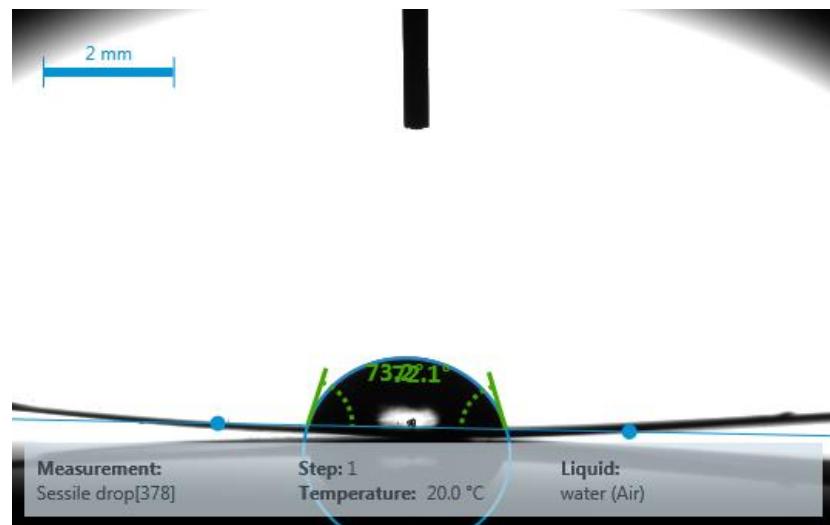


Figure 24: Contact angle of Kroll's etched, unanodized Titanium sample ~ 73deg.

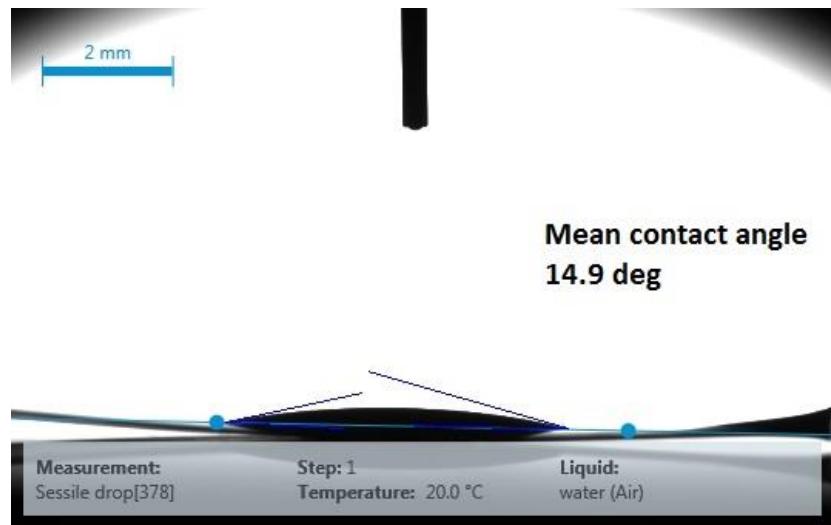


Figure 25: Contact angle of TNTs sample anodized with EG + HF electrolyte at 15V for 1.5 hours.

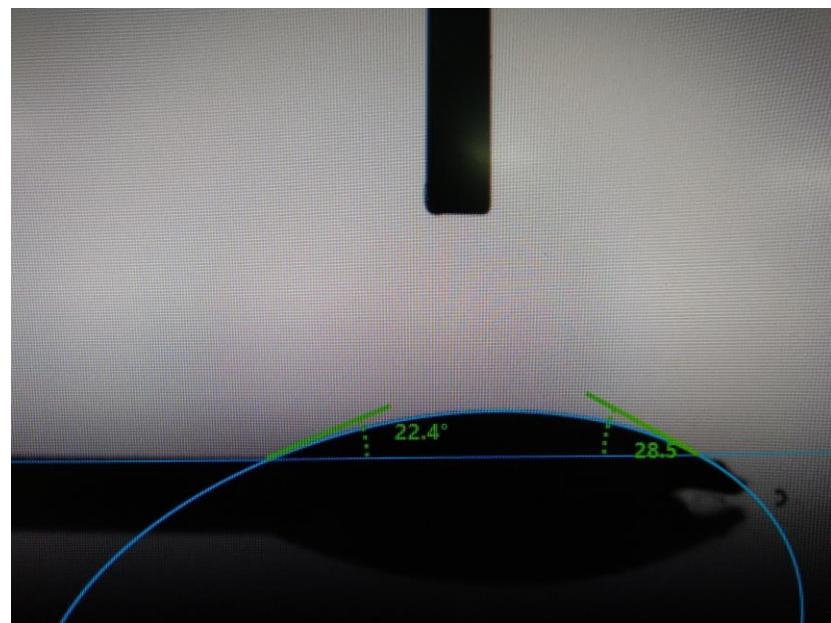


Figure 26: Contact angle of TNTs sample anodized with electrolyte containing EG (97 ml) + 3ml H₂O with 0.3 wt% NH₄F at 10V for 1 hour (Mean contact angle ~25deg).

4.3 CELL CULTURE STUDIES

4.3.1 Fluorescence imaging of cultures cells

Cell were cultured on five 1cm x 1cm samples:

1. Bare TNTs used as control.

2. TNTs loaded with ciprofloxacin and 0 w/v% PLGA.
3. TNTs loaded with ciprofloxacin and 0.375 w/v% PLGA.
4. TNTs loaded with ciprofloxacin and 0.75 w/v% PLGA.
5. TNTs loaded with ciprofloxacin and 1.5 w/v% PLGA.

