CHAPTER 1 INTRODUCTION

To most people, tooth-loss is a matter of great concern and the replacement of teeth with artificial substitutes to preserve and reinstate a comparable functional and aesthetic result, is essential to the continuance of a normal life. This attitude is by no means novel, since both archeological and histological records provide adequate evidence of such endeavors. The recent discovery of a cranium approximately 2000 years old featuring a metallic oral implant, serves to demonstrate that dental implantology is one of the oldest forms of artificial tooth replacement (Glantz, 1998).

Regarding metals, Titanium has been found to be an unusually suitable material for dental implants (Kasemo, 1983). The desire for restorative materials with superior mechanical properties, has motivated the use of Titanium-Aluminium-Vanadium alloys for the manufacture of implants.

Titanium and its alloy forms were limited to wrought appliances until the beginning of the 1980's (Miyakawa *et al*, 1996; Papadopoulos *et al*, 1999; Renner, 2001). Casting of Titanium was difficult due to the high melting point of the metal and its remarkable chemical reactivity at high temperatures (Al Mesmar *et al*, 1999). Employing the lost-wax casting technique and commercially available Titanium ingots (Darvell *et al*, 1995), casting of Titanium has been made possible by the advent of specifically designed casting machines (Ida *et al*, 1984; Hamanaka, 1989) and investment materials that are more stable than the conventional investments (Mori *et al*, 1994).

In the modern era, successful clinical employment of implants requires clear evidence of osseointegration. While the term has found considerable use in the clinical community as a means of describing the anchorage of an implant in bone able

to withstand functional loading, it has eluded satisfactory scientific definition. Thus, the use of the term osseointegration has provided virtually no insight into the mechanisms by which bone becomes juxtapositioned to an endosseous implant surface.

Fortunately, although there is still a paucity of such information, we now realize that the mechanisms by which endosseous implants become integrated with bone, can be subdivided into three distinct bone healing phases, each of which can be evaluated experimentally: The phenomenon of osteoconduction relies on the migration of differentiating cells to the implant surface. Implant design such as surface roughness and the chemistry of the surface have a profound influence on osteoconduction. The second phenomenon, de nova bone formation and bonding onto the implant surface, is generally accepted to be a function of chemical surface reactivity. The existence of micro-topographically complex implant surface, which allows micro-mechanical interdigitations, is essential for bone bonding to the implant surface. De nova bone formation results in a mineralized interfacial matrix. The third leading healing phase is that of bone remodeling (Davies, 1998).

The physiochemical and biological properties of the interface zone between the inorganic implant surface and most tissues constitute a largely unexplored focus of research. When criteria for selecting implant materials are reviewed, it becomes apparent that the entire groups of possible alloplastic implant material fall into one of three categories: metal and metal alloys; ceramics and carbons; and synthetic polymers. Although each of these categories has its own virtue, the choice for a particular purpose is always a compromise between different requirements (Glantz, 1998).

Over a period of approximately 20 years various experimental methods have evolved to predict and evaluate the long-term

success of implant materials. However further criteria for selection of specific dental implant materials remain to be identified or have only been partially delineated.

One group of significant factors that have been identified concerns the chemical characteristics of the implant surface. These factors, which determine the tendency to absorb foreign atoms or molecules onto the surface, are therefore inherently associated with the chemical aspects of biocompatibility (Glantz, 1998).

At a molecular and cellular level, the complex phenomenon of bio-adhesion of dental implants may be enhanced by mechanisms such as wetting and spreading (Baier *et al*, 1984; Kasemo & Lausmaa, 1986). Surface topography has been shown to have a definitive influence on both wetting and cell attachment (Buser *et al*, 1991; Brunski *et al*, 2000). In considering cell attachment and adhesion, it is of utmost importance to note that cells do not attach directly to the implant surface but through an intermediate conditioning film (Glantz, 1998).

Since treatment outcomes in dental implantology are critically dependent on these characteristics, the analytical consideration of the present study will be directed towards that level.

CHAPTER 2 REVIEW OF LITERATURE

2.1 Overview on Titanium

Wilheim Gregor was the first to discover the element we know as Titanium. Martin Klaproth named Titanium after the mythological Titans (the children of heaven and earth) after having discovered what he thought was "a new earth in rutile". He also found that ilmenite contained Titanium and that this element was identical to Gregor's discovery (William, 1981).

2.1.1 Titanium Development

Titanium is widely distributed in the earth's crust (ninth most abundant element) (Parr *et al*, 1985) but it took over 150 years for Titanium to become a commercially available metal due to its high reactivity. Presently the industrial process used for Titanium extraction is based on a reduction process developed by Dr Wilhelm Kroll in the 1930's. The reduction is carried out in an atmosphere of Argon, and the metal is obtained in the form of a sponge (Briquettes). This sponge is then fused into ingots. The conversion of the Titanium ingots into mill products is accomplished by conventional metalworking equipment. Although there have been modifications in the industrial process, essentially the same process is used today (William, 1981).

At present the metal is obtainable in rod, bar or sheet form of various dimensions and must be hand worked or machined into the forms desired - a time consuming and relatively expensive procedure (Beder & Ploger, 1959).

2.1.2 Pure/Unalloyed Titanium

Pure Titanium is a relatively soft non-magnetic material. The physical and mechanical properties of chemically pure Titanium can be greatly varied by the addition of small traces of other

elements such as Oxygen, Iron and Nitrogen. Based on the incorporation of small amounts of Oxygen, Nitrogen, Hydrogen, Iron and Carbon during the purification procedures unalloyed Titanium is available in four different grades I-IV (see Table 2-1) (Donachie, 1984).

Table 2-1: Maximum impurity limits of pure Titanium taken from Donachie (1984).

	Impurity Limits (WT%)				
Type	N (Max)	Fe (Max)	O (Max)	C (Max)	H (Max)
ASTM grade I	0.03	0.20	0.18	0.10	0.015
ASTM grade II	0.03	0.30	0.25	0.10	0.015
ASTM grade III	0.05	0.30	0.35	0.10	0.015
ASTM grade IV	0.05	0.50	0.40	0.10	0.015

ASTM = American Society for Testing and Materials

2.1.3 Titanium Alloy

Although pure Titanium is a very useful material, additions of alloying elements is known to improve the physical properties (William, 1981). Extensive research in the early 1950's resulted in several types of alloys, the most important being Titanium-6%Aluminium-4%Vanadium Alloy (Ti6Al4V) because of its desirable proportion and predictable producibility (William, 1981; Wang & Fenton, 1996).

Titanium alloys of interest in dentistry exist in three crystalline forms: alpha, beta and alpha-beta. These types originate when pure Titanium is heated, mixed with elements such as Aluminium and Vanadium in certain concentrations and then cooled. This treatment produces true solid solutions and the added elements are said to act as phase condition stabilizers (Guy, 1976). Elements that occupy the interstitial sites between the parent Titanium atoms in the lattice tend to stabilize the alpha phase. Oxygen, Nitrogen and Carbon come into this category while Aluminium is a further important

alpha stabilizer. Any of the transition elements is able to stabilize the beta phase i.e. Vanadium, Molybdenum, Niobium and Tantalum (William, 1981).

As Aluminium or Vanadium is added to Titanium the temperature at which the alpha to beta transformation occurs changes to a range of temperatures. In this range, both the alpha and beta form may exist. The alloy form desired is maintained at room temperature by quenching the alloy from the temperature at which the desired form exists. While Aluminium serves to increase the strength and decrease the weight of the alloy, Vanadium is called the beta phase stabilizer and once incorporated into the alloy, is said to inhibit corrosion (Renner 2001). Being an alloy it is 400% stronger and less susceptible to fracture under occlusal forces and stresses (Meffert, 1997).

The stability of the alpha and beta phases may be controlled by alloying additions, leading to a variation in the metallographic appearances of Titanium alloys (William, 1981). There are a number of thermal treatments that may give rise to varying microstructures. If the alloy is heavily worked above 900°C, a structure consisting entirely of transformed beta will be produced. The material will have low ductility and would normally be annealed. If the alloy is directly cast from the liquid phase, the beta phase is stable immediately upon solidification but has to partly transform to the alpha on cooling, leading again to an alpha and beta structure. However, in this case the acicular alpha phase is nucleated within the preexisting beta areas, which on cooling tend to give a much coarser structure. Annealing the cast alloy at 700 to 750°C will homogenize the structure (Williams, 1981).

2.1.4 Properties of Titanium

Titanium exists as a pre metal element listed in the periodic table with an atomic number of 22 and an atomic weight of

47.9. It is a silver gray, very light metal, having a density of 4.505gm/cm^3 at 25°C. It has a melting point of 1665°C though slightly variable data are reported due to the effect of impurities (William, 1981).

Titanium burns in air and it's the only metal that will burn in the presence of Nitrogen. Pure Titanium undergoes a crystallographic change on heating above 882.5°C, and can dissolve several other elements like Silver, Aluminium, Arsenic, Copper, Iron, Gallium, Uranium, Vanadium and Zinc to form alloys (Parr *et al*, 1985).

Titanium exists in two allotropic forms: the low temperature form, known as α(alpha) Titanium that has a close-packed hexagonal crystal structure and the body centered cubic structure called β(beta) Titanium that is stable above 882.5°C (William, 1981).

Environmental resistance of Titanium is dependant primarily on a thin, tenacious, and highly protective surface oxide film. Titanium develops stable surface oxides with high integrity, tenacity and good adherence. The surface oxide of Titanium will, if scratched or damaged, immediately reheal and restore itself regardless of its environment (Wang & Fenton, 1996). The protective passive oxide film on Titanium (TiO_2) is stable over a wide range of pH, potential difference and temperatures and is specially favored as the oxidizing character of the environment increases. For this reason Titanium generally resists mild reducing, neutral and highly oxidizing environments up to reasonably high temperatures. The major exceptions are strong solutions of some acids, particularly Sulphuric acid, Hydrochloric acid, Phosphoric acid, Oxalic and Formic acid and also solutions containing the Fluoride ion (William, 1981; Wang & Fenton, 1996).

2.1.5 Titanium Oxides

Titanium is a reactive metal (Parr et al, 1985) and within a millisecond of exposure to air, a 1nm oxide layer will be formed on the exposed surface of pure Titanium (Kasemo, 1983). Within a minute this layer can become 10nm thick. Within a few seconds the oxidation is often virtually completed, since the surface oxide growth is stopped as a result of the slow transport of Oxygen and unreacted metal atoms (Glantz, 1998). Passivity of Titanium is normally due to a few nanometers ($1nm=10^{-9}m$) thick film of amorphous Titanium dioxide (Schutz & Thomas, 1987).

A metal that is passive does not mean that it will not corrode but that the rate of corrosion is significantly reduced by the presence of the protective surface oxide film. Under physiological conditions in which implants function, even this slow rate may produce adverse tissue reactions (Parr *et al*, 1985).

The passivated oxide layer at the surface of Titanium has the physical properties of a ceramic and most metal oxides share this property (Albrektsson *et al*, 1983). The manufacturing procedure (air composition, impurities, temperature, coolant) influences the composition of the oxide layer and eventually the biocompatibility of the material. Therefore a strict standardization of the procedure and bulk metal is mandatory (Van Steenberghe, 1988; Young, 1988; Keller *et al*, 1989). Many of the Titanium alloys, in which Titanium is present in concentrations of 85% to 95%, maintain the passivity of pure Titanium. Although there is no universally accepted definition of the term "passivity" for our purposes, if an implant metal is oxidized and the oxide does not breakdown under physiologic conditions, the metal is said to be passive or passivated. The oxide layer will repair itself instantaneously on damage such as might occur during insertion of an implant (Parr *et al*, 1985).

Common Titanium oxides appear in three different crystalline forms (anatase, rutile and brookite), each with different physical properties. Rutile (TiO_2) is the most stable and widely used. Of special interest is the dielectrical constant - for anatase it is 48, for brookite 78 and for rutile it is between about 110-117 depending on orientation. No other metal oxides have such high dielectric constants (Albrektsson *et al*, 1983).

There are 7 different possible types of oxides that can form on Titanium materials and they include: (1) amorphous oxide, (2) cubic Titanium oxide (TiO), (3) hexagonal Titanium sesquioxide (Ti_2O_3), (4) tetragonal Titanium dioxide (TiO_2) (anatase), (5) orthorhombic TiO_2 (brookite), (6) tetragonal TiO_2 (rutile), and (7) nonstoichiometric oxides (Ti_xO_y). Normally, anodic oxidation with chromic acid forms amorphous Titanium oxide (Lim *et al*, 2001). The stoichiometric composition of cpTi allows its classification into four grades that vary mainly in the Oxygen content, with grade 4 having the most (0.4%) and grade 1 the least (0.18%) (Albrektsson, 1985).

The oxide layer on alloyed Titanium has a thickness approximately corresponding to pure Titanium. The major differences between pure and alloyed Titanium are (Kasemo & Lausmaa, 1991):-

1. The oxide on the alloy is enriched with the alloying elements Al and V,

2. The surface texture and microstructure are more complex and heterogeneous on the alloy,

3. Even thick anodic oxide films do not crystallize as those on pure Titanium.

The growth of the oxide film obeys the high field theory i.e. the thickness of the oxide film depends on the potential

difference across the film (Young, 1972). Accordingly the natural oxide growth under open circuit conditions has been suggested to be a weak function of time with a very low expected oxide growth rate (Mattsson, 1990).

Normally Titanium forms a passive surface oxide layer that is relatively stable and acts as a barrier against diffusion of elements from within the metal itself to the surface and from the outside onto the metal. In the passive state the rate of dissolution of the oxide layer is relatively low but occurs especially when the environment contains aggressive ions. The presence of contaminants modifies the oxide layer and influences its corrosion resistance. The oxide layers on Ti6Al4V have been found to be more amorphous and Ti6Al4V is generally less corrosion resistant than Titanium itself, which is normally covered by a stoichiometric oxide layer (Louw, 1997).

2.1.6 Corrosion of Titanium

Titanium is well tolerated by tissues; indeed it readily lends itself to the descriptive title of a physiologically indifferent metal (William, 1981) and in tissue Titanium is no chemical burden because of its inert reaction in solution (Steinemann, 1998). When Titanium is coupled with metals with greater corrosion potentials, the other metal may corrode by mechanism of galvanic corrosion (Parr *et al*, 1985) and release metallic species into tissues (Williams, 1982).

In the presence of chlorides, localized breakdown has been found to occur despite the highly passive oxide layer on cpTi and Ti6Al4V. Breakdown events are enhanced by increased acidity, while an alkaline environment may enhance a reaction by being negatively charged in physiological solutions, attracting the positively charged ions with in the body fluids and facilitating bonding leading to bio-acceptability (Shirkhanzadeh, 1996).

The precise amount of Titanium, Aluminium and Vanadium that is released by the implants has not yet been determined. Table 2-2 summarizes average normal values of these elements in humans (Vargas *et al*, 1992).

Table 2-2: Normal values of Titanium, Aluminium and Vanadium in Humans taken from Vargas *et al* (1992).

