ANTIBACTERIAL, BIOCOMPATIBILITY AND NANOMECHANICAL PROPERTIES OF TI-6AL-7NB ALLOY COATED WITH COPPER, HYDROXYAPATITE AND COPPER ION DOPED HYDROXYAPATITE FOR DENTAL IMPLANTS

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by

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
ANOVA	Analysis of variance
BHIB	Brain heart infusion Broth
CBA	Colombia Sheep Blood Agar
CFU	Colony forming unit
Cp-Ti	Commercialy pure titanium
DAE	Dual Acid Etching
DI	Deionized
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle Medium
EPD	Electrophoretic Deposition
EDX	Energy dispersive X-ray spectroscopy
Er	Reduced elastic modulus
FBS	Fetal bovine serum
H	Hardness
hFOB	Human fetal osteoblast
MHA	Mueller Hinton Agar
MSA	Mannitol Salt Agar
MHB	Mueller Hinton Broth
MD	Mean differences
MTT	3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide solution
nCu	Copper nanoparticles

nCu/HA	Copper ion substitute hydroxyapatite nanoparticles
nHA	Hydroxyapatite nanoparticles
OD	Optical density
PBS	Phosphate buffered saline
P.	Prophyromonas
Ra	Roughness
SEM	Scanning electron microscopy
S.	Staphylococcus
Ti	Ti-6Al-7Nb
Ti-6Al-7Nb	Titanium six aluminum seven niobium
Ti HA	Titanium six aluminum seven niobium coated with hydroxyapatite nanoparticles
Ti HA MSA	Titanium six aluminum seven niobium coated with hydroxyapatite nanoparticles with Mannitol Salt Agar
Ti HA MHA	Titanium six aluminum seven niobium coated with hydroxyapatite nanoparticles with Mueller Hinton Agar
Ti Cu	Titanium six aluminum seven niobium coated with copper nanoparticles
Ti Cu/HA	Titanium six aluminum seven niobium coated with copper ion substitute hydroxyapatite nanoparticles
Ti Cu/HA MSA	Titanium six aluminum seven niobium coated with copper ion substitute hydroxyapatite nanoparticles with Mannitol Salt Agar
Ti Cu MSA	Titanium six aluminum seven niobium coated with copper nanoparticles with Mannitol Salt Agar

Ti Cu MHA	Titanium six aluminum seven niobium coated with copper nanoparticles with Mueller Hinton Agar
Ti Cu/HA MHA	Titanium six aluminum seven niobium coated with copper ion substitute hydroxyapatite nanoparticles with Mueller Hinton Agar
Ti MSA	Ti-6Al-7Nb with Mannitol Salt Agar
Ti MHA	Ti-6Al-7Nb with Mueller Hinton Agar
V	Voltage
XRD	X-ray Diffraction

LIST OF SYMBOLS

Ag^{+}	Silver ion
Al	Aluminum
Al_2O_3	Alumina
Al-SL	Alumina sand blasted
Ar	Argon
α	Alpha
β	Beta
C	Carbon
Ce^{3+}	Cerium ion
CNTs	Carbon nanotubes
Cr	Chromium
CrN	Chromium nitride
Cu^{2+}	Copper ion
FA	Fluorapatite
Fe	Iron
Ga^{3+}	Gallium ion
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
Nb	Niobium
Ni	Nickel
O	Oxygen
PVP	Polyvinylpyrrolidone

SeO_3^{2-}	Selenium ion
SiO_2	silicate oxide
SS	Stainless steel
Zn^{2+}	Zinc ion
Sr^{2+}	Strontium ion
Ta	Tantalum
Ti^{4+}	Titanium ion
TiO_2	Titanium oxide
U	Uranium
V	Vanadium
YSZ	Yttria stabilized zirconia
Zr	Zirconium
ZrO_2	Zirconium oxide

SIFAT ANTIMIKROB, KESERASIAN BIO DAN MEKANIK NANO ALOI TI-6AL-7NB BERSALUT TEMBAGA, HIDROKSIAPATIT DAN HIDROKSIAPATIT ION TEMBAGA TERDOP UNTUK IMPLAN PERGIGIAN

ABSTRAK

Jangkitan berkaitan implan telah menjadi satu masalah klinikal yang serius. Ketidakhadiran jangkitan akibat pembedahan yang berkesan adalah salah satu kunci untuk terapi implan oral yang berjaya. Tujuan kajian ini adalah untuk menilai antibakteria, ketoksikan lekatan sel dan lekukan nano aloi titanium-6 titanium-7 niobium (Ti-6Al-7Nb) bersalut tembaga yang disintesis, hidroksiapatit dan hidrosiapatit ion tembaga terdop. Prestasi antimikrob terhadap sampel bersalut dan tidak bersalut pada *Staphylococcus aureus* dan *Staphylococcus epidermidis* telah dijalankan menggunakan dua jenis agar-agar (agar-agar 'Mannitol Salt' (MSA) dan agar-agar 'Mueller Hinton' (MHA)) yang telah dinilai selepas hari pertama, kedua dan ketiga menggunakan ujian resapan cakera. Sebagai tambahan, sifat antimikrob sampel bersalut dan tidak bersalut pada *Porphyromonas gingivalis, S. aureus* dan *S. epidermidis* telah dibandingkan selepas hari pertama, kedua dan ketiga menggunakan ujian resapan cakera dan ujian kultur broth. Analisa statistik telah dilakukan menggunakan ANOVA-berulang ($p<0.05$). Kesan ketoksikan sel dan fungsi sampel bersalut dan tidak bersalut telah dinilai menggunakan asai metil-thiazol-difeniltetrazolium (MTT) terhadap sel osteoblas fetus manusia (hFOB) selepas 24 dan 72 jam. Varians analisa sehala (ANOVA) diikuti oleh analisa perbandingan berganda post hoc menggunakan ujian Scheffe telah digunakan. Morfologi sel dan