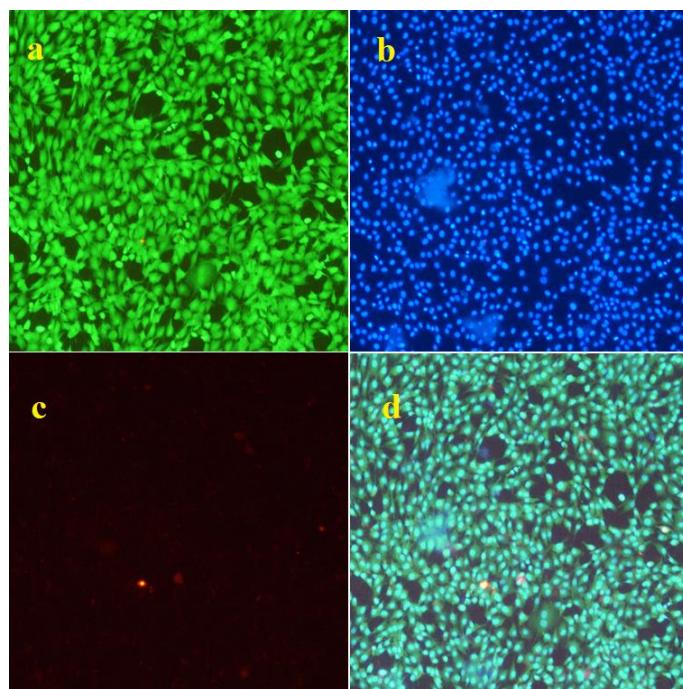


Figure 27: Fluorescence imaging of cells cultured on the control TNT sample having no drug or polymer on top. (a) Live cells (b) Total no. of cells (c) Dead cells (d) Merged image of (a), (b) & (c) for better visualization.

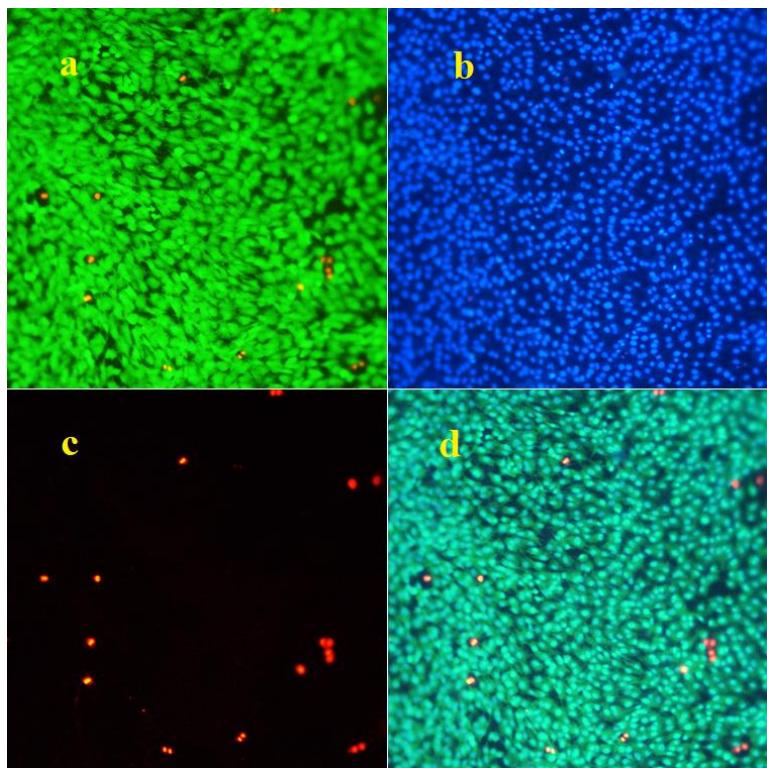


Figure 28: Fluorescence imaging of cells cultured on the TNTs sample loaded with ciprofloxacin and no PLGA coating. (a) Live & dead cells (b) Total no. of cells (c) Dead cells (d) Merged image of (a), (b) & (c) for better visualization.