Element	Total body burden	Normal daily intake	Normal daily excretion rate
Titanium	15 mg	0.3-1 mg	0.3 mg
Aluminium	300 mg	5 mg (mean)	N/A
Vanadium	0.1-0.2 mg	12-28 µg	9.3 µg

N/A – not determined

The normal value of Titanium in serum is still doubtful, but is probably less than $10ng.ml^{-1}$, that for Aluminium is below $10ng.ml^{-1}$; for Vanadium less than $1ng.ml^{-1}$. (Versiek, 1984; Lugowski *et al*, 1987). The concentration of Titanium in the contact tissue around implants is about 100 times higher than that of muscle tissue, but to date no case of local or systemic reaction for Titanium has been documented (Steinemann, 1998).

Based on its biological activity, release of Aluminium ions has the potential to be associated with neurological disorders such as Dialysis Dementia, Alzheimer's disease (Smith, 1988; Bruneel & Helsen, 1988; Pilliar, 1991) and Encephalopathy (Winship, 1992). Aluminium has also been implicated in Osteomalacia, Microcytic Anemia and bone diseases, which include Vitamin D Refractory Osteodydtrophy, and Hypercalcaemia (Smith, 1988; Bruneel & Helsen, 1988).

Toxicity of Vanadium ions is much less understood than the toxicity of Aluminium but is not considered to be very high. Aluminium is mainly associated with irreversible enzymatic

disturbance (Bruneel & Helsen, 1988) as well as inhibition of the mineralization process of bone (Albrektsson, 1989; Pilliar, 1991). Most Vanadium effects are associated with phosphate biochemistry by affecting the mechanism of Na^{+}, K^{+}, Ca^{2++} and H^{+} ATPases, since Vanadium acts as a Phosphorus analog (Boyd & Kustin, 1984).

2.2 Fabrication of Titanium

Titanium in its pure and alloy form was limited to wrought appliances until the beginning of the 1980's, when it was introduced into Prosthodontics as a cast material (Miyakawa *et al*, 1996; Papadopoulos *et al*, 1999; Renner, 2001).

2.2.1 Machining of Titanium

Titanium is available in bar or rod form of different diameters (ASTM, 1999). Fabrication of Titanium into different forms is achieved by use of lathe machines. The desired shape is cut and hand ground through 600- grit Silicon Carbide metallographic papers followed by a final polish with 1µm diamond paste. After polishing, the specimen is cleaned using a solvent (Methylethyl ketone), washed in double distilled water and acid passivated in 30% Nitric acid. Autoclaving is then done. Different companies have their own way of fabricating implants from Titanium but basically follow the above-mentioned process.

2.2.2 Casting of Titanium

Titanium casting is difficult because of its high melting point and remarkable chemical reactivity at high temperatures. To overcome these difficulties special equipment and systems have been developed for casting this metal (Al-Mesmar *et al*, 1999).

There are two ways to cast pure Titanium or Titanium based alloys for use in dentistry. One is to utilize the conventional dental casting technique by alloying and thus lowering the melting temperature of Titanium, and the other is to use a super high temperature resistant mould material and a casting machine suitable for casting of Titanium. To avoid contamination the casting procedure must be carried out in a vacuum or in an atmosphere of inert gas, hence modification of the established melting and casting techniques used for most metals (Beder & Ploger, 1959).

2.2.2.1 Casting Machines

Titanium is cast in specially designed casting machines using different technology compared with that used for conventional noble and base metal dental alloys. Available casting machines are classified into three types (Zinelis, 2000):

a) Inert gas arc-melting/gas pressure casting machines that consist of 2 chamber: an upper chamber (melting chamber) for arc melting under an inert atmosphere (Ar gas) and a lower chamber (mold Chamber) with a muffle in which the molten metal is forced into, under gravitational acceleration and inert gas pressure; (e.g. Castmatic system, Titaniumer machine, Tycast machine, Cyclarc system)

b) Inert gas arc-melting/centrifugal casting machines with vertical or horizontal centrifugal casting;

c) High-frequency induction-melting/gas pressure casting machines.

2.2.2.2 Cyclarc Titanium Casting Machine

Bessing & Bergman (1992) evaluated the castability of unalloyed Titanium in three casting machines and reported that

the Cyclarc machine produced the best results. The Cyclarc Titanium-Casting Machine[1] was produced especially to cast pure Titanium for the field of dentistry (it casts Titanium preserving its original properties). It is an Arc-type casting machine and the Cyclarc equipment contains the melting/casting unit, transformer, and protection gas. It has an extraordinary casting ability by using the pressure-differential method. The casting chamber consists of an upper melting chamber and a lower casting chamber, both of which are connected by a central hole with each other (see 4.1.2.2, p58). The casting ring is set at the bottom of the hole, and a crucible made of copper lies on top of it. An ingot or billet is placed in the centre of the copper crucible. First both the upper and lower chambers are evacuated by a built-in vacuum pump. The high degree of vacuum exhaust eliminates the reaction of molten Titanium with Oxygen, Nitrogen and moisture. After the air is exhausted, compressed Argon is fed into the upper chamber and an arc is generated between a Tungsten electrode (-) and the ingot/billet (+). Since the lower chamber is kept under vacuum (12Pa), a flow of Argon is maintained in the casting ring due to the pressure differences between the upper and lower chambers. Immediately after the billet has melted in the Argon environment, the molten metal falls down through the central hole and runs into the mould in the ring (Anony, 1991; J Morita Corp., Kyoto, Japan).

2.2.2.3 Investment Material

Two methods of casting have been developed which seem satisfactory. The first is shell molding and the second is investment casting with which the dental profession is familiar (Beder & Ploger, 1959). The properties required for an investment material for use in casting include controlled thermal expansion, hot strength to resist cracking and

[1] J Morita Corp., Kyoto, Japan

fracture, low hot deformation and minimal interaction with the metal to be formed (Curtis, 1998).

It is difficult to cast Titanium into a mould that is made of conventional investment materials. Therefore, more stable oxides than Titanium have been experimentally applied as refractory agents for its casting; such oxides include Calcia, Magnesia and Zirconia. In many dental laboratories, on the other hand, a Phosphate Bonded Alumina/Silica investment has been used for the same purpose (Miyakawa *et al*, 1989).

Phosphate bonded investment materials are extensively used in the lost wax casting technique because of their heat resistance at applicable casting temperatures. The thermal expansion of the refractory mould is used in the casting process to compensate for the solidification shrinkage of the cast alloy. Chemical interactions are known to occur between Phosphate bonded investment materials and cast metal surfaces during casting hence the necessity to grit blast cast metal surfaces to remove refractory material that adheres to the casting after devesting (Curtis, 1998).

2.3 Osseointegration

The process of "osseointegration" is considered of pivotal importance for establishing an intimate and stable connection between bone and implant surface (Branemark, 1983). Branemark *et al* (1985) describes "osseointegration" as a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant without soft tissue intervention.

According to Meffert (1997), osseointegration needs to be redefined into "adaptive osseointegration" in which the osseous tissue approximate the surface of the implant without any apparent soft tissue interface and "biointegration" is a direct biochemical bone interface.

How well an implant is fixated to the surrounding bone tissue is dependant on different implant and host related factors. The exact biological, biomechanical, and operative conditions which promote optimal osseointegration of implants are not known though six factors listed by Albrektsson *et al* (1981) and Matsuda *et al* (1998) have gained a general acceptance as being specially important: biocompatibility, design, surface quality, status of host tissue, surgical technique and loading conditions. The first three factors are implant related.

The mechanisms by which implants become integrated in bone can be subdivided into three separate phenomena, each of which can be tested experimentally (Davies, 1998). Bone may be formed on an implant surface either by distant or contact osteogenesis (Osborn & Newesley, 1980). In distant osteogenesis, new bone is formed on the surfaces of bone in the peri-implant site, similar to normal appositional bone growth. The existing bone surfaces provide a population of osteogenic cells that lay down new matrix, which as osteogenesis continues, encroaches on the implant itself - surrounding it. One can deduce that the implant surface is obscured from bone by intervening cells and connective tissue. In the second phenomena of contact osteogenesis, new bone forms first on the implant surface - de nova bone, which requires the recruitment of potentially osteogenic cells to the site of future matrix formation (see Fig 2-1, p17) (The Colgate Oral Care Report, 2000).

Puleo & Nanci (1999), suggest that bone is deposited directly on the surface of the implant, extending outwards from the biomaterial. Thus bone formation in the peri-prosthetic region occurs in two directions; not only does the healing bone approach the biomaterial, but bone also extends from the implant towards the healing bone. Interestingly the bone that forms away from the implant forms at a rate of about 30% faster than that moving towards the biomaterial.

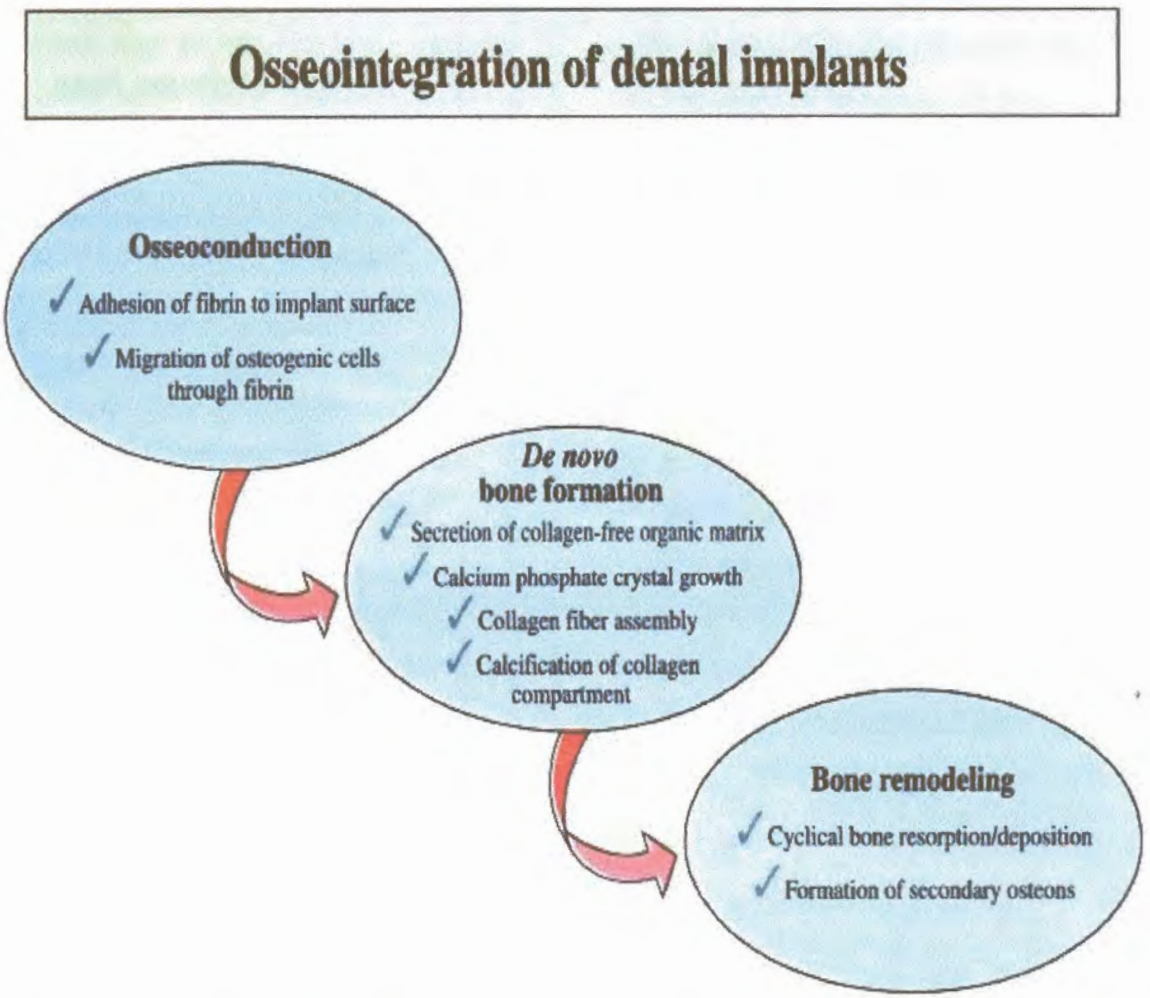

Fig 2-1: Three separate phenomena of osseointegration taken from The Colgate Oral Care Report, 2000)

In reality, one can assume that any implant healing compartment will display both the distance and contact osteogenesis phenomena. Subsequently, a third tissue response, that of bone remodeling will also, at discrete sites, create de nova bone formation at an implant surface (Davies, 1998).

If osseointegration is not successfully achieved, fibrous tissue is generated instead, leading ultimately to loosening and loss of the implant (Groessner-Schreiber & Tuan, 1992). A properly osseointegrated implant at the cortical passage should have a minimal direct bone contact of 90% to 95% of the implant surface (Albrektsson & Jacobsson, 1987).

Osseointegration is associated with intimate and long lasting contact between bone and the alloplastic tooth root

replacement material. There is as yet no generally accepted device or method for the objective clinical assessment of osseointegration (Zarb & Albrektsson, 1993).

2.4 Titanium and its Alloys as Dental Implants

Implant materials used by early practitioners of implant dentistry included Gold, Precious stones, Ivory, Irridium, Irridio-platinum, Vanadium and others (Balkin, 1988).

Even at very early stages of implantology it was acknowledged that it makes sense not to use materials that are frankly toxic, either to the tissues immediately surrounding the implant or systematically. Likewise, materials that are quite brittle or relatively soft were understood early on not to be adequate (Glantz, 1998).

Beder & Ploger in 1959 described the use of Titanium as intra-oral implants. Presently Titanium in various forms appears to be the most popular metallic implant material in use due to its high corrosion resistance against chemical attacks, desirable physical and mechanical properties and its excellent bio-acceptability (Buser *et al*, 1991; Glantz, 1998).

The dental implant differs from the typical orthopedic fixture, which heals and remains in a buried environment, in that the dental implant is intimately connected with the oral cavity, as it is semi-buried (Dmytryk *et al*, 1990).

According to the ASTM (1995) XI Rationale of the Standard Specifications for Unalloyed Titanium for Surgical Implant Applications, the choice of composition and mechanical properties is dependent upon the design and application of the medical device. Alloys possess many favorable mechanical and physical properties that make them excellent implant materials. The finer microstructure and two-phase alloy indicate a higher material strength. This condition improves

certain properties, such as fatigue strength, which is critical for some implant designs (Lemons, 1986).

The Titanium oxide or oxides never allow for any molecular contacts to be established between the true implant metal and the host tissue, but rather between the tissue and the surface oxide(s) of the implant. Even if surface oxides of metals and metal alloys contain both defects and impurities they still form stable oxides - TiO_2 (Glantz, 1998).