lekatan telah dinilai selepas 24 dan 72 jam, masing-masing menggunakan mikroskop songsang dan dicerap di bawah SEM. Selain itu, kesan penambahan nCu pada kekerasan dan modulus kenyal lapisan bersalut telah disiasat melalui lekukan nano. Analisa statistik telah dilengkapkan menggunakan ujian Kruskal-Wallis ($p<0.05$). Keputusan menunjukkan bahawa penilaian antibakteria menggunakan ujian agar-agar resapan dan kultur broth menunjukkan yang Ti Cu dan Ti Cu/HA merencat pertumbuhan *S. aureus, S. epidermidis* dan *P. gingivalis* secara signifikan manakala Ti dan Ti HA menunjukkan tiada kesan antibakteria. Sebagai tambahan, MSA menghasilkan keputusan sebanding MHA apabila digunakan sebagai medium untuk pengujian kerentanan bakteria menggunakan ujian agar-agar resapan. Asai MTT menunjukkan yang kandungan Cu pada permukaan aloi Ti-6Al-7Nb tidak mempunyai kesan sitotoksik pada kelangsungan sel. Kadar kelangsungan sel bagi Ti Cu/HA menunjukkan nilai yang tinggi secara signifikan pada hari ketiga berbanding pada hari pertama, menunjukkan pertumbuhan sel hFOB pada kadar proliferasi yang tinggi. Penilaian mikroskopik menunjukkan tiada perbezaan dalam morfologi sel untuk semua sampel. Di bawah SEM, sifat lekatan sel untuk semua sampel adalah memuaskan. Walau bagaimanapun, sel hFOB melekat dan membentuk lebih banyak sambungan pada Ti HA dan Ti Cu/HA berbanding kumpulan Ti dan Ti Cu. Keputusan lekukan nano mengesahkan kekerasan dan modulus kenyal HA telah bertambahbaik secara signifikan dengan penggabungan nCu. Sebagai kesimpulan, keputusan mencadangkan modifikasi permukaan aloi Ti-6Al-7Nb mungkin baik untuk kawalan setempat jangkitan untuk implan gigi dengan tiada kesan buruk terhadap ketoksikan sel hFOB. Sebagai tambahan, nCu juga boleh disyorkan untuk menambahbaik sifat mekanik nano lapisan salutan untuk aloi Ti-6Al-7Nb.

ANTIBACTERIAL, BIOCOMPATIBILITY AND NANOMECHANICAL PROPERTIES OF TI-6AL-7NB ALLOY COATED WITH COPPER, HYDROXYAPATITE AND COPPER ION DOPED HYDROXYAPATITE FOR DENTAL IMPLANTS

ABSTRACT

Implant-associated infection has been a serious clinical problem. An effective absence of surgical associated infection is one of the keys for a successful oral implant therapy. The aims of this study were to evaluate the antibacterial, cytotoxicity, cell attachment and nanoindentation of titanium-6 aluminium-7 niobium (Ti-6Al-7Nb) alloy coated with synthesized copper, hydroxyapatite and copper ion doped hydroxyapatite. The antibacterial performance of coated and uncoated samples on *Staphylococcus aureus* and *Staphylococcus epidermidis* was performed using two types of agar (Mannitol Salt Agar (MSA) and Mueller Hinton Agar (MHA)) that was evaluated after day 1, 2 and 3 by disk diffusion test. In addition, antibacterial properties of coated and uncoated samples on *Porphyromonas gingivalis, S. aureus* and *S. epidermidis* were compared after day 1, 2 and 3 by disk diffusion and broth culture tests. Statistical analysis was performed using repeated-ANOVA ($P< 0.05$). The effect of cell toxicity and function of coated and uncoated samples were assessed using methyl-thiazol-diphenyltetrazolium (MTT) assay on human fetal osteoblast (hFOB) cells after 24 and 72 hours. One-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons analysis using Scheffe test were used. The cell morphology and attachment were evaluated after 24 and 72 hours using inverted microscope and observed under SEM respectively. Furthermore, the effects of nCu addition on hardness and elastic modulus of coated layer was investigated by nanoindentation. Statistical analysis was completed using Kruskal-Wallis test ($P < 0.05$). The results showed that the antibacterial evaluation using agar diffusion and broth culture tests indicated that Ti Cu and Ti Cu/HA significantly inhibit the growth of *S. aureus, S. epidermidis* and *P. gingivalis* while Ti and Ti HA demonstrated no antibacterial effect. Additionally, MSA yielded comparable result to MHA when used as the medium for testing bacterial susceptibility using agar diffusion test. The MTT assay showed that Cu content on the surface of Ti-6Al-7Nb alloys has no cytotoxic effect on cell viability. The cell viability rate for Ti Cu/HA was kept at significantly higher value on day 3 as compared to day 1, indicating that hFOB cells grow at a high proliferation rate. Microscopic evaluation indicated no differences in the cell morphology among all samples. Under SEM, the cell attachment properties for all samples were favourable. Nevertheless, hFOB cells attached and formed more bridges on Ti HA and Ti Cu/HA compared to Ti and Ti Cu groups. The nanoindentation results confirmed that the hardness and elastic modulus of HA were significantly improved by incorporating nCu. In conclusion, the results suggest that the surface modification of Ti-6Al-7Nb alloy with nCu/HA may be good for local control of infection for dental implant with no adverse effect on the cytotoxicity of hFOB cells. Also, the addition of nCu may be recommended to improve the nanomechanical properties of the coating layer to Ti-6Al-7Nb alloy.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Replacing missing teeth without affecting the rest of the dentition, while imitating the physiology of a sound tooth for everyday function with good aesthetic appearance, is one of the goals in dentistry. One of the very popular treatment options towards the realization of this dream is dental implants.