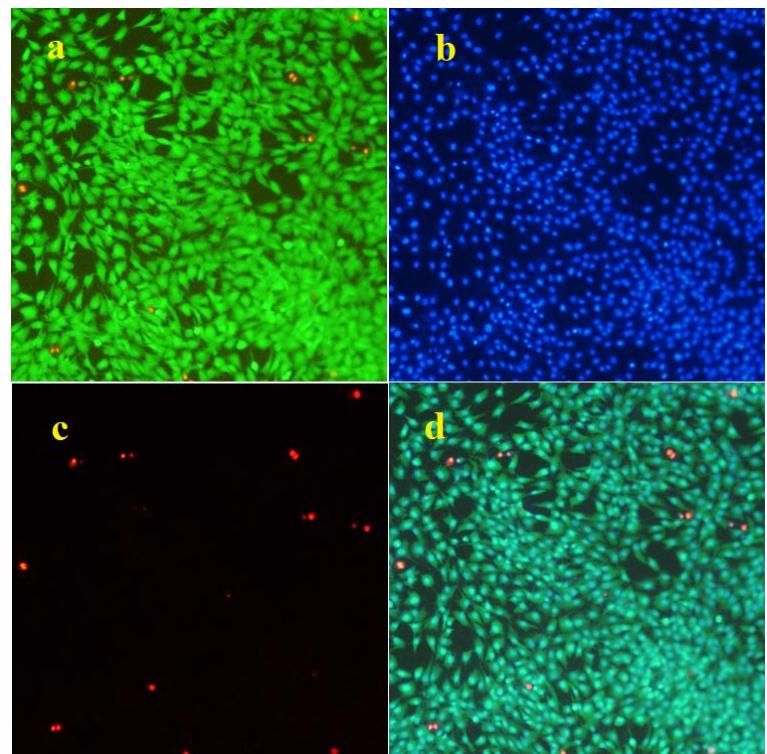


Figure 29: Fluorescence imaging of cells cultured on the TNTs sample loaded with ciprofloxacin and 0.375 w/v% PLGA. (a) Live & dead cells (b) Total no. of cells (c) Dead cells (d) Merged image of (a), (b) & (c) for better visualization.

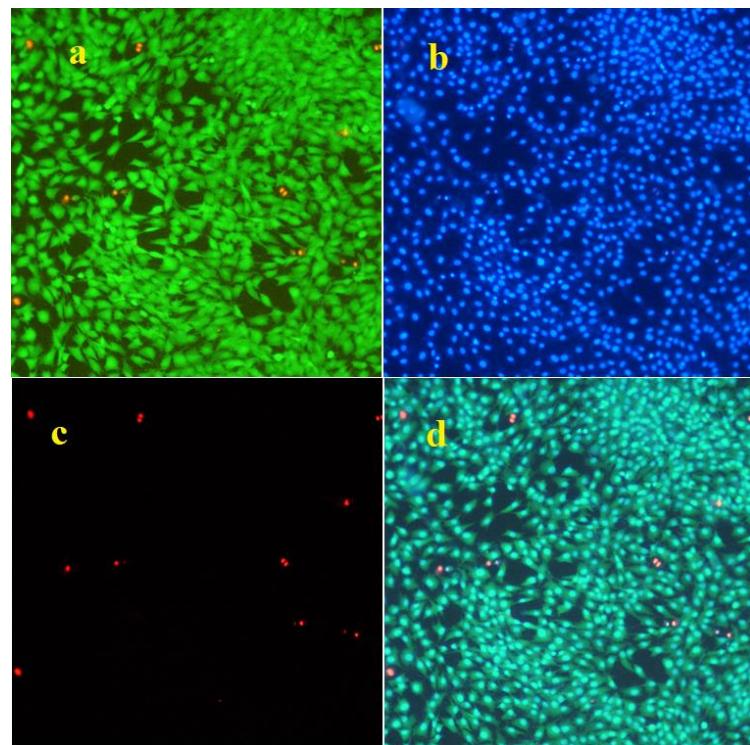


Figure 30: Fluorescence imaging of cells cultured on the TNTs sample loaded with ciprofloxacin and 0.75 w/v% PLGA. (a) Live & dead cells (b) Total no. of cells (c) Dead cells (d) Merged image of (a), (b) & (c) for better visualization.

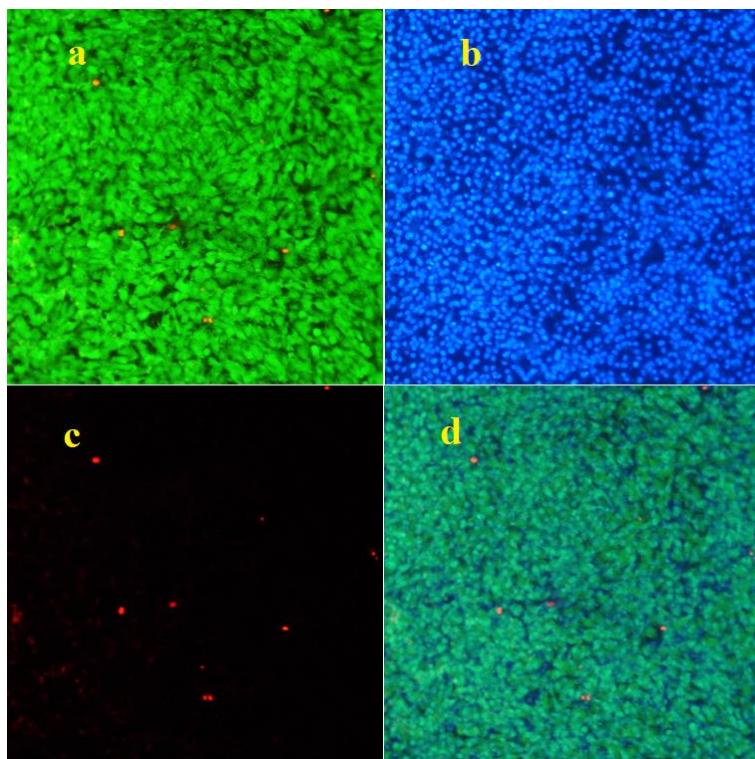


Figure 31: Fluorescence imaging of cells cultured on the TNTs sample loaded with ciprofloxacin and 1.5 w/v% PLGA. (a) Live & dead cells (b) Total no. of cells (c) Dead cells (d) Merged image of (a), (b) & (c) for better visualization.