2.5 Classification of Dental Implants

Dental implants may be classified according to their constituent material, their position, and their design or physical form.

a) By constituent material: Most implants are made of metal. The metals include commercially pure Titanium, Stainless Steel and other alloys like Ti6Al4V (Worthington, 1988).

b) By position, they may vary in that they may be placed (Worthington, 1988):

- Beneath the periosteum (subperiosteal implants)
- Through the full vertical thickness of the lower jaw (transosteal implants).
- They may be inserted into the bone of either jaw (endosteal implants)

c) By design: Titanium can be easily prepared into many different shapes and textures without affecting its biocompatibility (Schwartz *et al*, 1996). There are two types of retention for the endosseous implant: mechanical and direct bonding. Mechanical retention would consist of some type of macro-retention that is visible such as threads, pores, grooves, steps, holes, slots, dimples, etc. into which the bone would grow during the healing phase. Direct bonding

implies a chemical bonding of the bone to the implant material, such as would occur in ankylosis (Meffert, 1986).

The design of implants must suit the desired application, and in implant dentistry, screw and cylindrical implants are the preferred shape because they are relatively easy to place and remove (Gotfredsen *et al*, 1988; Gotfredsen *et al*, 1992; Albrektsson, 1993). Introduction of threads to the cylindrical implant improves the implant stability significantly (Frandsen *et al*, 1984).

2.5.1 The Implant Surface

The surfaces of currently available dental implants range from relatively smooth machined surfaces to those roughened by coatings, abrasions or blastings, acid etching, or combinations of these techniques (Buser, 1999).

Rough surfaces can be made using an addition or a subtraction technique. For instance, the addition of a hydroxyapatite (HA) surface coating on implants is popular because it is a highly biocompatible material. Subtraction techniques are simpler and provide a more uniform roughness and grit blasting is most commonly used (Mugnolo & Filliponi, 2001). The term "roughened" is erroneously used today to describe many different implant surface modifications, even though these modifications do not make the modified implant surface rougher than machined surfaces (Klokkevold, 2002).

2.5.1.1 Machined Surfaces

Threaded implants represent the most common design of dental implants, and although their primary form of fixation is macroscopic mechanical interlock with bone, much finer microscopic surface features exist that may influence the osseointegration potential of these designs (Branemark *et al*, 1977).

The primary mode of fixation of threaded implants relies on the macroscopic thread geometry. By appropriate selection of thread geometry, stresses dissipated in bone can be lowered and this presumably will reduce the possibility of localized crestal bone loss (Pilliar, 1998).

The microscopic features are related to the machining operation used for forming the threaded shapes and include fine machining lines, pits and gouges as well as some zones where metallic debris has been "cold welded" to the surface (Pilliar, 1998).

Differences in appearance due to variation in machining techniques do exist. It is however recognized that different implant companies have surfaces that are similar but not identical (Cochran, 1999).

The relatively smooth machined implant surface gained early popularity in the United States after its introduction in Canada in the early 1980's. Presently virtually every dental implant manufacturer markets implants that feature some form of roughened surface (Cochran, 1999).

2.5.1.2 Osseotite Surface

Chemical etching has also been used to develop textured bone-interfacing implant surfaces for enhanced implant fixation. This technique has been used for modifying surfaces of blade-type implants as well as threaded and cylindrical root-form implants subsequent to machining or other forming operations. As a result of controlled surface chemical attack that results from exposure to acid solutions, small pits form more or less regular arrays over the implant surface. Typically these are micron- to sub-micron sized and results in increasing the surface area of the implant (Pilliar, 1998). This implant is characterized by a hybrid design, a short, approximately 2- to 3-mm long machined surface in the crestal area, and an acid-

etched (Sulphuric-acid/Hydrochloric-acid) surface overall[2]. The Osseotite surface features micro-pits produced by the etching procedure, but its profile is flatter since no grit blasting procedure is used prior to the etching resulting in a Ra value of 1.3µm (Buser *et al*, 1998).

Lazzara *et al* (1999) assessed the Osseotite surface and concluded that the surface develops a statistically higher bone to implant contact and had an enhanced osteoconductive potential in poor quality bone when compared to the machined surface. Even if similar techniques are utilized to obtain a roughened surface, the conditions of the treatment could have an effect. For example, if an acid is used, the concentration of the acid, the time of exposure and the temperature of the acid bath can all influence the resulting surface characteristics.

2.5.1.3 Grit Blasted Surfaces

One approach for producing surfaces with irregularities is to grit-blast using an appropriate blasting medium. Typically SiC, Al_2O_3, glass or TiO_2 shot (particles) is used to erode a substrate to form irregular surfaces with pits and depressions that vary in size and shape depending on the blasting conditions (blasting medium, pressure, distance from blasting nozzle to implant surface, blasting time). Blasting also enlarges the surface area of an implant. Wennerberg *et al* (1996), demonstrated that the size of the blasting particles have a great effect on the amount of bone-implant contact, while the type of particle has no effect.

The Sand blasted, Large grit, Acid etched (SLA) surface was launched in mid June 1998 at the ITI world symposium in Boston and it is said to have a macro/micro double roughness (Scacchi, 2000). Pioneering work by Buser *et al* (1991), using

[2] Osseotite, Implant Innovations, Palm Beach Gardens, Florida, USA.

Scanning Electron Microscopy described the SLA surface as being characterized by a primary roughness produced by the sandblasting process that creates "valleys" whereas the acid-etching procedure removes remnants of grits and attacks the Titanium surface producing a secondary roughness that is apparent in the scanning electron micrographs. According to Buser (1998), the rough-blasted surface had superimposed micro-pits of 1- to 2-µm-diameter and a Ra value of 2.0µm.

The SLA is a solid screw implant (4.05mm diameter), characterized by a sandblasted (Large grit of 250 to 500µm and acid etched (sulphuric-acid/hydrochloric-acid) surface[3] and has consistently showed the best results both in histomorphometric and biomechanical testing. Aaboe *et al*, (2000) also showed that osseointegration of subperiosteal implants could be obtained when the surface was sandblasted and acid etched. However, long-term clinical studies on the survival of these implants have not been published.

2.5.1.4 Bioactive Coated Surfaces

The term bioactive or surface-active material is commonly used to describe those materials that generate direct biological or chemical bonding to their surfaces (Davies, 1989).

Plasma spray coating of implants to create a porous surface was first reported by Hann & Palich (1970). The Titanium sprayed surface is obtained by thermal spraying of Titanium on to the Titanium implant giving it a semi-porous structure. Plasma spray technique has also been used to coat Titanium implants with HA - hydroxyapatite (Buser *et al*, 1991).

Dental implants formed by sintering Ti6Al4V alloy powders to a machined Ti alloy substrate represents still another approach for achieving fixation by bone in-growth and micro-mechanical

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interlock (Pilliar *et al*, 1991). Following sintering, an integral implant structure is formed with a solid inner region providing overall mechanical strength and a porous surface region within which bone can form, thereby resulting in secure implant-to-bone fixation.

The uniqueness of this sintered porous structure compared with the other surface structures described previously, is the interconnectivity of the pores of appropriate size to allow rapid bone ingrowth during healing. The Ti6Al4V alloy powders used to form the porous surface zone are nearly spherical in shape by virtue of the atomization process used for their formation. Clinical experiences with sintered porous-surface dental implants indicate cumulative success rates over 8-year periods similar to those reported for other designs despite the fact that porous-surfaced implants of significantly shorter lengths were used (Pilliar, 1998).

In contrast to calcium phosphate coatings, biochemical surface modifications utilizes critical organic components of bone to affect tissue response. Coatings incorporating biomolecules are also being explored for delivering biomolecules to the tissue-implant interface (Brunski *et al*, 2000).

According to Rieger *et al* (1989), neither clinicians nor manufacturers should assume that bioactive coatings or bone bonding in general improve the biomechanical prognosis of implants.

2.6 Surface Characterization

Surface characterization is critical to biomaterials science because it provides the following information: unknown identification, reproducibility assurance, quality assurance, contamination detection and correlation between measured surface properties and biological responses (Baier & Meyer,

1988). The different techniques used for characterization are summarized with regard to their utility in Table 2-3.

Table 2-3: Surface analytic techniques used to study material interactions with host fluids and tissues taken from Baier & Meyer (1988).

Technique	Utility
Profilometry	Surface texture roughness
Electron Microscopy	Ultrastructure, surface morphology
Electron Spectroscopy for chemical analysis	Elemental and chemical bonding data for outermost 0.001 to 0.01 μm of surface
Scanning auger microprobe analysis	Elemental and Chemical state data for outermost 0.001 to 0.005 μm; depth profiling

Biomaterials research requires specific information about the quality of a surface utilized for experimentation or application. The most important contribution that surface characterization could make to the field of biomaterials science would be the direct correlation of surface structure or chemistry with the *in vivo* performance of materials in humans. This desirable goal, unfortunately, is still far from being realized. An intermediate goal might be to use physical surface characterization tools to perform preliminary screening on materials in order to eliminate unlikely samples from expensive biological evaluation (Ratner, 1983).

There are several ways to modify surface chemistry, topography and microstructure, although controlling all the parameters simultaneously is very difficult (Larsson, 2000). Various preparative procedures are known to introduce differences in surface topography and surface energy (Smith *et al*, 1991). Comprehensive surface characterization is therefore necessary to interpret cellular and, ultimately, tissue response to such surfaces (Smith *et al*, 1991).

2.6.1 Compositional-Considerations

The properties of an implant are determined, in part, by its chemical composition (Schwartz *et al*, 1996). The important fact that the implant surface consists of a relatively thick Titanium oxide must be remembered when the chemistry of the implant-tissue interface is considered. The chemical properties, and therefore the interface chemistry, are determined by the oxide layer and not by the metal itself (Mesaros, 1989). A dense oxide film with a thickness of approximately 10nm covers the bulk Titanium metal. Because surface oxides grow by inward transport of oxygen atoms (from O_2 molecules or H_2O in the atmosphere) and/or outward metal atom transport from the oxide-metal interface, there will be some concentration gradients for Oxygen and Titanium (Kasemo, 1983).

The chemical properties of the biomaterial surface play an important role for the tissue responses elicited by the material. This is at least one main reason why the tissues respond differently to different materials. A material with a surface that is accepted by the tissue seems to exhibit improved integration with bone, either due to passive growth, leading to a tight connection between implants and bone, or by stimulation that probably leads to bone implant bonding (Ellingsen, 1998).

It is now well understood that the surface composition of most implant materials is substantially different from the bulk, and a variety of techniques have been used to characterize such surfaces (Ratner, 1988; Smith, 1991). Klauber *et al* (1990) found considerable variation in surface contamination on as supplied dental implants with some showing high levels of Silicate in the oxide layer. Lausmaa *et al* (1990) found a dominance of Titanium and Oxygen peaks with relatively strong Carbon signal that they assigned as a normal observation for exposed surfaces. The Titanium and Oxygen signals show that

the surface consists mainly of Titanium oxide layer. Taborelli *et al* (1997), found negligible chemical compositional differences between samples they analysed and related it to the stability of the oxide layer formed upon exposure to air. Variable chemical composition from preparative procedures may also be accompanied by changes in surface topography and surface energy (Smith, 1991). Surface composition can be assessed at different depths - 1nm or greater than 5nm.

2.6.1.1 X-ray Photoelectron Spectroscopy (XPS)

XPS is used to elicit surface elemental and chemical information. The sample is exposed to a flux of X-ray photons, which excite electrons within the sample. The energy of the electrons is quantitized, and therefore discrete and the energy position of these (discrete) electronic energy levels is different for every atom (or solid) in the periodic table. Thus, the number of excited electrons with the energy close to each particular energy level will be higher than the number of electrons excited at other energies (Kittel, 1996). Moving through the solid towards the surface the electrons undergo (inelastic) collisions with ions and other electrons (Dowben, 1994). Therefore, only those electrons that were excited close to the surface (typically few electronic mean free paths away from the surface) have a reasonably high probability to reach the detector and be detected. Thus the information in XPS comes from a region close to the surface, at most 25nm below the outermost atomic layer (Krozer *et al*, 1999).

Surface analysis by XPS involves irradiating a solid *in vacuo* with monoenergetic soft X-rays and analyzing the emitted electrons by energy. The spectrum is obtained as a plot of the number of detected electrons per energy interval versus their kinetic energy. Each element has a unique spectrum. The spectrum from a mixture of elements is approximately the sum of the peaks of the individual constituents. Quantitative data

can be obtained from peak heights or peak areas and identification of chemical states often can be made from exact measurements of peak position and separation as well as from certain spectral features. Each element has a unique set of binding energies and XPS can be used to identify and determine the concentration of the elements in the surface. The electrons leaving the sample are detected by an electron spectrometer according to their kinetic energy. The analyzer is usually operated as an energy window referred to as the pass energy, accepting only electrons that are within the range of its window. A broad survey spectrum is obtained to identify the elements present, after which higher resolution scans can be performed around an elemental peak to identify chemical states. Curve fitting is normally applied to the high resolution peaks (Moulder *et al*, 1992).

A spectrum is used as a "fingerprint" of the surface (Kasemo and Lausmaa, 1988b). The atomic percent of each element on the surface can be calculated from the photo peak by marking and measuring the area by use of the XPS computer software. The detection limit of the machine for elements is ~ 0.1%. Identification of the different spectra can be done by use of the Handbook of X-ray Photoelectron Spectroscopy (Moulder *et al*, 1992).

Various photoelectron peak constituents can be obtained from peak separation measurements and full widths half maximum values (FWHM) using the Gaussian-Lorrentzian curve fitting for elements with atomic percentage greater than 3% (Kilpadi *et al*, 1998a; Kilpadi *et al*, 2000).

As mentioned the sample is irradiated with X-rays, most commonly XPS can be performed at angles of ~5° to 90° with the sample normal, depending on what depth one wants to extract data from. If the sample surface is not totally flat, screening of the irradiating source by surface features can occur and "dead spots" are created on the surface.

2.6.2 Surface Topography

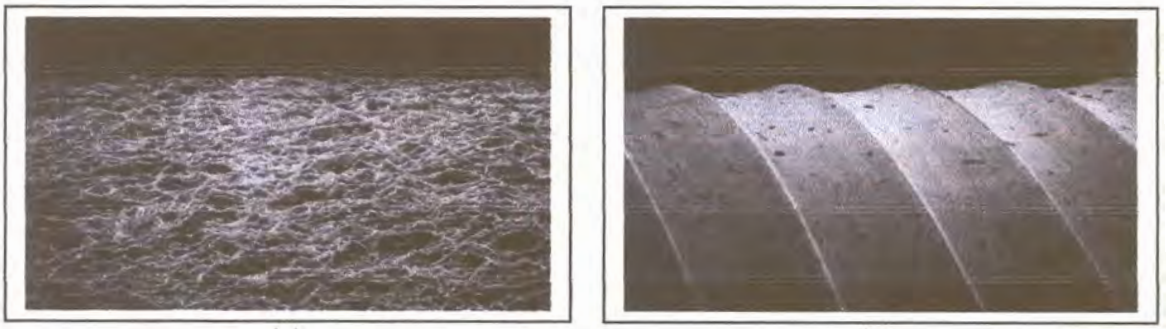
A surface is the boundary between two media and can be divided into two categories: functional and non functional. A non-functional area can be either mirror smooth or sandpaper rough without influencing the quality of the part while a functional surface is required to perform a function that is related to the users perception of the part's quality. Regardless of the function, the surface texture has a direct influence on the quality of the part. When the correlation between surface topography and part function is known, quality can be optimized. A surface is made by a combination of one or more formative processes. There are three broad categories of production processes that can influence surface topography:

1. Material removing processes
2. Material adding processes
3. Forming processes

Processes can also be classified as either random or periodic. A random process is one where there is no discernable pattern on the surface. This is called an isotropic surface (investment casting)(see Fig 2-2a, p30). A periodic process creates a surface that has evenly spaced irregularities on it (turning). This is called an anisotropic surface (see Fig 2-2b, p30). A particular manufacturing process is capable of producing a limited range of surface roughness values (Mummery, 1992).