Dental implantology offers a reliable and safer option to restore the missing teeth. Osseointegrated implants have recently become a viable option for treatment for totally and partially edentulous patients and furthermore as a single-tooth replacement option (Shimpuku *et al.*, 2003). Materials for tooth replacement are desired to exhibit biocompatibility, bioactive action, non-toxicity, non-allergic, and non-inflammatory. Biocompatibility and activity are strongly dependent on the material surface properties (Puleo and Nanci, 1999). Among many other metallic biomaterials options used for implants, cobalt-chrome alloy, stainless steel, titanium and titanium alloys are commonly used. However, the most commonly used dental material for dental implants are commercially pure titanium (Cp-Ti) and its alloys (Elias *et al.*, 2008).

Cp-Ti was initially designed to replace the 316L stainless-steel and Co-Cr alloys because of the comparatively better biocompatibility (Bannon and Mild, 1983). Despite of which, the mechanical properties of Cp-Ti were not enough to satisfy the necessities of biomaterials when strength is taken into consideration, as in the case of hard tissue replacement or in cases of replacement of structure with intensive wear. This deficiency may lead to implant failure like fracture of the implants that support partly edentulous restorations and could also lead to screw loosening (Oliveira *et al.*, 1998; Eckert *et al.*, 2000). In 1954s, titanium-6-aluminium-4-vanadium (Ti-6Al-4V) was produced to treat the deficiency in the implant mechanical properties (Semiatin *et al.*, 1997). Inspite of its common use as a metallic implant, Ti-6Al-4V began to lose its quality by the late eighties. This occurred when the toxicity of vanadium was recognized in an *in-vivo* study. The toxic effect of vanadium has been documented to cause cardiovascular and nephritic pathology. Apart from that, it has also been related to cardiovascular disease, Parkinson's disease and depressive psychopathy (Venkataraman and Sudha, 2005; Ngwa *et al.*, 2009; Manivasagam *et al.*, 2010). Therefore, the titanium 6-aluminium 7-niobium (Ti-6Al-7Nb) alloy was developed in late Nineteen Seventies. Vanadium was replaced with niobium to facilitate its implant application (Geetha *et al.*, 2009). This alloy displayed high corrosion resistance, with regards to the impressive strength, a lower weight and also the absence of carcinogenicity of vanadium (Hanawa, 2010). Various biological responses with the use of Ti-6Al-7Nb have been documented. Shimojo *et al.* (2007) concluded in their study that fibroblasts cells proliferation, adhering and spreading were equal on both Ti-6Al-7Nb and Cp-Ti. Additionally, short term implantation *in vivo* produced an exceedingly transient inflammatory response to Ti-6Al-7Nb that closely resembled the response to Cp-Ti. No obvious unfavorable biological effects have been reported for both. Also, Ti-6Al-7Nb elicited lower inflammatory response than the Ti-6Al-4V (Pennekamp *et al.*, 2006; Pennekamp *et al.*, 2007). These results suggested that Ti-6Al-7Nb has favorable biocompatibility and are considered as a promising material for oral implantology.

Although biocompatibility and mechanical properties of any biomaterial are among the primary issues for the choice of an implant material; still the success of dental implants is mainly reliant on bone implant osseointegration. To reinforce this bone-bonding mechanism, implants have been coated with osteoconductive biomaterials like hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$]. Currently, the process of HA coating is achieved by plasma spraying. HA acts as a bio-ceramic material which closely resembles the mineral composition of teeth and bones. The HA coating has been used to prevent the discharge of metallic ions by acting like a surfactant barrier, and consequently enhancing the bioactivity of bone owing to its chemical constituents (Chou and Chang, 2003; Shi *et al.*, 2007; Kwok *et al.*, 2009). Even though plasma-sprayed HA coatings are identified to be biocompatible, these coatings are not identified for its antibacterial properties. Additionally, higher occurrence of porosities, weaker bond strength, non-stoichiometric composition with trace amounts of amorphous phase have also been noted (Chen *et al.*, 1994; Eliaz *et al.*, 2005). To overcome these inadequacies, different techniques such as sputtering coating , dip coating, pulsed-laser deposition, sol–gel coating, and electrophoretic deposition have all been utilized to perform these coatings (Lusquinos *et al.*, 2002). Electrophoretic deposition (EPD) is a versatile and useful technique that can be used to fabricate medical specialty materials. EPD excels in producing uniform thickness of coating with meticulous control of coating thickness and a high deposition rate. EPD has exhibited the ability to deposit denser, thicker, and adherent coatings on a variety of shape and complex porous structures (Corni *et al.*, 2008). However, the applying of pure HA has presented several disadvantages, as well as its lack of antibacterial activity might affect the success of the implants to a certain extent. Bacterial infection is considered one of the rising complications after implant placement. The postoperative infection rate was reported to be 4-10% for patients receiving dental implants in spite of success rate of the dental implants was reported to be as high as 90-95% (Pye *et al.*, 2009; Camps-Font *et al.*, 2015). The recurrent incidence of this infection is also a concern, which is about 5-8% and is even more difficult to control and treated by antibiotics. The implant materials placed within the oral cavity can interfere with the host defense mechanism and it might influence the required clinical dose of antibiotics to safeguard against infections. Moreover, local antibiotics loaded on the implant surface gets quickly washed out and fail to protect against long term postsurgical infections (Bahadir *et al.*, 2009; Stanić *et al.*, 2011). Apart from that, repeated use of antibiotics to fight infection could also lead to the incidence of antibiotic-resistant bacteria. Once these implants associated infection happen, the risk for implant removal becomes higher. Apart from pain and suffering, implant associated infection bring significant economic burden to the patients and society (Ren *et al.*, 2014).

Interestingly, no single microorganism has been closely associated with colonization or infection that relates to dental implant. Some of dental peri-implantitis microflora look like those found in chronic periodontitis, showing predominantly anaerobic Gram-negative bacilli, especially *Porphyromonas gingivalis* (Lee *et al.*, 1999; Pye *et al.*, 2009). Also, microorganisms that is not usually associated with periodontitis or dental abscesses such as coliforms, Candida spp. in particular *Staphylococci* (*S. aureus and S. epidermidis*) have been reported to be isolated from peri-implant lesions (Salvi *et al.*, 2008; Mombelli and Décaillet, 2011). Due to this, the present study focus on investigating the antibacterial properties of implant coating materials against *P. gingivalis, S. aureus* and *S. epidermidis*.