From the above sets of images, it becomes clear that cells are able to adhere and proliferate on the TNTs without any significant loss in cell viability. There seems to be fewer dead cells in TNTs coated with higher amount of PLGA. The cells appear fully grown, healthy, flattened and in their natural triangular shape with well-defined edges. The cell proliferation seems to increase with increase in PLGA loaded onto the nanotubes. This was confirmed later with the MTT assay.

4.3.2 MTT Assay for cell cytocompatibility

The graph above shows the MTT Assay performed for comparison of cell viability in TNTs samples loaded with drug or drug and polymer against a control TNTs sample. There is a visible trend of increasing cell viability with increase in the amount of PLGA loaded on top of the nanotubes. Taking control sample as maximum cell viability and comparing other samples with it shows that the sample loaded only with drug shows least cell viability (28%) in comparison to control sample. The sample loaded with 0.375 w/v% of PLGA showed 49% cell viability in comparison to control sample. Similarly, the samples loaded with 0.75 w/v% and 1.5 w/v% PLGA showed 64% and 82% cell viability in comparison to the control sample.

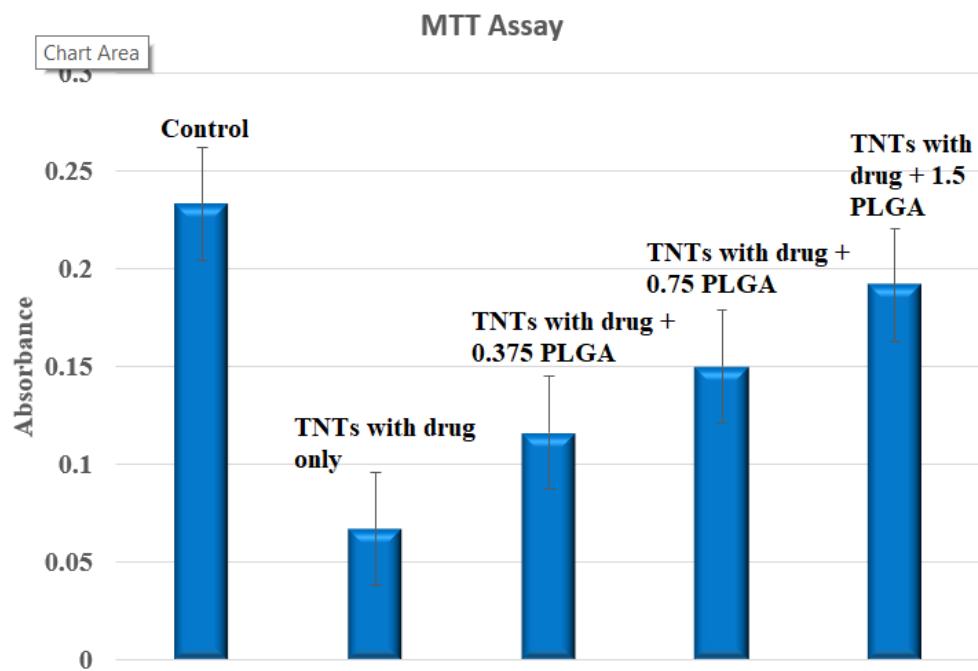


Figure 32: Graph showing absorbance values after performing MTT assay for the samples

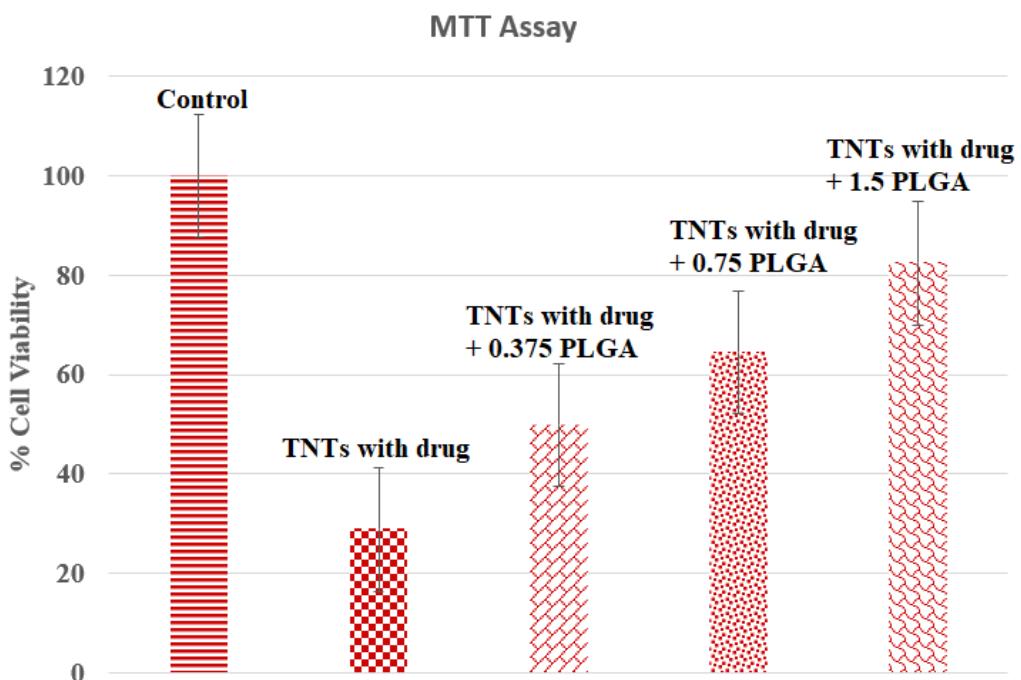


Figure 33: Graph showing the cell viability comparison obtained from absorbance values after performing MTT assay.