Surface geometric features are categorized as either macroscopic or microscopic. Macroscopic surface features can be readily characterized by unaided visual observation (magnification instruments are not needed). Typically, macroscopic features have dimensions measured in millimeters or greater. Microscopic variables describe features that can only be characterized fully with the assistance of optical or

electron microscopy. The dimensions of these features range from hundreds of microns (but less than a millimeter) to submicrons (Pilliar, 1998).



(a) (b)

Fig 2-2: a) Cast surface (An isotropic surface) and b) Machined surface (An anisotropic surface) taken from Mummery (1992)

Another important factor of the surface configuration is the microstructure of the implant surface probably in conjunction with macro-irregularities. This can vary considerably depending on the surface treatment of the implant. Variation of the surface microstructure has been reported to influence the stress distribution, retention of the implant in bone and cell responses to the implant surface. Surface roughness on a smaller scale was, however, found to be important for integration of the bone with the implant surface (Ellingsen, 1998).

Surface roughness on the atomic scale can, on the contrary, also influence the (local) chemical bonding through the particular geometric arrangements that can be established. When van der Waals interaction is prevailing, surface roughness will in principle also modify the interaction since it influences the local electromagnetic fields at the surface (Kasemo, 1983). The optimal surface topography for cell attachment to implants may differ for different cell types (Chehroudi *et al*, 1989). Abron *et al* (2001) in their study confirmed that alterations in cpTi implant topography alter the degree of bone formed at the implant surface. They associated a "rougher" implant surface with increased bone-to-

implant contact. Two surfaces with very different (Ra and Rp-v) parameters (non-ideal vs machined) did not show differing abilities to support bone formation. These results indicate the gross inadequacy of defining an implant in general terms of "rough or "smooth".

If two surfaces with different roughness characteristics are measured, the results for each surface should yield different values; this difference should be reflected by the roughness parameters. If the values do not show the difference, the measured data is meaningless and another parameter should be chosen. A roughness parameter should be used only if it is sensitive to the important surface characteristics. The selection of roughness parameters should be carried out with this criterion in mind (Mummery, 1992). The characterization of the surface topography was previously performed with stylus profilometry, a method frequently used to characterize Titanium surfaces with different roughness values (Wennerberg *et al*, 1992). The Atomic Force Microscope is another type of instrument that has been employed to access surface roughness.

2.6.2.1 Atomic Force Microscope (AFM)

The Atomic Force Microscope is a recent innovation that relies on a mechanical probe for generation of magnified images. The essential component in the AFM is a sensor with very high spatial resolution. The sensors can routinely measure distances with an accuracy of 0.01nm. The force sensor measures the deflection of a cantilever such that when the cantilever moves the light beam from a small laser moves across the face of a four-section photo detector. Imaging modes sometimes referred to, as "scan modes" or "operating modes" are the methods that are used to move the AFM probe over the sample surface and sense the surface in order to create an image. Imaging modes can be classified as "contact" or "non contact" depending on the net forces between the probe

and sample. In non-contact imaging mode the cantilever is pulled towards the sample by attractive forces while in the non contact imaging mode the cantilever is curved away from the sample due to repulsive forces. The force most commonly associated with atomic force microscopy is an inter-atomic force called the van der Waals force. The cantilever is held on the order of tens to hundreds of angstroms from the surface sample. The tip of the cantilevers for the AFM microscope are fabricated from Silicon Nitride and is located at the free end of a cantilever that is usually 100 to 200µm long (Anony, 1996).

There are several scanners available and the choice of scanner depends on the dynamic scan range required. Scanning Probe Microscopes were used solely for measuring surface topography and though they can be used to measure many other surface properties it is still their primary application (Anony, 1996).

The AFM microscope with its software is normally used for interpreting images created as a result of the AFM scanning probe. Depending on the required investigations either a 3D or 2D image is displayed after the removal of any scan line anomalies. Using the 3D image, values of area analysis can be measured namely Ra, RMS, maximum range, average height and projected surface area (see Area analysis, p33). For line analysis, required lines are drawn either on the horizontal, vertical or transverse axis on the 2D scanned image also after leveling and shading. Software is then used to define the values of the drawn lines namely Ra, Rp, Rpm, Rt, Rtm (see Line analysis, p33-34) (Anony, 1996).

The scanners come in different sizes ranging from 130µm to 7µm. Normally depending on the image required a corresponding scanner is used but smaller scan images can be zoomed from selected areas and analyzed (Anony, 1996).

Each of the values of area analysis and line analysis can be defined as follows (Mummery, 1992):

a) Area analysis

i) Area Ra - Ra is the average roughness of the scanned area.

ii) Area RMS - RMS is the root mean square of Ra. It is more sensitive than the numerical average of Ra.

iii) Average height - The highest point from the zero value on the whole surface is regarded as the Average height.

iv) Maximum range - Maximum range is the range between the highest to lowest point along the surface area, even below the zero value.

v) Projected surface area - calculated area increase due to surface topography.

b) Line analysis

i) Ra -Average roughness -The mostly used roughness parameters is Ra. In the past, Ra was known as the CLA - Center Line Average in England or AA - Arithmetic Average in the U.S.A. It is expressed in microinches or micrometers and does not differentiate between peaks and valleys. Since it is the average deviation of the profile from the mean line, defects in the surface do not influence the measured results greatly.

ii) Rt -Is a true indication of the Maximum peak height to the Maximum valley depth of the roughness profile over the total assessment length. The values of Ra (see (i)) and Rtm (see (iii), p34) are more useful than Rt.

iii) Rtm -Is the Mean peak-to-valley height within an assessed profile. It is the average of the Peak-to-valley heights. Since maximum profile heights and not averages are being examined, it is generally more sensitive to changes in surface finish than Ra (see (i), p33). Rtm values that are similar indicate a consistent surface finish while a significant difference indicates a surface defect in an otherwise consistent surface.

iv) Rp -Is the Maximum peak height (the highest single peak) within the line profile.

v) Rpm -Mean peak height is the distance from the highest peak to the profile mean line that is determined within each cut-off length. The mean of the peak heights is the parameter Rpm. It is a valuable tool in monitoring the variation of surface finish in a production process. Values that are similar indicate a consistent surface finish while a significant difference indicates a surface defect in an otherwise consistent surface.

Rpm is useful in predicting the bearing characteristics of a surface. A low Rpm and a large Rtm (see (iii)) indicates a plateau surface. The ratio Rpm/Rtm quantifies the asymmetry on a surface bearing area. Therefore a low ratio of Rpm/Rtm is preferred for bearing surfaces.

2.6.3 Depth Profiling

As already mentioned (see 2.1.5, p8) Titanium is a reactive metal. This means that in air, water or any other electrolyte, an oxide is spontaneously formed on the surface of the metal (Steinemann, 1998). The native oxide film has a thickness of about 4nm or 20 times the inter-atomic distance. Its mode of

growth is specific in that the Oxygen ions migrate towards the metal and react with the counter-ion Titanium at the base of the oxide. The specific mode of oxide growth on Titanium has the positive effect that no metal ion will reach the surface and be released into the electrolyte (Steinemann, 1998). An important consequence is that a biological molecule approaching the implant from the bone side sees a metal oxide and not the metal surface (Albrektsson *et al*, 1983). Larsson *et al* (1997) found that the oxide thickness of machined samples was about 3-5nm and increasing the oxide thickness of rough machined implants had no significant effect on bone response.

There is no absolute means of measuring Titanium oxide thickness, but oxide thickness can be estimated from depth profiles (Binon *et al*, 1992). Depth concentration profiles are maps of the concentration of various elements as a function of the distance from the outermost surface into the material. This is achieved by ion bombardment (sputtering) of the surface to remove layer by layer of surface atoms, thus exposing deeper lying atomic layers for analysis (Kasemo & Lausmaa, 1988a). In a depth profile the relative concentrations of the elements are displayed as a function of sputtering time (depth from the surface). Sputtering is often continuous and as the depth profiles pass through the oxide, over the oxide metal interface and into the bulk metal, the Titanium signal increases and the Oxygen signal decreases, as expected. The oxide thickness can be estimated from depth profiles as the depth at which the oxygen signal had decreased to half of its maximum intensity (Lausmaa *et al*, 1990; Larsson *et al*, 1997; Louw, 1997). Other researchers (Keller *et al*, 1990; Ong & Lucas, 1998) approximated the oxide thickness by multiplying the sputter rate by the crossover time obtained from the auger depth profile during continuous sputtering. Crossover time is the time corresponding to equal intensities of the Titanium and Oxygen auger signals.

The sputter yield or sputter rate is dependent on a number of factors, such as material being sputtered, crystallinity of the material and orientation to the ion beam. It is well known that metallic oxides sputter at different rates and many sputter more slowly than the pure metal. Although referenced literature assumes constant ion sputtering rates, the rate actually increases, as the oxide is sputter removed. Absolute sputter rates are unknown because of the continuously changing composition of the specimen with depth. In most instances where reliable and reproducible thickness measurements are required, the sputter rate is measured by sputtering a layer of standard material of known thickness for a known period of time (Ong & Lucas, 1998; Louw, 1997).

During 1993/1994, the first scanning X-ray photoelectron microprobe was introduced into the surface analytical market. It utilizes a scanned focused electron beam from a LaB_6 electron filament that generates X-rays on an Aluminium anode. The X-rays are focused using an ellipsoidal monochromator and produces a scanned, focused X-ray beam on a sample surface. The X-ray beam diameter is variable and X-rays can be rapidly scanned across a surface, generating secondary electrons that form an X-ray induced secondary electron image (SXI). An important advantage of the Quantum 2000 is that only the active areas defined on the image are irradiated, minimizing X-ray induced sample damage. Sputter depth profiles can be performed with compucentric Zalar rotation, where the sample is rotated around a center point defined by the selected analysis point. With Zalar rotation, buried interfaces can be examined with no loss of depth resolution, as surface roughening and cone formation are minimized (Louw, 1997).

Depth profiling is normally conducted with the photoelectron analyzer set at the same resolution as for the high-resolution spectra. The sputter depth profiling is usually conducted with a preferentially pumped focused ion gun in the "alternating"

mode, where the sample is sputtered; then the ion gun is turned off and the photoelectron spectra is recorded (Louw, 1997).

2.6.4 Cleaning and Sterilization of Implants

A surface that looks perfectly clean to the eye may still be extremely dirty (contaminated) in a chemical sense, since it may be covered by hundreds of molecular layers of foreign, contaminating elements, invisible to the naked eye. In the strictest sense a clean surface means that the bulk material extends all the way to the surface, without change of chemical composition, and with no foreign elements attached to the surface (Kasemo & Lausmaa, 1988b).

Unsaturated chemical bonds will attract air impurities and it will take a few seconds at most before the surface is contaminated by several percent of a molecular monolayer. After about one minute most of the unsaturated chemical bonds are saturated. The nature of the contamination layer is also determined by the composition of the environment to which the implant is exposed (Kasemo & Lausmaa, 1988b).

Surface contamination must not be regarded a *"priori"* as having a negative effect on biological responses. The effects could actually be beneficial in some cases, but the significance is unknown (Aronsson *et al*, 1997).

Probably the most common source of both inorganic and organic superficial contaminating deposits on implant devices is from sterilization procedures. It has been noted previously that conventional processes of steam autoclaving, ethylene oxide gas sterilization, glass bead immersion, and cold solution exposure all leave disabling residues on the implant surface (Baier *et al*, 1982; Doundoulakis, 1987).

National and international standards on the properties of metallic biomaterials for surgical implants are fairly broad,

allowing for several possible procedures prior to implantation - cleaning, passivation and sterilization that may include exposure to elevated temperatures (Albrektsson *et al*, 1981).

ASTM (1997)- The Standard Guide for Descaling and Cleaning Titanium and Titanium Alloy Surfaces states that it is not intended that these procedures be mandatory for removal of any of the indicated contaminants but rather serves as a guide when Titanium and Titanium alloys are being processed in the wrought, cast, or fabricated form.

Several techniques have been suggested to produce a clean and sterile Titanium implant surface with minimal contaminants. A common procedure used in the past for the Branemark implant was to clean the Titanium fixture with residue free detergent, rinse with distilled water and clean ultrasonically with a series of butanol and ethanol (Hobo *et al*, 1989) followed by autoclave sterilization at 2psi and 120^{0}C for 20 min (Hobo *et al*, 1989; Hartman *et al*, 1989; Keller *et al*, 1990;). Another method of cleaning was ultrasonic cleaning for 20 min in 99% ethanol and air dried in a laminar flow hood (Kononen *et al*, 1992). Baier *et al* (1982) found that steam sterilization compromised the properties of carefully prepared biomedical implants by depositing hydrophobic organic and hygroscopic salt contaminants over the implant surfaces.

In addition to contaminated surfaces by adsorption of organic molecules from ambient air (Kasemo & Lausmaa, 1988b; Mittal, 1979) impurities due to specific preparation methods on biomaterials are residues from grit blasting media (e.g. Silicon and Aluminium) residues or reaction layers from the processing fluids (electrolytes, etchants, and cleaning solvents), and residues from sterilization procedures (e.g. autoclaving or ethylene oxide) (Kasemo & Lausmaa, 1988b).

The ultimate goal of a cleaning procedure should be to remove the contaminants and restore the elemental composition of the

surface oxide without changing the surface topography (Mouhyi *et al*, 2000). Bone tissue response seems to be disturbed by contaminated implants as compared to clean controls (Ivanoff *et al*, 1996).

2.6.4.1 Radio Frequency Glow Discharge Treatment (RFGDT)

Plasma treatments have for some time been a relative common method for increasing the surface energy and for surface cleaning of biomaterials before using them in biological evaluation studies (Kasemo & Lausmaa, 1988b; Baier & De Palma, 1970). Baier *et al* (1984) found RFGDT to be an effective method for cleaning and sterilizing inorganic surfaces and elevating them to a high energy state correlated with greater cellular – adhesion potential.

The criterion for efficient plasma cleaning is that the process removes chemical impurities without adding new ones (Aronson et al, 1997). RFGDT technique basically utilizes a vacuum chamber and a radio frequency generator to take a gas into a "plasma" state. A characteristic of a plasma is a visible glow discharge caused by the electrically excited elements returning to lower energy levels (Fassel, 1978). After a vacuum has been obtained, Argon gas is allowed into the chamber and the radio-frequency generator activates the gas into a plasma state. Impaction of the plasma's ion and electrons on objects within the chamber acts to sputter away and then microash any surface contaminants from inorganic objects (e.g. implants) leaving them in sterile, high surface energy states that improve their wettability, thereby facilitating adhesion by organic materials (Baier & De Palma, 1970).