The incorporation of antibacterial agents, which includes metal ions consisting of copper (Cu^{2+}), silver (Ag^{+}), and zinc (Zn^{2+}) in HA is proposed to resolve the problem of implant related infections that have been associated with deficiency of antibacterial activity in HA (Borkow *et al.*, 2010; Grass *et al.*, 2011). Numerous *in vitro* researches reported that the coated implant with above metallic ions play an important role in minimizing or preventing preliminary bacterial colonization (Kim *et al.*, 1998; Yang *et al.*, 2009; Stanić *et al.*, 2010). Unfortunately, it is observed that applications of the inorganic antibacterial agents carrying silver are avoided due to high price and discoloration issues. Consequently, copper represents a greater promise coating because of its decrease toxicity and higher biocompatibility (Radovanović *et al.*, 2014). Moreover, copper is a metabolizable agent while silver tends to reside inside the human body and is also known to increase the serum levels (Masse *et al.*, 2000; Shirai *et al.*, 2009). Beside its antibacterial properties, Cu is an essential trace element in human beings as it is a enzymatory release stimulatant and also responsible for the bone collagen and elastin crosslinkage (Radovanović *et al.*, 2014). The antibacterial properties of nCu are not its only benefit as its particle size also facilitates greater surface contact area which further increases its action. The smaller dimensions and consequently increase surface contact ratio contributes to the increased interaction with the bacterial membranes as the microbial action takes place at the surface of any intended material (Morones *et al.*, 2005; Martinez-Gutierrez *et al.*, 2010).

Additionally, the nanosize particles not only influence the antibacterial properties, but also influence the mechanical properties of particles such as hardness, rigidity, high yield strength, flexibility and ductility (Puzyn *et al.*, 2010). Hussain *et al.* (2006) and Rajabi-Zamani *et al.* (2008) reported that the mechanical properties of composite coatings were improved when using nanoparticles (NPs). From mechanical point of view, the HA that is used as bioactive surface modification has poor mechanical properties, which is shown by its brittle nature. The HA coated layer is prone to wear and displayed weak mechanical adhesion to the substrate, and thus more prone to crack and fracture (Filiaggi *et al.*, 1991; Fernández-Pradas *et al.*, 2002; Mohseni *et al.*, 2014). To enhance the mechanical properties of the HA coating itself, HA composite coatings particularly nanocomposite coatings were introduced. To achieve this purpose, the HA is combined with alternative materials like carbon nanotubes (CNTs) (Hu *et al.*, 2004), yttria-stabilized zirconium (YSZ) (Evis and Doremus, 2005), and alumina (Al_2O_3) (Evis and Doremus, 2005). Based on the above, this study was aimed to modify the surface of Ti-6Al-7Nb alloy with copper ion hydroxyapatite in NPs using electrophoretic deposition technique to improve its antibacterial, biocompatibility and nanomechanical properties.

1.2 Problem statement

Ti-6Al-7Nb is considered a biologically inert element. It has been widely used in the fabrication of biomaterials notably in the implant technology. Ti-6Al-7Nb demonstrates high fatigue strength, low weight, a suitable Young's modulus and corrosion resistance. Ti and ti alloys display good biocompatibility related to formation of a compact layer of oxide. In spite of the acceptable biocompatibility of Ti-6Al-7Nb alloy, it remains troublesome to satisfy all the necessities of a biomaterial, like osseointegration, antibacterial and mechanical properties. Among the serious complication of dental implant is bacterial infection and this complication usually could not be solved by traditional ways like using antibiotics. Therefore, the modification of the surface of Ti-6Al-7Nb alloy by coating it with metals with antibacterial properties to reduce the number of microorganisms and to prevent their adhesion which can in turn lower the incidence of infection and therefore improve the implant longevity.

1.3 Justification of the study

Nowadays, Cp-Ti is commercially available and currently used as biomaterials for dental implant. Nevertheless, these Cp-Ti displays one main disadvantage that is poor mechanical properties and thus makes it not too favourable for use on its own. To improve the mechanical properties, the use of Ti-6Al-4V alloy had been advocated. However, studies found that this Ti-6Al-4V alloy induced some inflammatory responses which is related to the release of vanadium. In this instance, vanadium has been reported to be toxic and affect the proliferation of periimplant cells. As a result, Ti-6Al-7Nb alloy have been recommended as alternative to Cp-Ti and Ti-6Al-4V alloy.

With increasing insertion of dental implant number per year, the implant failure also increases due to different causes which are periimplantitis and implant mobility due to absence of osseintegration. Hydroxyapatite nanoparticles (nHA) has been commonly designed as osteoconductive coating material for implant. It has been reported that the reduction in HA material size particles could improve their bioactivity and their antibacterial activity (Zhou and Lee, 2011; Mathew *et al.*, 2014). Studies on the antibacterial property of nHA found that it had no antibacterial effect (Li *et al.*, 2010; Stanić *et al.*, 2010; Gopi *et al.*, 2014; Huang *et al.*, 2015a). To overcome this shortcoming, several studies have been performed by doping the HA with antibacterial materials like gold and silver to improve antibacterial behavior and to control the implant associated infection. The results found that gold and silver are expensive and silver is more toxic in low concentration. Therefore, an alternative material for reducing infection is to use synthesized copper ion doped hydroxyapatite to determine if they possessed some antibacterial behaviour or not. Up to our limitation of knowledge, the present study is the first that has been conducted to investigate the antibacterial properties of copper ion doped hydroxyapatite as a coating on Ti-6Al-7Nb against *P. gingivalis*, *S. aureus* and *S. epidermidis*.