Thus, from both the fluorescence imaging and the MTT assay, it can be concluded that the samples show good biocompatibility promoting cell adhesion and proliferation. Another important observation is the increase in cell viability with increase in presence of PLGA on the TNTs.

4.4 DRUG LOADING AND RELEASE STUDIES FROM THE TNTs

4.4.1 SEM Imaging of TNTs loaded with Drug

It can be seen from images below that the drug and polymer has filled spaces in the inter tubular gaps as well as the nanotubes. Some places in the images appear white due to charge deposition on the non-conducting PLGA deposited on the TNTs.

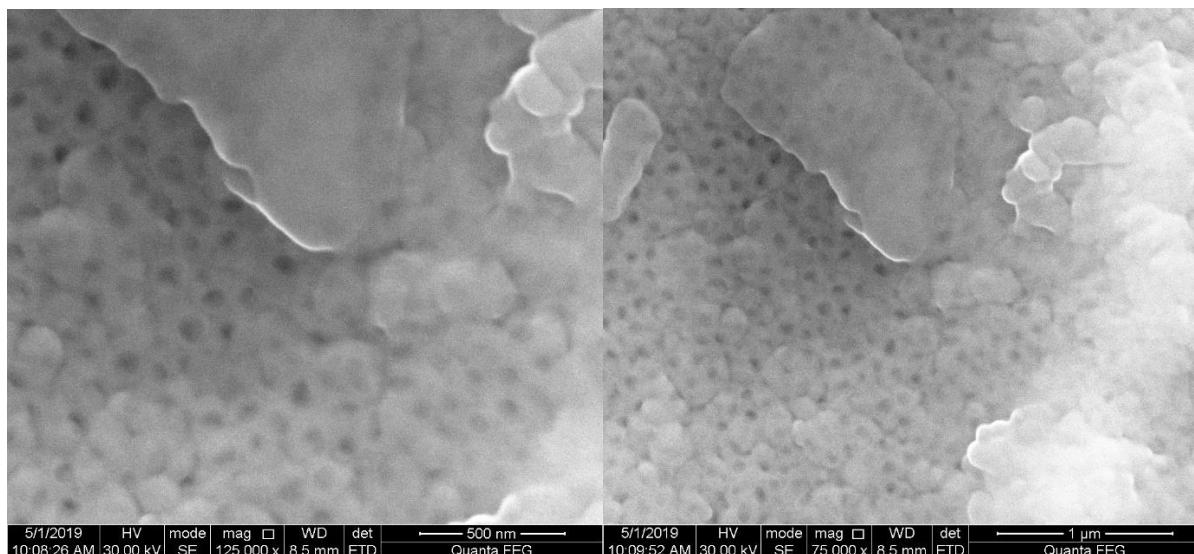


Figure 34: Images of TNTs loaded with ciprofloxacin and 1.5 w/v% PLGA showing TNTs covered by the polymer and some patches of PLGA all over the substrate.