Vargas *et al* (1992) hypothesized that the specific glow discharge treatment applied, modified the superficial oxide layers of the implants in a two-step process. First by removing the contaminated over-layers rich in hydrocarbons and

other elements, and then "leveling" the subjacent, irregular oxide layer remaining from prior manufacturing and sterilizing procedures. This removal of both superficial contaminants and oxide protrusions (e.g. oxide needles, as proposed by Solar *et al*, 1979) resulted in a clean, more uniform oxide layer that reacts with Oxygen present in the environment to create a new, more coherent and corrosion resistant "passive" surface. Sterilization by RFGDT for 3 to 5 minutes was found to produce an extremely high surface energy with minimal contaminants for samples with flat surfaces (Doundoulakis, 1987; Baier & Meyer, 1988).

A drawback of RFGDT is that the very clean surface is also very reactive and will react with even minute impurities in the process gas, or with impurities that are desorbed from the walls of the process chamber. As the energetic ions hit surfaces other than the sample under treatment, material from these other surfaces may thus be deposited on, and contaminate the sample. After RFGDT is terminated the clean, reactive, high-energy surface will then be exposed to the remaining atmosphere in the vacuum chamber depending on how fast the atmosphere is evacuated. Finally the removal of the sample from the RFGDT apparatus will definitely cause exposure to a new environment (Kasemo & Lausmaa, 1988b).

Redeposition of sputtered material is frequently reported for different plasma systems. It becomes a serious problem if the sputter rate is too low, sputtered material is not pumped away fast enough, or if the initial surface contamination is large. Upon readsorption on the surface, the particles react with the surface atoms, and then reaction layers such as carbides and/or nitrides are formed. These layers can be difficult to sputter away and therefore often remain after the plasma treatment (Baier & Meyer, 1988).

Carlsson *et al* (1989) and Hartman *et al* (1989) found glow discharge to be a suitable preoperative cleaning and sterilization method for Titanium implants.

2.7 Cell Culturing

Irrespective of their use and destination, all materials (metallic or non metallic) designed for biomedical application in humans must be submitted to bio-acceptability *in vitro* and *in vivo* tests (Davies, 1989) aimed at verifying the response and the behavior of the cells interacting with them, according to the rules of the ISO 10993.

A general *in vitro* approach applicable to all types of medical devices is represented by the cytotoxicity method performed by incubating nearly confluent monolayers of mammalian cells in the presence of extractables from the device material (indirect method). In the case of devices projected and destined to be colonized by tissue in which they are implanted, the *in vitro* biocompatibility test can be accomplished more usefully by a direct contact method. In this approach the cells are cultured directly onto the material under investigation and observed over a period of days or weeks relative to their morphologic and functional features. In the direct contact method, biomaterials should be tested with cell populations typical of the implant site (Calandrelli *et al*, 2002).

In vitro assays are relevant to *in vivo* behavior but the choice of *in vitro* assay must critically depend upon both the type of information sought and the geometry of the testing system (Davies et al, 1989). When designing either an *in vivo* or an *in vitro* cell culture experiment it is essential to define the aims of the experiment and which aspects of the tissue reaction to the implant are to be investigated (Davies, 1989). Investigation of continuous cellular and tissue reactions to a material using *in vivo* studies is impossible

because of the wide deviation due to the existence of a number of unknown factors. *In vitro* methods using the techniques of tissue culture allow a more easily controlled test situation and higher statistical accuracy than when an *in vivo* system is used and has become one of the important methods for the biological standardization of dental materials (Kawahara *et al*, 1968).

In vitro methods can potentially be specifically designed to investigate the reactions of single cell types to implant surfaces. *In vitro* assays are usually more rapidly carried out than *in vivo* assays and almost inevitably cheaper (Davies, 1989). Assessing the attachment and growth of various cell types to a biomaterial *in vitro* offers a well controlled, quantitative, and cost effective model of interactions at the tissue-implant-interface. Not only can the biocompatibility of the implant be determined, but also the physical and chemical characteristics of the implant surface can be modified to maximize the attachment and growth of selected cell populations (Kamen, 1989).

Since materials which are destined for human implantation should, ideally be tested with human cells rather than animal cells, it is only by developing methods *in vitro* that there is a chance that these methods may be adapted for use with human cells (Davies, 1988).

2.7.1 Types of Cells used in Culture

Of particular importance is the use of cultured cells that are as close as possible in origin and phenotype to the cells of interest (Kamen, 1989). Connective cell and bone cell models are increasingly employed to study bone-biomaterial interactions. Primary and passaged cells from several species and anatomical locations have been used, as well as several cancerous, transformed, clonal and immortalized cell lines (Puleo & Nanci, 1999).

According to Mustafa *et al* (1998) and Mustafa *et al* (2000) the importance of using the relevant cell systems when evaluating dental implant materials has been clearly established and they recommend the use of human oral fibroblast and osteoblast-like cells derived from human mandibular bone.

i) Osteoblast cells

Bone is the only tissue of higher vertebrates that differentiate continuously, remodel internally, and regenerate completely after injury (Urist *et al*, 1983). The mechanical stimuli are the main mode for controlling bone turnover since osteoblast differentiation is a stress/strain responsive process (Mesaros, 1989).

Bone tissue comprises three cell types. Osteoblasts arise from undifferentiated mesenchymal cells and are responsible for the formation and mineralization of bone matrix. Osteocytes, osteoblasts that have become surrounded by the matrix they have elaborated, are responsible for maintaining the matrix. Osteoclasts resorb bone matrix and are thus essential for the process of bone remodeling (Davies *et al*, 1989).

Osteoblasts, osteoid, and mineralized matrix have been observed adjacent to the lamina limitans, (Linder 1985; Davies *et al*, 1990; Murai *et al*, 1996) suggesting that bone can be deposited directly on the surface of the implant, extending outward from the biomaterial. Thus bone formation in the peri-implant region occurs in two directions: not only does the healing bone approach the biomaterial, but bone also extends from the implant towards the healing bone (Puleo & Nanci, 1999)(see 2.3, p16).

Osteoblasts are ideally positioned to function as cellular mediators of hormonally regulated skeletal metabolism and evidence suggests that the effects of osteotropic hormones, cytokines and prostaglandins on the skeleton including

osteoclast activation are mediated through the osteoblast (Rodan & Martin, 1981). Osteoblast-like cells demonstrate a cell morphology with a central spherical body with filopodial-like processes extending away from the central area in all directions (Bowers *et al*, 1992).

A variety of methods have been developed to grow osteoblast-like cells. Many studies employ the method of using enzymatically-digested cells from bone fragments (Matsuda *et al*, 1987; Ishaug *et al*, 1997) consequently bone-forming cells have been obtained from newborn calvaria of different animals. Bellows *et al* (1986); Keller *et al* (1994); Ishaug *et al* (1997), used stromal osteoblastic cells isolated from calvariae of Sprague-Dawley rats by collagenase digestion. Bowers *et al* (1992) and Lumbikanonda & Sammons (2001) used parietal plates from 3 day old Wistar rats calvarial explants as osteoblast while Sudo *et al* (1983) and Cooper *et al* (1993) established the clonal MC3T3-E1 cell line from new born calvaria that resembles osteogenic cells *in vitro* in many ways (Ham, 1969) and a number of studies employed the culture system using this MC3T3-E1 cell line (Itakura *et al*, 1988; Ito *et al*, 1995; Kurachi *et al*, 1997). Transformed osteoblast-like cells (Schwartz *et al*, 1996), chick embryonic calvaria (Groessner-Schreiber & Tuan, 1992) and immature New Zealand rabbits (Cheung & Haak, 1989) have been used as a source of most cell types and have also been a favorite model for studying the interaction of an implant with "bone" cells.

Human osteoblast-like cells from human osteosarcoma have also been used (Martin *et al*, 1995; Kieswetter *et al*, 1996). Schmidt *et al* (2002) used collagenase treated human explants from bone tissue of femur or femoral head while Anselme *et al* (2000) used trabecullar bone from the iliac crest. Mustafa *et al* (2001) used human mandibular bone to harvest osteoblasts.

ii) Fibroblasts

In human gingiva, the fibroblasts lay down and maintain the dense fibrous connective tissue enmeshing the vascular plexus and sensory nerve fibres of the lamina propria (Takarada *et al*, 1975).

Considerable progress in the field of connective-tissue research has brought about detailed information on the behavior of fibroblastic cells under culture conditions and *in vivo* situations. Well-characterized biological functions of fibroblasts include cell migration, cell attachment, cell proliferation and biosynthesis as well as degradation of multiple connective tissue components. An exact control of these functions is essential in physiological processes of tissue development and repair, while on the other hand, fibrotic disease or poor wound healing are associated with functional deviances of fibroblasts (Heckmann & Krieg, 1989).

An acceptable bone to implant interface is equally dependent on the development and maintenance of a suitable soft connective tissue implant interface in the coronal implant region (Pilliar, 1998).

Fibroblasts cells from procine periodontal ligament were isolated and sub-cultured by Brunette *et al* (1976). Since then most studies have employed the use of human gingival fibroblasts (Inoue *et al*, 1987; Kononen *et al*, 1992; Oakley & Brunnette, 1993; Botha, 1995).

Another source of human fibroblasts was that obtained from fore skin explants - this cell model has been used to study the effect of implant surface modifications on cellular responses and there is no evidence to indicate that these cells are phenotypically different from fibroblasts derived gingival connective tissue (Keller *et al*, (1989).

Few studies have used Mouse fibroblastic L929 cells (Morra & Cassinelli, 1997; Orsini *et al*, 2000) or 3T3 mouse fibroblasts (Saltzman *et al*, 1991).

2.7.2 Cell Attachment

Rate of cell attachment characterizes cells and indicates their viability. Cellular attachment and regeneration are influenced by the method of disaggregation (Weiss & Kapes, 1966). Therefore passage of cells by enzymatic digestion or any other method influence subsequent cellular attachment, spreading and proliferation for an extended period of time after the actual process has been inactivated (Weiss & Kapes, 1966; Davies, 1989; Clavin *et al*, 1990)

In order for normal embryonic or adult cells from avian or mammalian organisms to survive and divide *in vitro*, they stringently require adherence to a specific type of tissue culture substrate. Malignant cells, however, frequently loose this stringent anchorage dependence and can be adapted to grow as single cells in suspension (Culp, 1978). Cells transformed with oncogenic viruses or derived from tumors have been shown to move more aggressively across the substrate (Gail & Boone, 1972).

It is apparent from the literature that osteoblast-like cells and other anchorage-dependent cells, such as fibroblasts, show similar morphologic behavior in attachment studies (Brunette, 1988; Zreiqat *et al*, 1996). The cell membrane is the first contact a cell has with its surrounding environment and as such must play an important role in determining cellular activity and function (Shelton *et al*, 1987). The strength of adhesion of a cell settling on the substratum would increase progressively as the area of its contact with the substratum increases (Grinnell *et al*, 1973).

The process of cell adhesion and spreading consists of four events (Rajaraman *et al*, 1974)

- Attachment of cells at point of contact with the substratum - this was effected by microvilli-like cell processes.
- Centrifugal growth of filopodia - the microvilli like processes elongate into processes called filopodia
- Cytoplasmic webbing - filopodia growth always precedes cytoplasmic webbing during cell spreading
- Flattening of the central mass.

Cell spreading starts immediately after adhesion of cells to the surface has taken place. The process of cell attachment and spreading cannot be divided by specific time intervals but is an inter-twining process (Chen, 1981; Grinnel & Bennett, 1981; Brunette, 1986a). Most studies have used tissue treated polystyrene as a standard against which to measure cell attachment to the surface of biomaterials (Kamen, 1988).

2.7.3 Cell Detachment

The usual approach to studying the process of cell attachment is to detach cells from their growth surface by trypsinization and to place them on the test surface. The number of cells attached to the surface is then measured as a function of time (Brunette, 1988).

Weiss & Kapes (1966) indicated that crystalline Trypsin gave the best results as a cell detachment agent and a study by Clavin *et al* (1990) suggested that a mixture of Trypsin and EDTA (Ethylene-Diamine-Tetraacetic-Acid) yielded the most desirable single cell suspensions. Thom *et al* (1979) used Tetracaine (local anaesthetic) as a cell detachment agent and results of Botha (1995) support Tetracaine as an alternative cell detachment agent. Tetracaine causes paralyses of the

cells thereby lifting them off the surface. It requires a much longer time interval for action compared to Trypsin-EDTA.

2.7.4 Inoculation/Seeding of Cells

According to Ishaug *et al* (1997) cell-seeding density affected initial cell attachment and proliferation rate, but differences became less significant over time. Different studies have used different cell concentration or densities for inoculation or seeding. Morra & Cassinelli (1997) and Brunette *et al* (1976) used a seeding concentration of $1X10^5$ cells.ml^{-1} while Botha (1995) seeded cells at a concentration of $3\text{-}5X10^5$ cells.ml^{-1} and Kononen *et al* (1992) seeded cells at a density of $2X10^4$ cells.cm^{-2}.

2.7.5 Estimation of Cell Number

Several methods exist for the quantification of cell numbers in a specific culture, namely, surface measurement, counting chambers, electronic particle counter and many others. Groessner-Schreiber & Tuan (1992) used the method of measuring cellular protein content and level of cytosolic enzyme to compare cell numbers between cultures while Guy *et al* (1993); Cooper *et al* (1993) used tritiated thymidine to determine cell attachment.

Electronic particle counters[4] have been used for decades. It has obvious advantages of simplicity, accuracy and rapid operation but has the disadvantage that it counts all cells in suspension regardless if they are viable or not (Harris, 1959).

The method of using counting chambers such as the Neubauer haemocytometer has also been used in haematology for several decades with great success. Hemocytometer counts do not distinguish between living and dead cells but a number of

[4] Coulter Cell Counters

stains are useful to make this distinction. Trypan blue among others[5] is excluded by the membrane of the viable cells where as the nuclei of damaged or dead cells take up the stain. Although this distinction has been questioned, it has the virtue of being simple and giving a good approximation (Hakkinen *et al*, 1988; Saltzman *et al*, 1991; Botha, 1995).

In previous investigations, the numbers of attached cells have usually been determined indirectly from counts of detached cells (Bowers *et al*, 1992; Keller *et al*, 1994). Botha (1995) determined the number of attached cells by counting an aliquot of the cell suspension using a haemocytometer.

The viable cell concentration in the cell suspension can be calculated per ml by the following formula:

Viable cells per ml = average viable cell count per square X dilution factor X 10^4

The non-attached cells can also be counted to confirm the accuracy of the method (Marmary *et al*, 1976).

2.7.6 Cell Proliferation

Proliferation of cells represents the final stage of the cellular processes involved in the viability and survival properties of cells and can be summarized in sequential stages of contact, attachment, spreading and proliferation (Kamen, 1988). The efficiency of cell attachment and cell proliferation can be calculated into a percentage by the following method (Botha, 1995):

$$\% \text{ Attachment efficiency and proliferation} = \frac{VT}{VI} \times 100$$

VT = Number of viable cells per ml at a given time

VI = Number of viable cells per ml in initial suspension

[5] Erythrosin B, Nigrosin

2.8 Bio-acceptability

The cycle of events when a cell is placed on a biomaterial surface indicate that the whole process of attachment, spreading and proliferation are important criteria for the evaluation of surface bio-acceptability (Davies, 1988).