Additionally, several *in vitro* studies evaluated the biological responses of Ti-6Al-7Nb alloy without surface modifications using different types of cells like human gingival fibroblasts and osteoblast like cells (Osathanon *et al.*, 2006; Shimojo *et al.*, 2007). These results revealed that Ti-6Al-7Nb is biocompatible and supports early osteoblast-material interaction. In this study, the human osteoblast cells were used to evaluate the toxicity of nCu because these cells responsible for bone formation and osseointegration around dental implant (Insua *et al.*, 2017). The human fetal osteoblast cell line hFOB was chosen as a type of osteoblast cells because these cells have several advantages when compared with human osteoblast cells that originated from adult, like high proliferation rates, well survival throughout cryopreservation and better response for stimulations of environment (Christodoulou *et al.*, 2005). Additionally, to the best of our knowledge, there is no information on the human fetal osteoblast cells proliferation associated with Ti-6Al-7Nb alloy coated with copper ion doped hydroxyapatite using electrophoretic deposition.

Coating method is one of several procedures that is used to improve osseointegration and antibacterial properties of implant materials. Therefore, the mechanical property of coating layer is one of the main factors that can affect the service life and the performance of coating components. This could be due to the susceptibility of the coating layer to fracture due to poor mechanical properties and thus making it unsuitable to load bearing implants. Not many attempts have been made to understand the surface and bulk mechanics of HA and nCu at the nanoscale. Therefore, the current study evaluated if the nCu materials can improve the hardness and elastic modulus of the coating layer.

The results of this study could be used in the medical and dental fields. Also, information and the results of this study may be used to reduce the potential failure of dental implant due to infection and may enhance the biocompatible properties of implant and the mechanical properties of coating layer. In addition, it may give information and help to increase the lifespan of implants or even reduce the implant failure. In addition, the outcome of this study will provide the clinician in oral implantology with some knowledge that will help them to choose better treatment modalities in order to provide longer lasting implant to their patients.

1.4 Objectives

1.4.1General objective

- To synthesize and investigate the antibacterial, biocompatibility and nanomechanical properties of Ti-6Al-7Nb alloy coated with copper, hydroxyapatite and copper ion doped hydroxyapatite using electrophoretic deposition method for dental implants.

1.4.2 Specific objectives

1. To synthesize the copper, hydroxyapatite and copper ion doped hydroxyapatite in nanosize by wet chemical, sol gel and ion exchange method in aqueous solution, respectively.
2. To assess the crystal structure and surface morphology for nCu, nHA, nCu/HA, Ti, Ti Cu, Ti HA and Ti Cu/HA using X-ray diffractometer (XRD) and scanning electron microscope (SEM), respectively. Also, to evaluate the elemental composition of Ti Cu, Ti HA and Ti Cu/HA using energy dispersive X-ray spectroscopy (EDX).
3. To compare the surface roughness for Ti Cu, Ti HA and Ti Cu/HA before and after sintering. Also, to compare the surface roughness of coated samples with those uncoated using profilometer
4. To compare the antibacterial performance of Ti, Ti Cu, Ti HA and Ti Cu/HA on *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) using two types of agar (Mannitol Salt Agar and Mueller Hinton Agar) by means of disk diffusion test. Also, to compare the antibacterial performance of Ti, Ti Cu, Ti HA and Ti Cu/HA on *Prophyromonas gingivalis* (*P.*

gingivalis), *S. aureus* and *S. epidermidis* using disk diffusion and broth culture methods.

5. To investigate and compare the influence of Ti, Ti Cu, Ti HA and Ti Cu/HA on cell cytotoxicity, proliferation, cell attachment and morphology of human fetal osteoblasts cells cultured *in vitro*.
6. To compare the hardness and elastic modulus of Ti Cu, Ti HA and Ti Cu/HA using nanoindentation test.

1.5 Research questions

1. Does the copper, hydroxyapatite and copper ion doped hydroxyapatite synthesized by wet chemical, sol gel and ion exchange method in aqueous solution, respectively, produce a high purity copper nanoparticles powder?
2. Does the assessment of crystal structure for nCu, nHA, nCu/HA match well with the standard peaks of nCu and nHA and are there any phases transformation when compare Ti with Ti Cu, Ti HA and Ti Cu/HA using XRD during phase component identification after deposition and sintering processes? Does the evaluation of surface morphology and microstructure of nCu, nHA and nCu/HA show similarity to standard morphological properties of the nCu and nHA and does the evaluation of surface morphology Ti, Ti Cu, Ti HA and Ti Cu/HA show uniform deposition of coating when observed using SEM? Also, does the evaluation of elemental composition by EDX show a homogeneous distribution of elements?
3. Are there any significant differences in surface roughness for Ti Cu, Ti HA and Ti Cu/HA before and after sintering? Also, are there any significant differences the surface roughness of coated samples with those uncoated using profilometer?

4. Are there any significant differences in the antibacterial performance of Ti, Ti Cu, Ti HA, and Ti Cu/HA on *S. aureus* and *S. epidermidis* upon using two types of agar (Mannitol Salt Agar and Mueller Hinton Agar) by means of disk diffusion test? Also, are there any significant differences in the antibacterial effect between Ti, Ti Cu, Ti HA and Ti Cu/HA against *P. gingivalis, S. aureus* and *S. epidermidis* using disk diffusion and broth culture methods?
5. Are there any significant differences of Ti, Ti Cu, Ti HA and Ti Cu/HA on cell cytotoxicity, proliferation, cell attachment and morphology of human fetal osteoblasts cells cultured *in vitro*?
6. Are there any significant differences in hardness and elastic modulus among Ti Cu, Ti HA and Ti Cu/HA groups?

1.6 Research hypotheses

1. The synthesis of the copper, hydroxyapatite and copper ion doped hydroxyapatite by wet chemical, sol gel and ion exchange method in aqueous solution, respectively, produces a high purity powder.
2. The assessment of the crystal structure of the nCu, nHA and nCu/HA matches well with the standard peaks of nCu and nHA, and there are no phases transformation when comparing Ti with Ti Cu, Ti HA and Ti Cu/HA using XRD during phase component identification after deposition and sintering processes. Also, the SEM shows similarity to the standard morphological properties of the nCu and nHA, and Ti, Ti Cu, Ti HA and Ti Cu/HA shows uniform deposition of coating when observed using a SEM. Additionally, EDX evaluation shows a homogeneous distribution of elements.