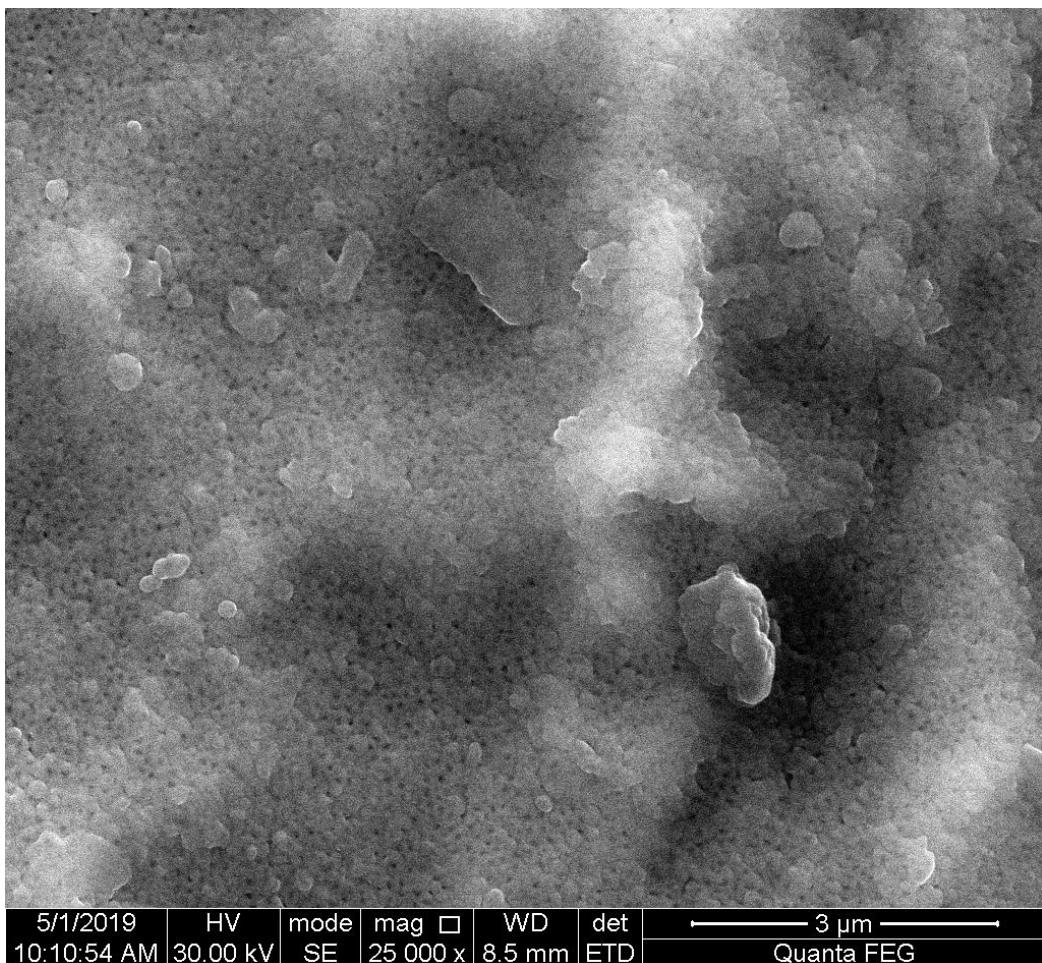
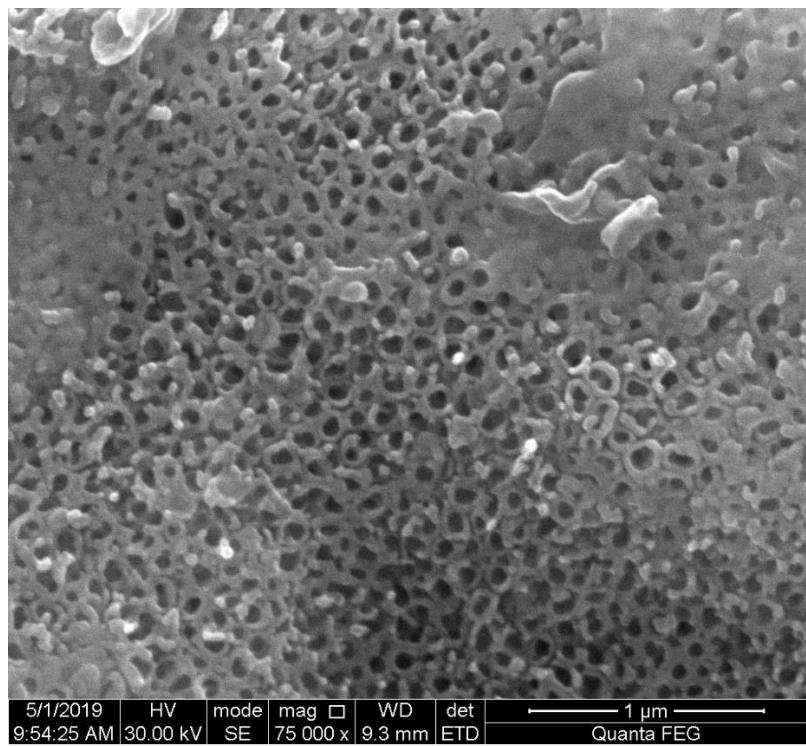
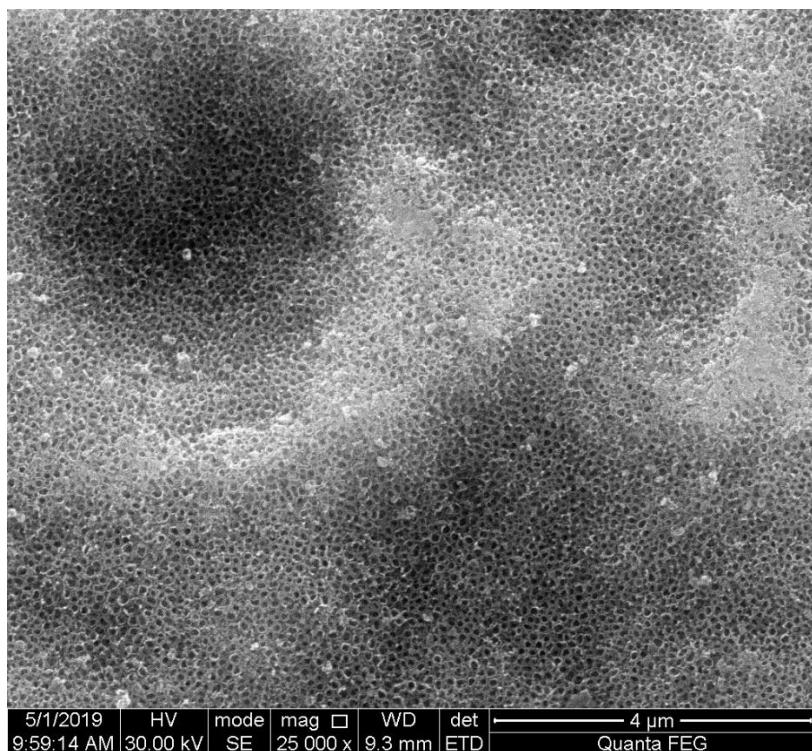


Figure 35: Image at lower magnification showing uniformity of PLGA coating over all the nanotubes with some patches of PLGA present in certain regions.



**Figure 36: Image showing the sample with only ciprofloxacin loaded onto the TNTs.
Many uncovered tubes can be observed here.**



**Figure 37: Image of the sample loaded with only ciprofloxacin at a lower magnification
showing clear TNTs with small, rare patches.**

4.4.2 Drug Release Studies

Drug release profile was developed for 192 hours of release for all the different drug + polymer loading combinations. The release shows two phases: 1. Burst release and 2. Sustained release. Burst release is the immediate release of the ciprofloxacin from the sample in the first 6 hours of study. This is followed by slow, continuous release over the next 186 hours of study.