Many surfaces of biomaterials, including metals and polymers, could provide adequate mechanical support for the attachment of cells. The differences in cell behavior following attachment to different surfaces must be related to physico-chemical properties of the surface (Saltzman *et al*, 1991).

Titanium has an excellent bio-acceptaility due to the fact that it is highly insoluble in body fluids and forms a protective oxide layer on the surface (see 2.1.4, p7; 2.1.5, p8; 2.1.6, p10). In addition to its chemistry, however surface topography of the bulk material is important (see 2.6.2, p29). An analysis of cell behaviour carried out under well-controlled conditions allows conclusions about the bio-acceptability of a material (see2.7, p41) and cellular morphology is an accepted parameter of bio-acceptability tests (Schmidt *et al*, 2002). *In vitro* studies with cells from target tissues are useful tools for the investigation of implant behaviour, because these cells react differently on various surface structures and chemical properties (Schmidt *et al*, 2002). Several *in vitro* experiments and animal studies have demonstrated the importance of the implant surface to host response during healing and the quality of implant anchorage in bone (Buser *et al*, 1991; Bowers *et al*, 1992; Gotfredsen *et al*, 1992; Buser *et al*, 1998). From the animal studies, results showed that the interaction between implant material and the surrounding tissues was affected by the surface structure and that better bone contact was achieved to implants with rougher surfaces than with polished surfaces (Buser *et al*, 1991; Gotfredsen *et al*, 1992; Wennerberg *et al*, 1996). In most studies the type of surface preparation and/or

characterization methods used prevent any firm conclusions being drawn as to which surface properties were the determining factors for the observed differences in biological response (Larsson *et al*, 1997).

2.8.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy can be used to quantitate cell adhesion to implant surfaces hence enabling the evaluation of cell attachment to different surfaces (Kasten *et al*, 1990). Keller *et al* (1994) using the scanning electron microscope found that in general cells appeared spherical in shape with early signs of filopodial extensions extending to adjacent areas of the prepared surfaces. Over time considerable spreading had occurred on rough surfaces, however it was not clear how intimately the cells had adapted to the irregularities on the respective surfaces. There were no significant differences in cell morphology between cpTi and Titanium alloy surfaces. Mustafa *et al* (2000) used the SEM to quantify the percentages of attached cells on each of their specimens and they confirmed that in accordance to other previous studies that the type of roughness on a Titanium surface affects cell attachment and spreading. Brunette (1986b) found that fibroblast cells had adopted an elliptical shape and increasingly aligned with the direction of the grooves and cells on smooth Titanium surfaces were circular in shape. Kononen *et al* (1992) found that fibroblast attachment and spreading was not so good on sandblasted Titanium surfaces as on electropolished Titanium surfaces and the fibroblasts on sandblasted surfaces grew in clusters, suggesting that their ability to migrate was impaired. Lumbikanonda & Sammons (2001) found that on smooth Titanium implants, cells were polygonal or circular in shape with complete cytoplasmic extensions of the cell body on the substrate surface forming intimate contact with the implant surface such that the underlying topography of the surface was visible beneath the cell. On rougher surfaces cells spanned across pits, grooves and pores, mostly contacting prominent features of the surface.

CHAPTER 3 AIM OF THE STUDY

A great interest in the use of cast Titanium for dental prosthesis has evolved in the dental community. Because of its high melting point and strong tendency to oxidize, Titanium is cast in specially designed dental casting machines. The use of cast Titanium restorations in dentistry are still very low and probably related to the lack of knowledge among dentists or long term clinical follow-up.

Rehabilitation treatment utilizing dental implants is not a readily available service to most in the southern hemisphere due to its high cost. The relatively high cost of implants could be related to its fabrication and the different methods employed for surface enhancement that is regarded as a "priori" for bio-acceptability. Depending on its bio-acceptability an alternative to the high priced available implants in the market could be cast Titanium and Titanium alloy implants, which could then be manufactured into custom made implants and implant superstructures.

The Aim of this study was:

1. To determine and compare the elemental and chemical composition of Machined and Cast Titanium and Titanium alloy before and after surface enhancement by using

    - Atomic Percent Concentration of elements on the surface of samples

    - Chemical composition by curve fitting

2. To determine and compare the surface topography of the differently fabricated samples by using

    - Area Analysis

    - Line analysis

3. To determine and compare the oxide thickness formed on samples as a result of the different fabrication procedures by using

    - Depth Profiles

4. To evaluate the effects of RFGDT on the different fabrication procedures by

    - Analyzing samples before and after RFGDT

5. To access the degree of bio-acceptability of the above mentioned variables with *in vitro* fibroblasts and osteoblast-like cell cultures by determining

    - Count of viable cells and

    - Cell % attachment efficiency and proliferation

CHAPTER 4 MATERIALS AND METHODS

4.1 Fabrication of Specimen Discs

4.1.1 Preparation of Machined Discs

Custom-manufactured experimental discs measuring 6.35mm in diameter and 2mm in thickness were manufactured from rods of both commercially pure Titanium (cpTi) Grade 3 and Titanium alloy (Ti6Al4V) Grade 5 (see Table 4-1).

Table 4-1: Description of materials used in this study for sample fabrication

Material	Batch No.	Source
cpTi	BN 3720	T. Wire, Weissenstrasse 8, CH 8034 Zurich, Switzerland
Ti6Al4V	000XY07	L. Klein SA, Lanfeldweg 110, 614 Bienne/biel, Switzerland
President Putty	KJ390	Coltene® A G Feldwiesenstrasse 20, 9450 Altstatten, Switzerland
DuraLay Resin	022900	Dental Mfg.Co. 5805 W, 117th Pl. Worth, Illinois, U.S.A.
Rematitan Plus	029527	75104 Pforzheim, Federal Republic of Germany

The discs were manufactured using machining procedures that were identical to those used for manufacturing commercially available machined-surface, threaded Titanium implants. Southern Implants[6], carried out these commercial production procedures according to proprietary specifications.

The prepared discs were cleaned using alternating ultrasonic cycles of ethanol and N-hexane and were sterilized using gamma radiation with a dose level of 30kGy as used for dental implants. Packing was done under a laminar flow hood with air filtered through a 2µm Hepa filter to remove dust and microorganisms. Fig 4-1 (see p55) shows the packaged samples as received from Southern Implants.

[6] Southern Implant, P.O. Box 605 Irene 0062, South Africa

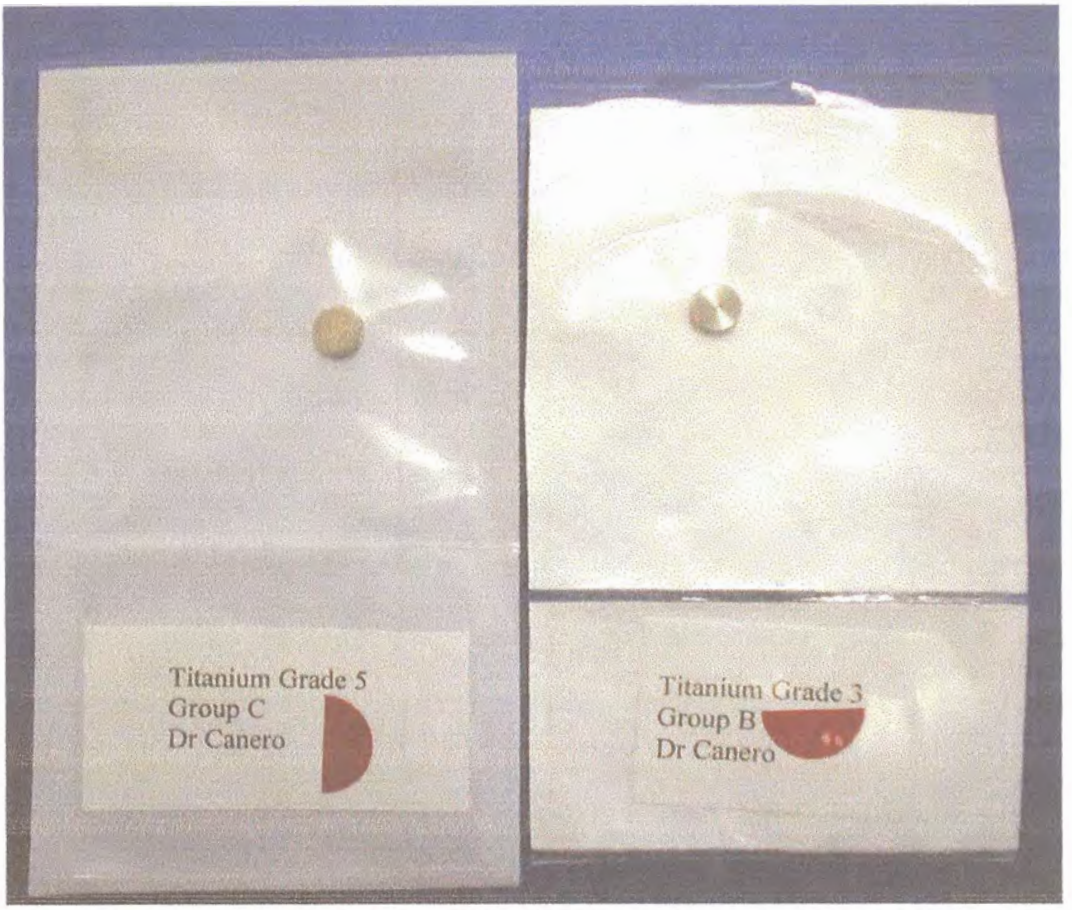


Fig 4-1: Sample discs of cpTi (Grade 3) and Ti6Al4V (Grade 5) as received from Southern Implants

4.1.2 Fabrication of Cast Discs

Ingots with identical chemical compositions as those used for the machined discs were used to cast discs of similar dimension.

4.1.2.1 Preparation of Resin Disc Patterns

A duplicating mould using a glass pipette measuring 6.35mm in diameter and 100mm in length was made from President impression putty (see Table 4-1, p54). After removal of the pipette, standardized acrylic resin rods were fabricated using DuraLay, a Chemical polymerizing Inlay Pattern Resin (see Table 4-1, p54). The resin was prepared by mixing a ratio of 1ml monomer with 0.95gm polymer in a mixing bowl. The mixed resin was then poured into the duplicating mould and allowed to polymerize at room temperature. After curing, the resin

rods were removed from the mould and inspected for processing defects. Fig 4-2 shows the duplicating mould used and the obtained cured resin disc.

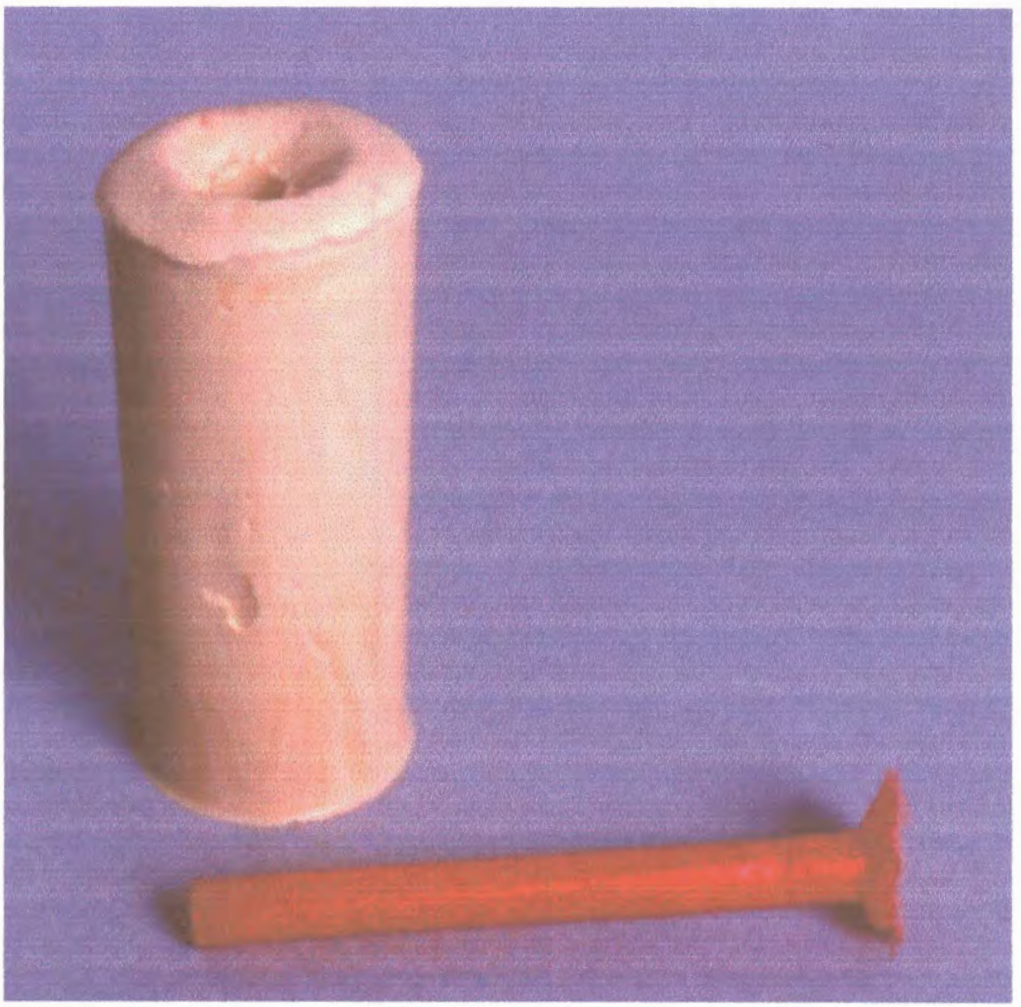


Fig 4-2: DuraLay resin rods as fabricated from the duplicating mould

These rods were then sectioned into discs of 2mm thickness using a Slow Speed Cutting Saw (Isomet^{FM})[7] (see Fig 4-3, p57).

Dimensional accuracy of all discs was checked by a digital caliper[8] and corrected using Silicon Carbide 600 grit sandpaper[9] with water.

[7] Buehler Lake Bluff, Illinois, USA
[8] Laboratory & Scientific Equipment Company (Gauteng) (Pty) Ltd. P. O. Box 1296, North Riding 2162, South Africa
[9] IMP Scientific and Precision Equipment, P. O. Box 1110, Boksburg 1460, South Africa.