3. There are significant differences in surface roughness for Ti Cu, Ti HA and Ti Cu/HA before and after sintering. Also, there are significant differences in surface roughness of coated samples with those uncoated using profilometer?
4. There are no significant differences in the antibacterial performance of Ti, Ti Cu, Ti HA and Ti Cu/HA on *S. aureus* and *S. epidermidis* when using two types of agar (Mannitol Salt Agar and Mueller Hinton Agar) tested by means of disk diffusion test. Also, there are significant differences in the antibacterial effect against *P. gingivalis*, *S. aureus* and *S. epidermidis* between Ti, Ti Cu, Ti HA and Ti Cu/HA using disk diffusion and broth culture methods.
5. There are no significant differences in cytotoxicity, proliferation, cell attachment and morphology of human fetal osteoblasts cells cultured on Ti and Ti Cu when compared with Ti HA, and Ti Cu/HA.
6. A significant difference exists in the hardness and elastic modulus of Ti Cu and Ti Cu/HA when compared with Ti HA.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 History of dental implants

Dental implantation has been thought about for over 5000 years with archaeologic proof revealing that ancient Egyptians experimented implantation of precious stones and metals into the jaw bones of corpses wherever teeth had been lost; this was performed as a ritual for the hereafter (Saini *et al.*, 2015). The earliest case of a functional implant from history has been dated to the 1-2 A.D. when a Gallo-Roman man was orally examined to find a wrought iron device embedded in his right second maxillary bicuspid region (Crubzy *et al.*, 1998). It absolutely was however not till the nineteenth century that endosseous (inside the bone) dental implants were designed, once Maggilio, a French dental practitioner at the University of Nancy documented using customized gold implants placed directly into an extraction socket (Ring, 1995a; Ring, 1995b).

By mid-20th century, transosseous (through the bone), subperiosteal (top of bone), and endosseous (within the bone) implants were developed and were composed from a range of different materials; but, they were unpredictable in terms of their stability and reactions with soft tissue (Caswell and Clark, 1991). Additionally, throughout these early years, infection was a relentless drawback and it absolutely was not till the fortunate discovery of osseointegration with the Ti that dental implants started prospering as a treatment modality for replacement of missing teeth.

2.2 Materials used for dental implants

Implant materials have been classified according to the biological responses upon implantation or the chemical composition (Sykaras *et al.*, 2000). According to chemical composition, dental implants can consist of metals, ceramics or polymers.

2.2.1 Polymers

Polymers have lower elastic modulus and strength but better resistance to fractures as compared to the other categories of biomaterials. Polymers act as thermal and electrical insulators and are comparatively not susceptible to biodegradation. When placed next to bony structure they need a lower elastic modulus with magnitudes close to soft tissues. Porous and solid forms of polymers are made for tissue attachment, augmentation and replacement. They are also fabricated as coatings for force dissipation and distribution to soft and hard tissue regions. As a general rule, polymers and their composites are particularly sensitive to sterilization and manipulation techniques. Polymeric implants were initially introduced in Nineteen Thirties. However, they did not find intensive use in implant dentistry due to the inherently low mechanical strength and lacking osseointegration capability (Chauhan *et al.*, 2011). In addition, if they were meant for implantation, most of the products cannot be sterilized by any method. Polymers have electrostatic surface properties and show a tendency to harbor dirt or other particulate if exposed to non hygienic oral environments (Ananth *et al.*, 2015).

2.2.2 Metals and metal alloys

Metals have biomechanical properties that promote their acceptability as an implant material. Besides these properties, metals are also very simple to fabricate and have easily achievable finish. Metallic implants can be readily sterilized by the most normal sterilization procedures which allow a very straightforward use of this material. However with time, the documented low success rate of certain metals (like gold, stainless-steel, cobalt-chromium) and their adverse tissue reactions have undermined their clinical application as a permanent treatment and are presently changed by newer materials which are Ti and its alloys (McCracken, 1999; Sykaras *et al.*, 2000).

According to the clinical reports, the long term success of Ti implants have deemed Ti alloys, the “gold standard material for dental implants fabrication” (Adell *et al.*, 1990; Niinomi, 1998).

2.2.2 (a) Commercially pure titanium

Titanium was once in early days considered a rare metal, however now a day it's one among the foremost necessary metals within the biomedical business. The primary elemental metal “Titanium” was 1st discovered by William Gregor in 1790 in England, however Martin Heinrich Klaproth gave it the name of “Titanium” in 1795. A combination of high strength to weight ratio, low density, excellent biocompatibility, plasticity and improved corrosion resistance and remarkable mechanical properties verify the application of Ti and its alloys in various industries such as, aviation, power, automotives, shipbuilding, architecture, medicinal field and sports equipment (Stadlinger *et al.*, 2012).

The commercially pure titanium (Cp-Ti) is classified into four grades on the basis of difference in their oxygen content. Grade four has the highest (0.4%) and grade one has the smallest amount (0.18%) of oxygen content. The mechanical variations that exist between the various grades of Cp-Ti is primarily as a result of the contaminants that are present within. For increased strength, aluminum is added, for corrosion resistance and decreased density iron is added, whereas to prevent corrosion vanadium acts as an aluminum scavenger. Hexagonal compact lattice of Ti is termed the α -Ti (α -phase). On heating at 883 ℃, phase transformation happens from hexagonal close packed to body-centered cubic lattice or β - phase. Ti is reactive because it forms spontaneously a dense oxide film at its surface. Ti could be a dimorphic metal i.e. below 882.5 ℃ it exists as α-phase and above this temperature it changes form α- phase to β phase.