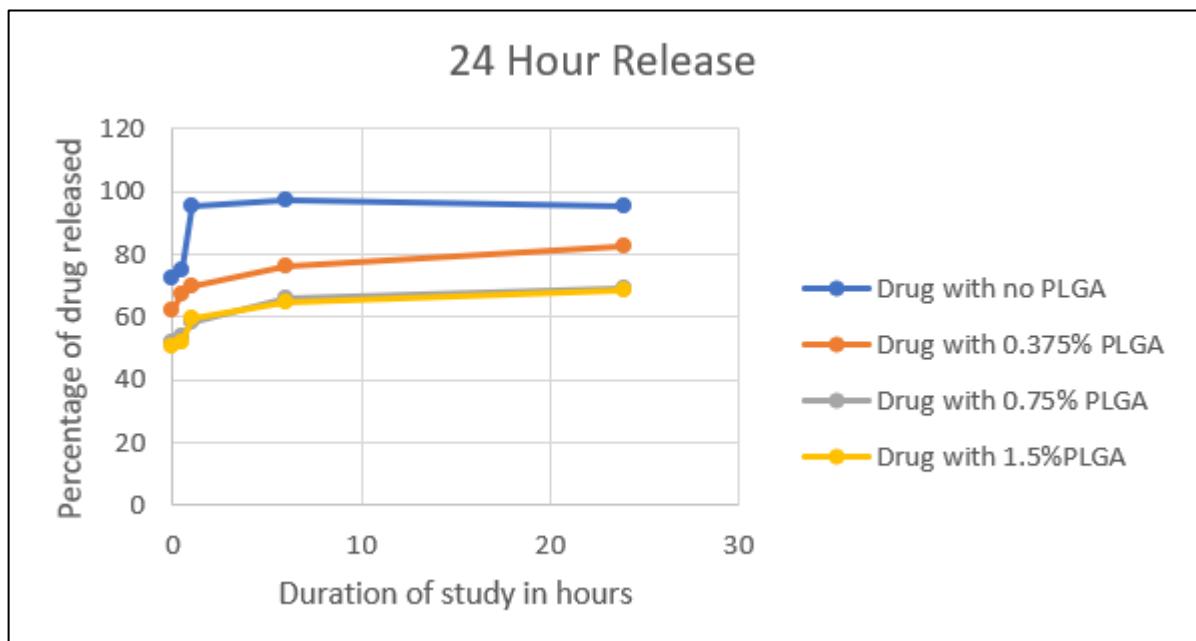


Figure 38: Trendlines showing drug release from the samples over 24 hours

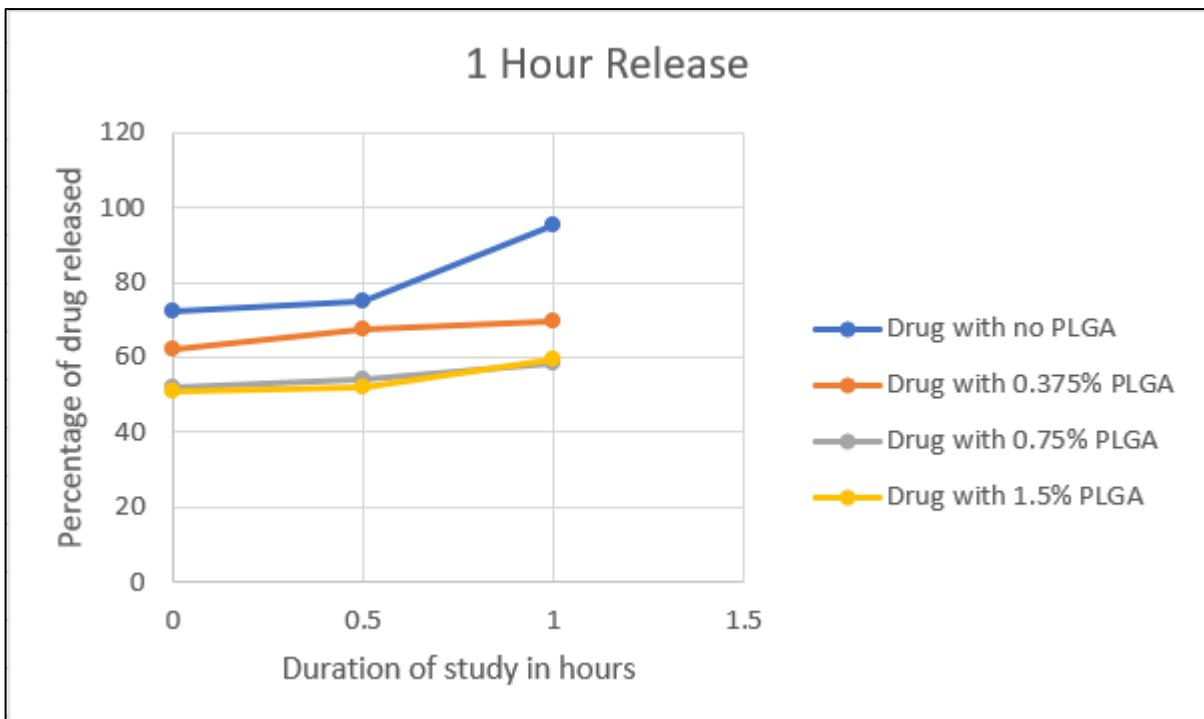


Figure 39: Trendlines showing drug release from the samples over 1 hour

From the above graphs, it is evident that there is significant reduction in percentage of drug (ciprofloxacin) released when PLGA is used to control the release rate. In the sample containing 0.375% PLGA, there was over 30% reduction in the amount of drug released over the first day of studies. There was further 10% reduction in total drug released from the sample containing 0.75% PLGA in comparison to the sample containing 0.375% PLGA. At the end of 24 hours, the percentage of drug released was 95, 83 and 66% for 0, 0.375 and 0.75% PLGA loaded samples respectively. The graph showing 1 hour release gives a better picture of how PLGA limits the burst release of the drug from the TNTs.