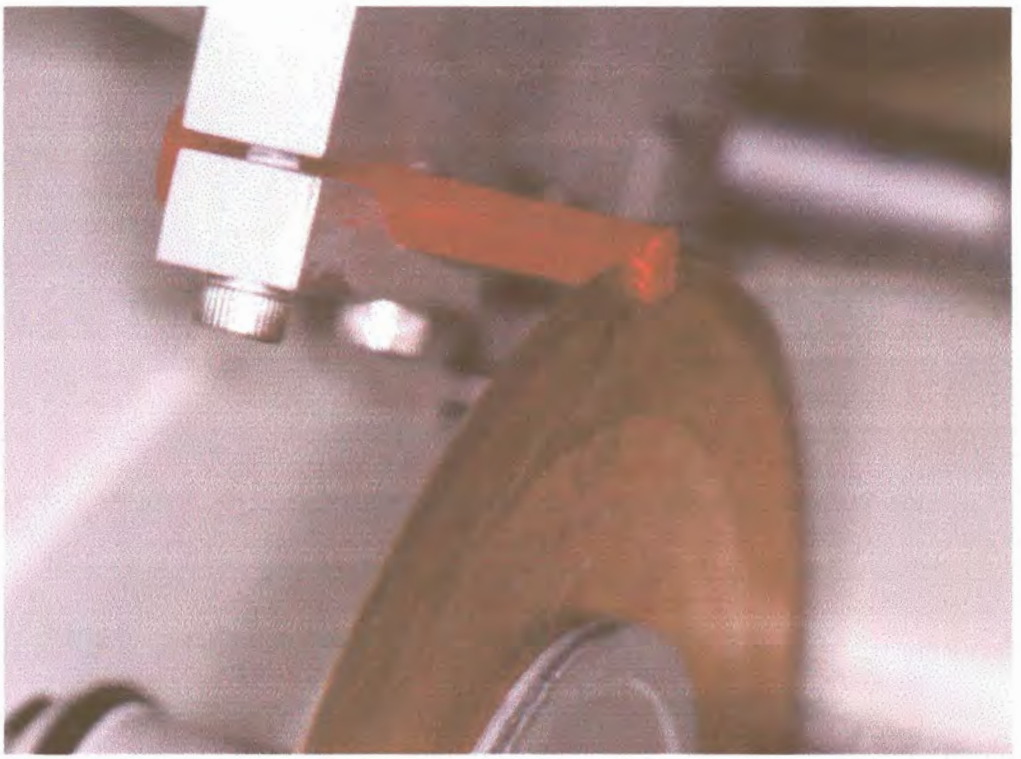


Fig 4-3: Slow speed cutting saw sectioning resin rods into discs

4.1.2.2 Casting Procedures

Twelve groups of 12 resin disc patterns were sprued and invested with a Phosphate bonded investment material, Rematitan Plus (see Table 4-1, p54). The powder and liquid were mixed at prescribed ratios in vacuum for 30 seconds prior to pouring into 60mm in diameter and 50mm high cylindrical moulds. After bench drying for 24 hours, the mould with the resin patterns underwent a three-stage thermo-cycle: 250°C for 20 minutes, 500°C for 20 minutes and finally 920°C for 60 minutes. The heating rate between the hold times at the respective temperatures was 5°C per minute. The invested discs were made from DuraLay resin because it volatizes in 40 minutes at approximately 300°C leaving, no residue. Prior to casting, the mould was cooled down to 500°C at a rate of 5°C per minute.

A Centrifugal Titanium Casting System[10] (see Fig 4-4, p58) was used to cast cpTi and Ti6Al4V into the moulds. The casting

[10] Morita Cyclarc Casting Machine, Kyoto, Japan

chamber was evacuated to a pressure of 13.33Pa three times and back filled with 99.99% pure Argon gas.

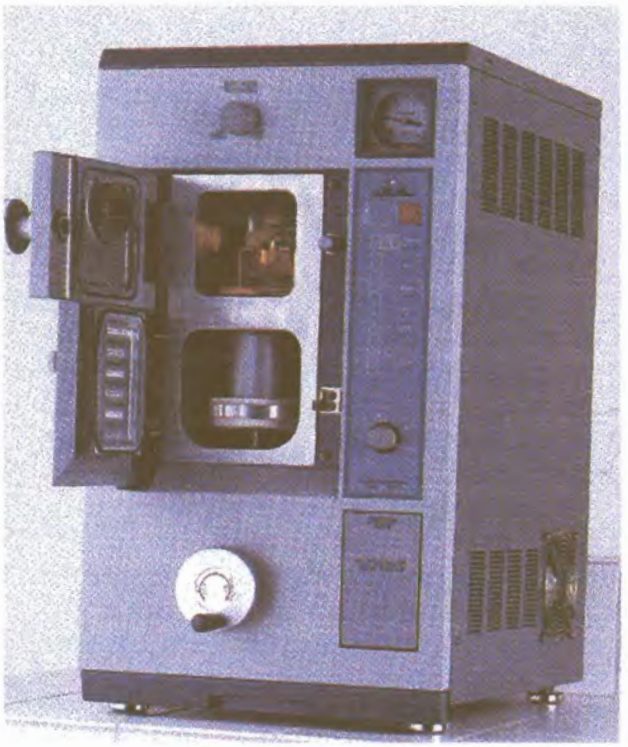


Fig 4-4: The Morita Cyclarc Casting Machine (J.Morita, Europe, GMBA)

Surgical ingots, each of 25mm diameter and 11mm in height of cpTi and Ti6Al4V were arc-melted in the copper crucible in an Argon atmosphere under gas pressure of 0.69MPa. Heating was facilitated by Tungsten electric arc melting with an electric arc current of 250A until the major portion of the ingot was liquidified. The liquid Titanium was then cast using a vertical centrifical system. The Argon supply continued for about 2 minutes after the molten metal was cast into the mould. Hereafter the machine stopped automatically and air was allowed to enter the casting chamber (Anony, 1991).

After the casting had solidified in the moulds, the partially cooled moulds were immersed in water at room temperature. The cast patterns were sectioned and divested using a small hammer. The remaining closely adhered investment material was removed by grit blasting the casting with 100µm Aluminum particles at 5-6bar for about 3 minutes at a nozzle distance

of approximately 60mm. The cast patterns were detached from the sprue with separating disc, specifically grouped for the envisaged experimental procedures and stored in labeled Argon filled sterile plastic containers (Anony, 1991).

4.2 Preparation of Different Surfaces

Specific predetermined topographies were introduced on selected surfaces of the machined and cast fabricated disc groups of cpTi and Ti6Al4V (see Table 4-2).

Table 4-2: Summary of the designation of sample material, fabrication procedures adopted and introduced surface enhancement.

Material Employed (N=144)	**Method of Fabrication** (N=72)	**Types of Surface Characterization** (N=24)
Commercially Pure Titanium (cpTi)	Machined Grade 3	As is (Control surface)
		110 sandblasted and etched (SI)
		250 sandblasted and etched (ES)
	Cast Grade 3	As is (Control surface)
		110 sandblasted and etched (SI)
		250 sandblasted and etched (ES)
Titanium Alloy (Ti6Al4V)	Machined Grade 5	As is (Control surface)
		110 sandblasted and etched (SI)
		250 sandblasted and etched (ES)
	Cast Grade 5	As is (Control surface)
		110 sandblasted and etched (SI)
		250 sandblasted and etched (ES)

SI – surface enhancement according to Southern Implants, Irene, South Africa
ES- experimental surface enhancement

Depending on the specific type of surface enhancement, the non selected surface or opposite base of the disc was coded by engraving (see Fig 4-5).

Fig 4-5: Coded sample discs

4.2.1 Non-enhanced Surfaces

The first group features samples of cpTi and Ti6Al4V as obtained from both machined and cast procedures - These samples were used as controls (see Table 4-2, p59).

4.2.2 Enhanced Surfaces

The second and third test groups also featured samples of cpTi and Ti6Al4V from both machined and cast procedures that were further enhanced.

4.2.2.1 Surface Enhancement of Samples according to Southern Implants (SI)

The second group featured samples of cpTi and Ti6Al4V as obtained from both machined and cast procedures that were surface enhanced according to production procedures of Southern Implants. Enhancement entailed grit blasting using

110µm Aluminum particles and acid etching at room temperature (see Table 4-2, p59).

4.2.2.2 Experimental Enhancement of Samples (ES)

The third group featured samples of cpTi and Ti6Al4V as obtained from both machined and cast procedures that were enhanced by grit blasting with 250µm Aluminum particles and acid etched using a 20% concentration of Hydrochloric-acid/Sulphuric-acid for five minutes at room temperature (see Table 4-2, p59).

4.3 Sterilization by Radio Frequency Glow Discharge Treatment

RFGDT was used as a method of cleaning and sterilizing samples. Twenty-one of the 24 samples from each of the specified groups underwent RFGDT[11]. The remaining three samples from each group were the controls to treatment rendered.

After the different samples were placed into the vacuum chamber - the chamber was backfilled with Argon gas (99.9% pure) to a partial pressure of $1X10^{-2}$ mbar and the radio - frequency generator activated the gas into a plasma state using a current of 200W for 10 minutes. Impaction of the plasma's ion and electrons on objects within the chamber acts to sputter away and then subsequently microash all the surface contaminants. The flow of pure Argon gas through the chamber sweeps the contaminants off the sample surface and out of the chamber. Controlled oxidization of the clean surfaces was then obtained using the same 200W current and an Oxygen pressure of $5X10^{-3}$ mbar for one minute. The background pressure was maintained at $\leq 5X10^{-4}$ mbar. After RFGDT sample discs were stored in sterile plastic containers filled with pure Argon

[11] Department of Physics, University of Pretoria, Pretoria 0001, South Africa

gas. A pair of sterile pure Titanium tweezers was used to handle the sample discs.

4.4 Analysis of Surface Characterization

4.4.1 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy, also known as Electron Spectroscopy for Chemical Analysis (ESCA) was used to elicit surface elemental and chemical information associated with the different preparative procedures adopted for the twelve different sample groups (see Table 4-2, p59). The XPS analysis using the Perkin-Elmer - PHI 5400 X-ray Photoelectron Spectrometer SYSTEM[12] was conducted using an Aluminuim anode as an X-ray source and an X-ray monochromator (produces X-rays with narrow frequency range).

After samples were placed onto the stage using a pair of sterile pure Titanium tweezers they were secured and immediately placed in the pre-vacuum chamber of the XPS machine to avoid contamination of the surface. The samples were then located in the main chamber with the stage adjusted to an analyzing angle of 65° and a large round aperture of 1.1mm was employed. X-rays generated with a primary acceleration voltage of 14kV, yielding 300W Al-X-rays was used. Attempts were made to maintain the chamber vacuum at approximately 10^{-7}Pa.

The binding energies for all photopeaks were corrected by using the Carbon (1s) photopeak at 284.6eV for referencing. This is a common procedure for correcting the binding energies of XPS spectra.

A spectrum of all identified elements was obtained as a plot of the number of detected electrons per energy interval versus their kinetic energy. Survey scans for each sample were run

[12] Perkin-Elmer Corporation, Norwalk, CT

for 10 minutes using a pass energy of 44.75 with an eV/step of 0.5 and a range of 0 to 1100 eV. Atomic concentrations of identified elements on the sample surface were determined from a subsequent multiplex spectral scan with a pass energy of 17.9 with an eV/step of 0.1 and run for 15 minutes. The atomic percent of each element on the surface was calculated from the photopeak by selecting the peak area of elements from beginning to end of spike and atomic percent was calculated by use of the XPS computer software. The detection limit of the technique for elements is said to be ~0.1 atomic percent. Each element has its own sensitivity factor (see Addendum H, p200-209) for quantification.

Peak separation measurements and full width half maximum (FWHM) values were standard fitting parameters determined using the Gaussian-Lorrentzian curve fitting for the elements with atomic percentage greater than 3% (Moulder et al, 1992).

Identification of the different spectra was performed using the Handbook of X-ray Photoelectron Spectroscopy (Moulder et al, 1992).

4.4.2 Atomic Force Microscope (AFM)

Surface roughness of samples was determined using the TopoMetrix TMX 2010 Discovery Atomic Force Microscope[13] from the Department of Materials Science and Metallurgical Engineering[14]. Samples that had been used for chemical and elemental composition analysis were re-used for surface roughness evaluation.

Samples were fixed with glue (cyanoacrylate) to tin foil squares of 5mm X 5mm using a sterile Titanium tweezers. Each glued sample was probed to ensure that it was firmly attached

[13] Santa Clara, USA.
[14] Industrial Metals and Minerals Research Institute, Department of Materials Science and Metallurgical Engineering, University of Pretoria, Pretoria 0001, South Africa

to the tin foil before mounting them on the magnetic stage of the microscope.

A 130µ Z AFM Scanner was used to quantify surface roughness of the different samples. Five scans were randomly performed for each sample to achieve a more accurate determination of surface roughness. The scan range was set at 20µm. A 5µm scan was then magnified from a randomly selected area of the 20µm scan. As the size of a cell is approximately 20 microns the scan area of 20 X 20µm was chosen specifically as a representation of cell size. The smaller scan of 5 X 5µm was specifically chosen for the evaluation of the primary topography.

The scan rate was set to be three times higher than the scan range. The AFM probe was a V shaped cantilever with a 0.032N/m force constant and an arm length of 200µm. The tip of the cantilever was fabricated from Silicon Nitride (Si_4N_3).

Images were shaded and flattened with a zero-order line fit to remove scan line anomalies prior to being placed into a quadratic plane in the x and y directions.

The TopoMetrix software[15] was used to interpret each corrected image. Surface roughness values were determined in terms of area analysis and line analysis.

For area analysis scanned images were depicted as 3D images and analyzed values were reported as Projected surface area, Area Ra, Area RMS, Maximum range and Average height (see 2.6.2.1, p33).

For line analysis scanned images were depicted as 2D images on which three pre-determined lines (*ca.* line 50, 100 and 150) were drawn. Standard roughness values for each of the three pre-determined lines were determined as Ra (Average roughness), Rp (Maximum peak height), Rpm (Mean peak height), Rt (Maximum

[15] Microsoft Windows SPM LAB V3.06.06 software,Santa Clara, USA.

peak valley for assessment length) and Rtm (Mean peak to valley height) (see 2.6.2.1, p33-34) (Anonymous, 1996).

4.4.3 The Quantum 2000 Scanning ESCA Microprobe

The same samples used for the above characterization procedures were employed to determine the oxide thickness on the surface. Since depth profiling is a destructive technique, it was done last.

Measurements of oxide thickness were done by depth profiling using the Quantum 2000 Scanning ESCA Microprobe with a hemispherical analyzer (HMA) at a base pressure of $<5X10^{-7}$ Pa. Depth profiles were recorded with a monochromated, 100µm beam diameter, 450W Al-Kα-X-ray beam. The sputter depth profiling was conducted with a preferentially pumped focused ion gun in the "alternating" mode, where the sample was sputtered, the gun was turned off and the signal measured. The chamber pressure rose to $<2X10^{-6}$ Pa during sputtering. Primary beam energy of 1 KeV was used with a sputter rate of $3.5nm.min^{-1}$ for all samples. The sputter rate was calibrated by performing depth profiles through (200 ± 20) nm Ti layer on Si substrate (Louw, 1997).

The oxide thickness for samples was estimated according to the method described by Lausmaa et al, (1990), Larsson et al (1997) and Louw (1997). On the depth profile two lines were drawn, one at the maximum Oxygen concentration and one at the Oxygen background level. A line was then drawn to intersect the Oxygen profile at half the value of the height between maximum and background. The point of intersection of this line with the sputter time was then used to calculate the oxide depth using the calibrated sputter rate of $3.5nm.min^{-1}$.