The formation of oxidation layer on the Ti surface has several properties like the ability to repair itself when any cracking occurs, chemical resistance, catalytic activity for a few chemical reactions and suitable modulus of elasticity comparable to the human bone promote the use of Ti and its alloys as a biomaterial. Ti is the material of selection for intraosseous applications (Wennerberg *et al.*, 1996; Sykaras *et al.*, 2000; Tschernitschek *et al.*, 2005; Stadlinger *et al.*, 2012). However, they're neither bioactive nor bactericidal, although upon investigation a reduced microbial colony formation has been discovered on Ti surfaces when compared with stainless steel (SS) surfaces, thanks to its higher osseointegration (Harris and Richards, 2006).

2.2.2 (b) Titanium alloys

Experiments have been carried out to develop Ti alloys constituting various compositions to attain higher performance in terms of biomechanical compatibility by excluding non-toxic elements. Ti reacts with many other elements like zinc (Zn), silver (Ag), argon (Ar), aluminum (Al), iron (Fe), copper (Cu), uranium (U), and vanadium (V) to create alloys (Geetha *et al.,* 2004).

Ti alloys are divided into 5 classes based on their microstructure at room temperature, which are (1) α, (2) near-α, (3) α+β, (4) metastable β and (5) stable β. However, the alloying elements have been categorized as (1) α stabilizer, (2) β stabilizer and (3) neutral (Long and Rack, 1998; Leyens and Peters, 2003). α-stabilizers include aluminum and interstitial elements like O, C, and N. Increasing the α-stabilizer concentration results in a larger α-phase region within the phase diagram, and consequently lowered growth of β transus temperature. However, β stabilizers alloying elements are classified depending on the solubility into 2 groups, (1) β isomorphous (Ta, V, Nb, Mo) and (2) β eutectoid (Ni, Cr, Fe, Mn) (Leyens and Peters, 2003). In comparison with the α stabilizer, β transus temperature is reciprocally proportional to the increase in β stabilizer content. An increase in β-stabilizing elements leads to the formation of smaller β phase region. Also, α+β region can be expanded by increasing the substances of each α and β alloying elements. Zirconium and stannum (Sn) are classified as neutral because no effect on β-transus temperature has been noted for these elements (Long and Rack, 1998; Leyens and Peters, 2003).

2.2.2 (b) (i) Ti-6Al-4V alloy

Ti-6Al-4V has been extensively used in dental and medical applications because of its suitable corrosion resistance and high specific strength (Soboyejo *et al.*, 2002). These types were formulated by controlled heating and cooling down of pure Ti with Al and V at fixed concentrations. These elements act as different types of phase-condition stabilizers. Aluminium acts as an α -phase stabilizer and additionally decreases the weight and increases the strength of the alloy. Vanadium acts as β phase stabilizer. The α+β pattern alloy is frequently used for dental implants fabrication (Sykaras *et al.*, 2000; Saini *et al.*, 2015). The major limitation of this material is the lack of bioactivity, which makes it less stable and prone to aseptic loosening and eventually failure of the implant (Lee *et al.*, 2000).

During insertion of the implant in the strongly corrosive biological environment, there is hazard of transportation of ions of vanadium to the tissue that surrounded the implant with local corrosion process. This may produce an adverse immune response (Williams, 2008). After releasing of ions from the corroded surface they have a tendency to migrate to adjacent soft tissues or might become protein-bound which can trigger an adverse reaction. Vanadium is taken into account as probably harmful agent, as it has been known to affect many physiological processes of human body and has also been suspected as a culprit in etiopathogenesis of Parkinson's disease (Venkataraman and Sudha, 2005; Ngwa *et al.*, 2009). This hazard may be removed by using niobium instead of vanadium.

2.2.2 (b) (ii) Ti-6Al-7Nb alloy

Comparable to the morphology of Ti-6Al-4V, the Ti-6Al-7Nb alloy is α+β combination alloy. Ti-6Al-7Nb alloy has been chemically optimized for its use in biomedical field (Semlitsch *et al.*, 1992; Niinomi, 1998). This alloy exhibits an excellent strength with a low weight, high corrosion resistance, and inherently lacks any biotoxic element (Hanawa, 2010).

According to the results of *in vitro* experiments, Ti-6Al-7Nb exhibited similar cellular responses to Ti (grade four). Whereas statistically superior performance in terms of cellular proliferation, attachment, morphology, viability, and spreading were noted when compared with Ti-6Al-4V alloy (Osathanon *et al.*, 2006; Challa *et al.*, 2013). Additionally, *in vivo* experiments also noted that Ti-6Al-7Nb evoked a lesser inflammatory response when compared with Ti-6Al-4V (Kraft *et al.*, 2005).

Ti-6Al-7Nb has almost half of the modulus of elasticity when compared to SS, however the yield stresses of both materials (Ti-6Al-7Nb and SS) are comparable. Ti-6Al-7Nb has higher resilience and a lower density than SS, which makes it a material of choice for dental implants (Gotman, 1997; Lavos-Valereto *et al.*, 2001).

2.2.3 Ceramics

Ceramics have been initially used as implant devices due to their excellent physical properties, inertness, and minimal thermo-electrical conductivity. However their brittleness and low ductility have limited their use in these fields (Sykaras *et al.*, 2000).

2.2.3 (a) Ceramics as dental implant coatings

Ceramics were first used in dental implant fields as a variety of coatings over metallic endosseous implants to boost up osseointegration (Lacefield, 1998). This concerned the employment of various bioactive ceramics, like calcium phosphates ($Ca_3(PO_4)_2$), bioglasses, inert ceramics like aluminum and zirconium oxide. Depending on the coating technique, coatings may be dense or porous, and the thickness ranges from one to one hundred μm (Lacefield, 1998; Nicholson, 2002). Bioactive ceramics tend to release $Ca_3(PO_4)_2$ ions when coated over implant surfaces, resulting in an increased bone apposition compared with other inert materials (Lacefield, 1998; Barrere *et al.*, 2003; Le Guéhennec *et al.*, 2007). Inert ceramic materials that lack bioactivity are uncommonly used when compared with bioactive ceramic materials which are able to produce $Ca_3(PO_4)_2$ ions. 97.8% clinical success has been reported for HA coated implants, however considerations regarding degradation and debonding of those coatings are raised (Yu-Liang *et al.*, 1999; Tinsley *et al.*, 2001).

