CHAPTER 6 DISCUSSION

Bio-acceptability is a complex issue involving all the phenomena that contribute to the interfacial reactions between host tissues and implant. The bio-acceptability of Titanium has been attributed to its physical and chemical characteristics and the stable oxide layer that forms on its surface (Kasemo, 1983).

Most metals form oxide layers when exposed to the atmosphere and Titanium and its alloys are no exception. Many of the Titanium alloys in which Titanium is present in concentrations of 85% to 95% maintain the passivity of pure Titanium (Parr *et al*, 1985).

Due to the reactive nature of Titanium (see 2.1.5, p8) the oxide formed on its surface is dependent on the conditions under which it gets oxidized. Any substance that comes into contact with the implant surface has the potential to modify the oxide layer (Lausmaa *et al*, 1990; Hellsing, 1997).

Assuming that a true clean metal surface is available, the first stage is adsorption of the Oxygen on the metal surface, usually considered as a chemisorption process, which is assumed to involve a process of dissociation and at least partial ionization of the Oxygen leading to the formation of ordered superlattice domains or sub-oxide platelets within the metal. So called "oxide nuclei" form at the surface of many metals and these may grow together to form a continuous layer of oxide. These initial stages of the oxidation process depend on the cleanliness of the surface, which in turn depends on the gaseous environment and the purity of the metal. It also critically depends on the surface orientation and the roughness of the surface. Further complexities are introduced after the growth of a continuous layer of oxide on the metal surface, since the oxide provides a barrier between reactants. If the layer is compact, diffusion processes dominate and in

the case of a porous oxide, the reaction may be controlled by phase-boundary processes. The initial stage of oxidation, if a continuous oxide layer is already present, is still adsorption of gaseous species (Louw, 1997).

The incorporation of Oxygen into the oxide generally depends on the defect structure of the oxide. Diffusion of cations and anions across the oxide film is much slower than the electron transfer and can lead to space charge layers that may modify the transport process. The driving force for the diffusion of metal or Oxygen ions may be either the strong electric field set up across thin films of oxides, and/or the chemical potential gradient across thicker oxide films or scales. The reaction will also normally be a function of temperature, Oxygen pressure and the crystal structure and physical properties of the oxide on the metal (see 2.1.6, p10) (Louw, 1997).

Before the advent of Titanium Casting machines, Titanium products were available in the wrought form and either machined or plastic formed to the desired shape (see 2.2, p12). Casting of Titanium opened up a new era in the fabrication of implants. Pure Titanium has a hexagonal close-packed structure to a body-centred cubic phase that remains stable up to the melting point. Titanium alloy microstructure is made up of two phases the alpha phase and smaller amounts of beta phase (see 2.1.4, p7). Casting involves heat treatment and with the increase in temperature, a different crystalline structure is produced to the wrought form (Louw, 1997). Miyakawa *et al* (1996) claims that surface contaminants introduced as a result of casting or surface processing may influence Titanium's biocompatibility and resistance to corrosion negatively. Chemical interactions are known to occur between investment materials and cast samples and it is therefore necessary to grit blast cast surfaces to remove refractory material that adheres to the casting after

devesting (Curtis, 1998). According to Miyakawa *et al* (1989) the surfaces of Titanium castings exhibit a layered structure. Grit blasting with larger size particles may be considered to improve the mechanical bonding of cast Titanium (Papadopoulos *et al*, 1999) and grit blasting with 250µm Alumina (Al_2O_3) has the potential to remove significant amounts of substances that could affect the end result of the prosthesis, in this instance its bio-acceptability (Watanabe *et al*, 1999).

As it is the oxide layer of implants that makes contact with body tissues (see 2.4, p19), allowing direct apposition of bone to the implant surface enhancing osseointegration (Branemark *et al*, 1977), different treatments including tailoring of the oxide thickness, chemical composition and roughness, have been developed by implant companies according to proprietary preparations (Buser *et al*, 1991; Martin *et al*, 1995; Boyan *et al*, 1996; Schwartz *et al*, 1996; Arys *et al*, 1998). However as yet, overall *in vitro*, animal and clinical studies do not yield compelling conclusions about the role of surface composition and texture with respect to bone response at the interface (Brunski *et al*, 2000).