4.5 Cell Culturing

4.5.1 Cell Cultures

Two different cell lines namely Fibroblasts and Osteoblast-like cells were used for *in vitro* culture of cells onto the differently fabricated and enhanced samples. The different cell lines were maintained to confluence in 75cm^2 Tissue Culture Flasks[16] (Botha, 1995).

4.5.1.1 Fibroblasts

Healthy gingival tissue that was acquired from patients during periodontal surgery was used as a primary source of Human Gingival Fibroblasts cell line[17]. The medium used to maintain the cells was EMEM (Eagles Minimum Essential Medium) with NEAA (Non-Essential Amino Acids)[18] and 10% Fetal Calf Serum and 100U/ml Penicillin and 100 MCG/ML Streptomycin Sulphate[19].

4.5.1.2 Osteoblast-like Cells

A cell line of Human Osteosarcoma ACC 439, CAL-72 was obtained from DSMZ[20]. Medium used to maintain the cells was 90% Dulbecco's MEM[21] + 2mM L-glutamine[21] + 1X insulin-transferrin-sodium selenite[21] + 10% Fetal Bovine Serum[19].

4.5.2 Cultivation of Cell cultures

Confluent cultures were used for plating after cultivation. The culture media was removed from the confluent cultures and cells were washed twice with Phosphate Buffer Saline (PBS). Detachment of cells from the tissue culture plastic was by

[16] AEC-Amersham (PTY) LTD, P. O. Box 1596, Kelvin 2054, South Africa.
[17] Center for Stomatological Research, School of Dentistry, University of Pretoria, P. O. Box 1266, Pretoria 0001, South Africa
[18] National Institute of Virology, Private Bag X4, 2131 Sandringham, South Africa
[19] Highveld Biological (PTY) LTD, P. O. Box 1456, Lyndhurst 2106, South Africa
[20] German Collection of Microorganisms and Cell Cultures. Department of Human and Animal Cell Cultures, Mascheroder Weg 1b, D-38124 Braunschweig, Germany
[21] Sigma-Aldrich (Pty) Ltd, 1630, Aston Manor, South Africa)

trypsinization with 3ml of 0.5% Trypsin and 0.2% ethylene-tetra-acetic-acid (EDTA). Detachment of the cells was confirmed using an inverted microscope and the trypsinization process was stopped by adding 3ml of media + 10% fetal calf serum[19]. Cells were then harvested by centrifugation[22] at a speed of 1600rpm for 5 minutes (67.1 xg). The cells that were obtained after centrifugation were seeded into measured tissue culture media (Botha, 1995).

4.5.3 Cell Concentration

The concentration of the cell suspension was determined by Trypan blue[23] exclusion counts using a Neubauer haemocytometer[24]. The cell concentration was determined by mixing 0.5mls of the cell suspension into 0.5mls of Trypan blue. The inoculating cell concentration used was standardized to 3-5 X 10^5 cells per ml of media (Botha, 1995).

4.5.4 Inoculation of Cells onto Sample Discs

Using a sterile Titanium forceps, the differently prepared Titanium disc samples were placed into the 6.4mm diameter 96 tissue culture wells[25] with utmost care to avoid contamination of the surface by unwanted molecules or elements (see Fig 4-6, p68).

Fibroblasts and Osteoblast-like single cell suspensions of cells were then seeded by placing 0.25ml of the cell suspension onto the Titanium discs. Wells without any material were considered as controls and consisted of Tissue Culture Plastic (TTC) that was also inoculated with the same concentration of viable cells.

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[22] Sorvall, Newtown, Connecticut, 06470, USA
[23] Sigma Chemical Company, U.S.A. Batch 68F-50355
[24] American Optical Corporation, Buffalo, New York, 14215, USA
[25] Corning, Adcock Ingram Scientific, P. O. Box 6888, Johannesburg 2000, South Africa

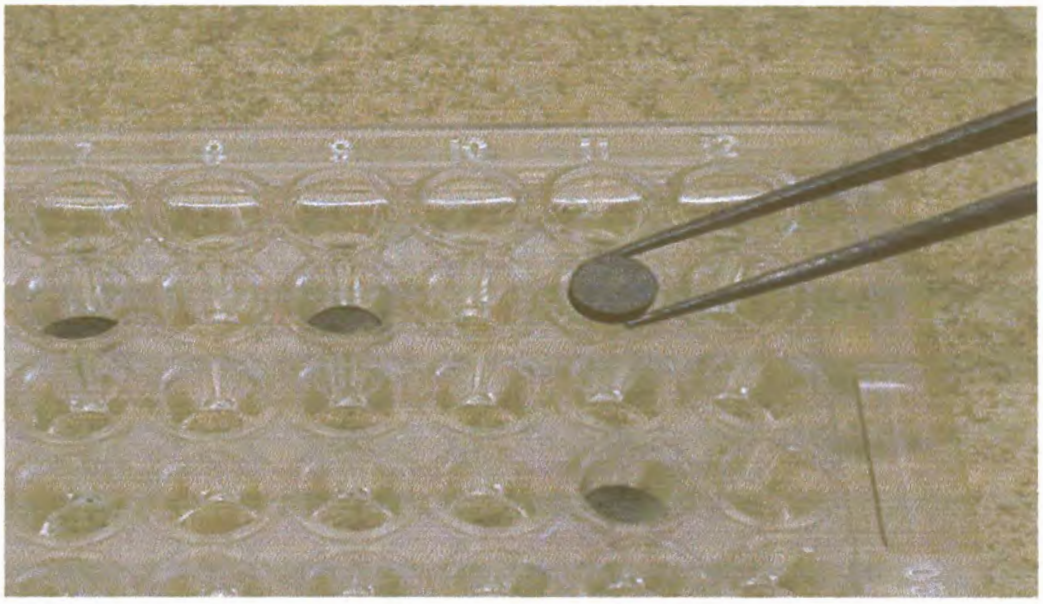

Fig 4-6: Placing of sample discs into tissue culture wells

4.5.5 Incubation

Inoculated tissue culture wells with the Titanium discs and controls were incubated at 37°C in a 95% relative humidity and an atmosphere of 5% CO_2 and 95% air in a CO_2 incubator[26]. Feeding using cultivating media was done twice weekly. The old media was removed and new media was introduced.

At time intervals ranging from 1day, 2days, 14days and 28days the cells on the different surfaces were counted.

4.5.6 Counting Procedures

4.5.6.1 Preparation of Trypan Blue

In all instances the Trypan blue exclusion method[27] was used for the determination of viable cell count. Viable cells remain unstained while non-viable cells stain blue. The Trypan blue was prepared by mixing of 0.5mls of Trypan blue stock solution in 4.5mls of PBS. It is essential to have a 1:1 ratio of Trypan blue to cell suspension. Required volumes of Typan

[26] ShelLab, CO_2 Incubator, Model TC2323, ELMULAB (Pty) LTD, P. O. Box 124, Roodepoort 1725, South Africa
[27] Sigma Cell Culture Reagents, 1992

blue were placed in blue-topped tubes. Once the equivalent amount of cell suspension was present in the Trypan blue the Cell-Trypan blue mixture was placed on Hemocytometer and cell counting was done within five minutes. This is due to the fact that the dye penetrates into non-viable cells and viable cells can absorb the dye on long exposure to Trypan blue resulting in inability to distinguish between viable and non-viable cells (Botha, 1995).

4.5.6.2 Cell Detachment for Osteoblast-like Cells

All media with unattached cells was withdrawn using a pipette. Attached cells were washed twice with PBS prior to detachment. Detachment was facilitated by adding 0.125ml of 4mM Tetracaine free-base[28]. The Tetracaine and cells were then placed in the incubator for 30min. Detachment of cells from control surfaces (Tissue Culture Wells) was confirmed with a Nikon Stereo Microscope[29]. When cells had rounded on the tissue culture wells indicating detachment it was assumed that they had also detached from the Titanium discs. Cells were then carefully pipetted to ensure a single cell suspension. The detached cells were then collected using a pipette and placed into the test-tube containing 0.125ml of Trypan blue.

4.5.6.3 Cell detachment for Fibroblasts

All media was withdrawn using a pipette and to get rid of unattached cells, the cells were rinsed twice with PBS. Attached cells were detached by placing 0.125ml of 0.25% Trypsin and 0.1 % EDTA. Detachment of cells from control surfaces (Tissue Culture Wells) was confirmed with a Nikon Stereo Microscope[29]. When cells had lifted from the tissue culture wells it was assumed that they had also lifted from the Titanium discs. To stop the trypsinization reaction

[28] No. T-7383, Sigma®, Chemical Company, St Louis, USA
[29] Research Instruments cc, P. O. Box 1148, Gallo Manor 2052, South Africa

0.125ml of respective media was added and cells carefully and pipetted to ensure a single cell suspension. The detached cells and media were then collected using a pipette and placed in to the test-tube containing 0.25ml of Trypan blue (Botha, 1995).

4.5.6.4 Neubauer Haemocytometer

Using a pipette, a small volume of the Trypan blue-Cell suspension was transferred to both chambers of the Neubauer haemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowing each chamber to fill by capillary action (see Fig 4-7).

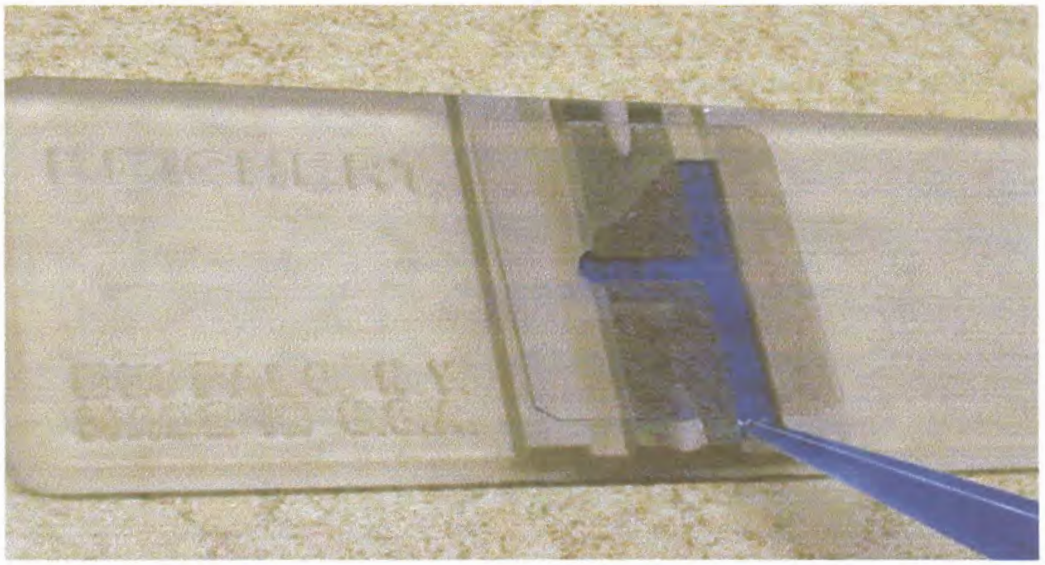

Fig 4-7: Neubauer haemocytometer being filled by capillary action prior to cell counting.

Counting was done using an ordinary light microscope (100X) with a 10X ocular and 10X objective lens. Starting with one chamber, all the viable cells in the center $1mm^2$ and the four corner $1mm^2$ of the Haemocytometer were counted. This procedure was repeated for the other chamber. Counts were in duplicate and results were tabulated (see Addendum A and B, p178, 179).

4.5.6.5 Estimation of Cell Number and Proliferation

At time intervals of 1 day, 2 days, 14 days and 28 days viable cell counts were determined. The viable cell concentration was then calculated per ml by the following formula (Botha, 1995):

Viable cells per ml = average viable cell count per square X dilution factor X 10^4

The efficiency of cell attachment and proliferation was calculated in percent by the following formula:

$$\% \text{ Attachment efficiency and proliferation} = \frac{VT}{VI} \times 100$$

VT = viable cells per ml at a given time interval

VI = viable cells per ml in initial suspension

4.5.7 Fixation Procedures

At 2 days and 28 days samples and controls with attached cells were washed twice with PBS to remove the culture medium and unattached cells. Fixation of cells was done using 0.25ml of 2.5% Glutaraldehyde in 0.075M Phosphate buffer at a pH of 7.4-7.6 for one hour. The wells were then rinsed three times each for 5min with 0.25ml of 0.075M phosphate buffer. Post-fixation using 0.25ml of 0.25% Aqueous Osmium Tetroxide[30] was also done for half an hour under a fume hood. All samples were then rinsed three times each for 5min with distilled water whilst in the fume hood. Dehydration of the cells was then undertaken on a bench by exposing each of the samples to varying concentrations of ethanol, 30%, 50%, 70% and 96% three times for a period of 5min respectively. Cells were transported to the Scanning Electron Microscopy Department immersed in 96% ethanol for further processing (Bullock & Petrusz, 1985).

[30] Merck Chemicals (Pty) LTD, Halfway house 1685, South Africa

4.6 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to study cellular morphology as a function of time exposed to the differently treated Titanium surfaces. Samples were removed from ethanol and placed in a critical point dryer using liquid CO_2. The dried specimens were mounted on viewing stubs. Carbon was placed on sample sides to ensure conductivity before sputtering with gold. The sputtered samples were viewed and analyzed using the JEOL JSM-840 Scanning Electron Microscope[31] with an accelerated voltage of 5.0KV, probe current of $6X10^{-11}$, and the working distance of 10mm. Representative photomicrographs of cell morphology on the different sample surfaces were taken at X500, X2000 and X5000 magnification. Other magnifications taken were specifically for viewing certain morphological characteristics.

4.7 Statistical Analysis

The data obtained were coded, tabulated and analyzed using a linear model doing a General Analysis of Variance (ANOVA)[32]. The factors were Material (cpTi = 1; Ti6Al4V = 2), Method of Fabrication (Machined = 1; Cast = 2), Surface enhancement (Control = 1; SI enhanced = 2 and ES enhanced = 3) and Treatment rendered (not RFGD Treated = 1; RFGD Treated = 2).

The response variables for oxide thickness and chemical were analysed as follows: OXI_DEP, M2C, M2O, M2Ti, M2Zn, M2Pb, M2Na, M2Ca, M2N, M2V, M2Zr, M2Al (see Addendum F, p193).

The response variables for surface topography were analyzed as follows: M3Ra, M3RMS, M3HEIG, M3RAN, M3SUR, M3Ra_1, M3RMS_1, M3HEI_1, M3RAN_1, M3SUR_1, LARA, LARTM, LART, LARPM, LARP, LARA_1, LARTM_1, LAART_1, LARPM_1, and LARP_1 (see Addendum F, p194).

[31] JEOL Ltd, Tokyo, Japan
[32] The statistical package employed was Statistix for Windows Version 7

The growth of osteoblasts (OSTEO) and fibroblasts (FIBRO) were analyzed in a two factor ANOVA with Surface at 13 and 12 levels and Time at 3 (2, 14 and 28 days) and 4 (1, 2, 14, 28 days) levels respectively (see Addendum F, p195).

Testing was done at the 0.05 level of significance.