2.2.3 (a) (i) Nano-hydroxyapatite

HA is a naturally occurring mineral form of calcium, that is abundantly located in mineralized tissue (Cao and Hench, 1996; Sebdani and Fathi, 2011). HA is additionally one amongst the key parts of dentin. Because of its bone-bonding ability, HA has been widely used as a coating material for dental implants and grafts. In addition, HA is extremely biocompatible and might speedily osteointegrate with bone tissue. HA can be utilized in various forms, like powders, coatings, and composites for dental restoration (Sung and Kim, 2003; Sung *et al.*, 2004; Fathi and Hanifi, 2007).

As compared to standard microsized HA, nanophase HA properties differs in surface grain size, pore size and wettability. These properties may contribute to its influence in protein interactions like adsorption, configuration and bioactivity. HA modulation subsequently increases osteoblast adhesion and long-term functionality of a material. Webster et al discovered that these increased osteoblast functions are

Ca containing mineral deposition and alkaline phosphatase synthesis (Webster *et al.*, 2000; Webster *et al.*, 2001). Nanometer grain size and surface wettability not solely promotes the exaggerated selective vitronectin adsorption (a protein that mediates osteoblast adhesion), however additionally it has an enhancing effect bone-forming cell functions. These nanometer-sized needle-like crystals are roughly 5-20 nm in width and 60 nm in length (Ferraz *et al.*, 2004).

HA demonstrates remarkable bioactivity and biocompatibility with regards to bone cells and tissues, most likely because of its close similarity with the hard tissues of the body. Therefore, phosphate biomaterials have been widely used in clinics (Ferraz *et al.*, 2004).

2.2.3 (a) (ii) Zirconia

In 1789, Martin Klaproth discovered zirconium (Zr) (Denry and Kelly, 2008; Tsuge, 2009). Zr is not naturally found in a pure form, however it has been identified to coexist with silicate oxide in the form a mineral (Zircon ($ZrO_2.SiO_2$)) or as a free oxide (ZrO_2) (Piconi and Maccauro, 1999).

These minerals can't be used as primary biomaterials in dental medicine due to the inherent impurities of many metallic elements that partly effect on coloration of final product. The presence natural radionuclides like uranium and thorium also make these materials radioactive (Porstendörfer *et al.*, 1996). A meticulous and time consuming process is carried out to separate and obtain pure ZrO_2 powder, after which the product can be readily used for ceramic biomaterial synthesis (Christel *et al.*, 1988; Piconi and Maccauro, 1999). ZrO_2 has showed a great potential as a suitable material for dental restoration owing to its naturally esthetics, superior mechanical properties, and ability to inhibit plaque accumulation (Olsson *et al.*, 2003; Bremer *et al.*, 2011; Bateli *et al.*, 2014). In 1789, a chemist named Martin Heinrich introduced ZrO_2 (Raigrodski, 2004). ZrO_2 is a non-cytotoxic compound, which is insoluble in water and to which microbial adhesion is not possible. It's radiopacity and high corrosion resistant properties have been documented (Dion *et al.*, 1994; Scotti *et al.*, 2007).

Due to the excellent chemical properties and dimensional stability the ZrO_2 has been used as a biomaterial extensively (Qian *et al.*, 2003; Samuel *et al.*, 2010; Rosalbino *et al.*, 2011). ZrO_2 is resistant to different types of acidic chemicals and no adverse effect to human living tissue has been reported. It should be noted that Ti-Zr alloy have exhibited an increased cellular response (Gómez-Florit *et al.*, 2014). ZrO_2 has recently been researched for its use in surface modifications (Duygulu *et al.*, 2007; Sollazzo *et al.*, 2008; Zhang *et al.*, 2013).

2.2.3 (b) Ceramics as dental implant materials

The principal disadvantage of Ti is its dark greyish color, which frequently is visible through the peri-implant tissue layer, thus impairing esthetic outcomes within the presence of a thinner membrane genotype. Recession or unfavorable gingival conditions might cause compromised esthetics. This is a pertinent issue particularly in the case of maxillary incisor involvement (Heydecke *et al.*, 1999). Novel implant technologies are being developed and researched to manufacture ceramic implants which can overcome these disadvantages (Kohal and Klaus, 2004). Ceramic has always had a high risk for fracture (Andreiotelli and Kohal, 2009). However, ceramics have been found sensitive to tensile and shear stress loads, and surface cracks can lead to early failure.

Ceramic implants are not a new option in dentistry. Sandhaus was one amongst the first researchers to report about ceramic implants (Sandhaus, 1967). However, ceramic based implants showed a success rate of a mere 25%, after calculating a mean of observation of five years (Strub *et al.*, 1987). Since the ceramic based implant system failed to meet the long-term clinical expectations it had been discontinued. Since then, high strength zirconia ceramic became enticing as a new material for dental implants. Yttria-stabilized tetragonal zirconium dioxide polycrystals (Y-TZP) seem to offer benefits over the alumina or aluminum oxide for dental implants thanks to its higher fracture resilience and better flexural strength (Sennerby *et al.*, 2005).

2.3 Osseointegration

"Osteointegration" or "Osseointegration" refers to a direct junctional interface of bone-to-metal without any soft tissue involvement. Branemark explained a concept that the load carrying implant forms of an extremely differentiated tissue junction with the bone (Branemark, 1983). Branemark observed that Ti implants might become permanently incorporated inside bone. Then the living bone might become therefore united with the titanium oxide layer of the implant and therefore the separation without fracture could not be possible. Thus it was clear that the union of Ti screws and bone may well be helpful for supporting dental prostheses on a permanent basis (Branemark, 1983; Mavrogenis *et al.*, 2009). The inflammatory cells colonize the implant surface primarily, and most of the investigators refer this