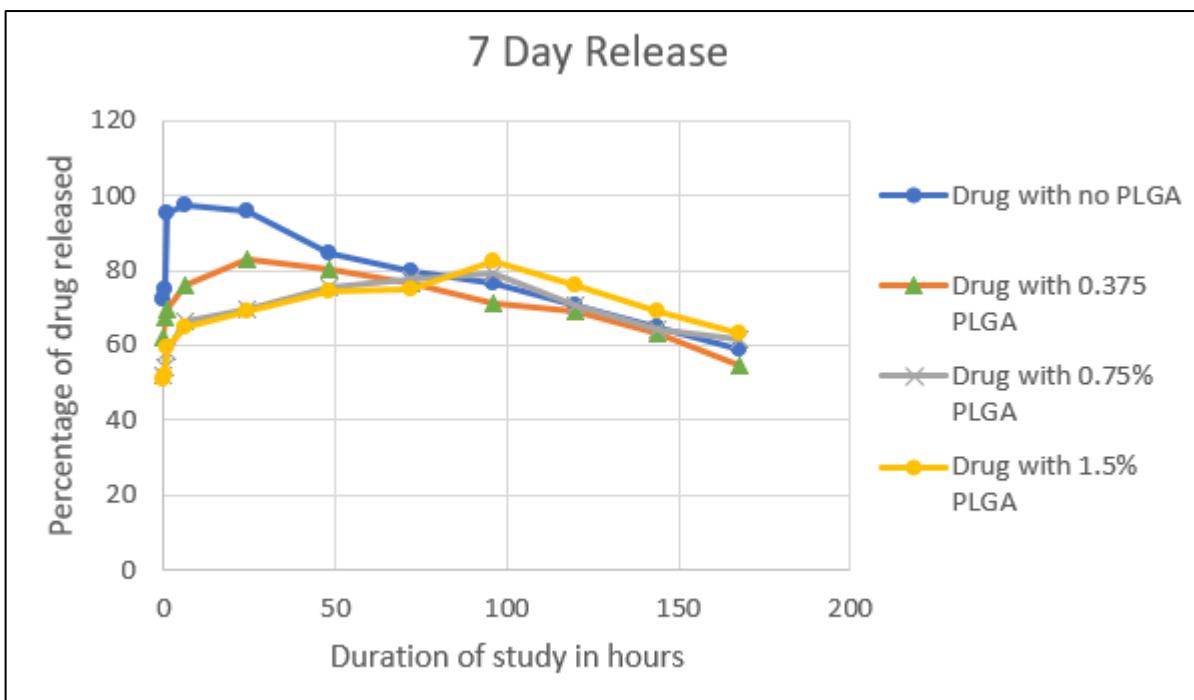


Figure 40: Trendlines showing drug release from the samples over 7 days.

In the graph showing the drug release over 7 days, it can clearly be seen how the peaks of release occur at increasing durations with increase in PLGA concentration coated onto the TNTs. Surprisingly, there was a decline in absorbance observed after peaks in each of the trendlines. This could possibly be due to photocatalytic degradation of ciprofloxacin in PBS after release. Further studies are warranted to confirm the same. However, the major conclusion from this study is the confirmation of steady drug release from TNTs and the PLGA layer acting as a drug release rate inhibitor in the samples.

CHAPTER 5

CONCLUSIONS

The major conclusions drawn from this project are:

1. Self-organization and arrangement of the TNTs improved on switching from acidic to organic electrolytes. The most self-arranged nanotubes were formed by anodization in EG containing fluoride salt with 6% volume of water in the electrolyte.
2. Length of the nanotubes increased with the duration of anodization time. This supports the theory of fluoride ions etching with higher rate at the bottom of the tubes, thus increasing the length of the hollow tubes as time goes on.
3. Anodization at higher voltages with acidic electrolytes led to breaking of the nanotubes during growth and left behind precipitate and an oxide layer on the surface. The aggressive nature of the electrolyte causes the breaking of the nanotubes.
4. The fabricated TNT samples showed increase in cell viability with increase in percentage of PLGA loaded on top of the nanotubes. Cell proliferation and adhesion was heavily promoted by the presence of nanotubes and PLGA on the surface.
5. The drug release rate was lowered in the presence of polymer coating on top of the nanotubes. With increasing concentration of polymer in the solution used for loading, the burst release of the drug was better controlled. Thus, the concentration of the PLGA used for loading could be varied to obtain a drug release profile which would be suitable for post-operative care such as controlling inflammation and sustained treatment or prevention of infections.
6. Having shown success with controlling the drug release rate, this technique of sustained drug release could be further tested with small animals before moving to clinical trials.

REFERENCES

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