Bio-acceptability of the different samples in terms of materials used, fabrication procedures employed and surface preparations adopted will be discussed in relation to the different surface characterizations.

6.1 Chemical Composition

cpTi and Ti6Al4V showed no significant differences in their surface chemical composition in quantitative (atomic %) and qualitative (composition) terms in disagreement to Kasemo & Lausmaa, (1991) who mentioned major differences between cpTi and Ti6Al4V.

Chemical analysis of sample surfaces showed had dominant signals for Carbon, Oxygen and Titanium, in agreement to other

studies (Lausmaa *et al*, 1990; Keller *et al*, 1994; Placko *et al*, 2000). The atomic % concentration of Carbon reflects the amount of residues or contamination on the surface (Hellsing, 1997) and can be assigned mainly to surface contamination by adsorbed carbon containing (organic) molecules, which is a normal observation for air-exposed surfaces (Lausmaa *et al*, 1990). RFGDT significantly reduced Carbon indicating that the majority of it was present as a surface contaminant and that its presence was not related to the materials employed, fabrication procedures or surface enhancement. Baier & De Palma (1970) also reported that strongly bound hydrocarbon layers, the most common residual contamination on solid surfaces, could be efficiently stripped by RFGDT.

The concentration and XPS binding energy determination of the different oxygen peaks are important in determining the chemical make up of the oxide. Similar to findings by Placko *et al*, (2000) the O1s peak had a characteristic peak for TiO_2 at ~531eV. Other peaks were of metal oxides of Aluminium and other oxygen containing species. The significant increase in Oxygen concentration with RFGDT could be attributed to the process of plasma cleaning itself as immediately after the Argon plasma treatment the samples were oxidized *in situ* in pure Oxygen at room temperature (Aronsson *et al*, 1997). In this study the atomic percent concentration of Oxygen was significantly influenced by RFGDT and was not related to cpTi or Ti6Al4V material employed or the machining and casting procedures or the differently enhanced surface preparations.

The dominant Titanium peak at 457-458eV indicates that the oxide is mainly made up of TiO_2 and some Ti_2O_3, as found in other studies (Lausmaa *et al*, 1989; Machnee *et al* 1993; Sittig *et al* 1999; Kilipadi *et al*, 2000). The absence of a Titanium metal peak at 454eV indicates that the oxide thickness is greater than the escape depth of photoelectrons from the underlying Titanium substrate and intermediate layer, meaning

that the bulk material was not exposed (Lausmaa *et al*, 1990). A lower Titanium surface concentration is measured on the cast and surface enhanced samples. This can probably be attributed to the remnants of grit blasting over surfaces of samples. The Titanium surface concentration detected on cpTi and Ti6Al4V samples was similar but the significant increase in Titanium surface concentration after RFGDT can be explained by the consequent significant decrease of Carbon on the surfaces of samples.

The relatively high Aluminium concentration detected on the surfaces of samples can be attributed mostly to the Aluminium oxide used in the grit blasting procedures of surface enhancement. RFGDT was not able to influence the Aluminium concentration indicating that it was either strongly embedded into the surface or because the particles were too huge to be sputtered of the surface (see 2.6.4.1, p39). In agreement with results of this study, Wennerberg *et al* (1996) also found relatively high atomic % levels of Aluminium (17%) on the surfaces of blasted implants. Darvell *et al* (1995) indicated the presence of up to 10% atomic concentration of Aluminium on the surfaces of castings after blasting was done. According to Kononen *et al*, (1992) Aluminum is generally known to be biocompatible though it has been associated with irreversible enzymatic disturbance (Bruneel & Helsen, 1988) as well as inhibition of the mineralization process of bone (Pilliar, 1998).

The presence of Sodium on samples is related to the process of fabrication and surface enhancement and its concentration was not affected by RFGDT. Small amounts of Sodium on implant surfaces do not cause problems in implant use (Klauber *et al*, 1990) and its presence may be important in the initial attachment of cells to the surface of the implant, especially due to its interaction with other inorganic elements within body tissues.

Most of the other inorganic contamination (Calcium, Lead, Zinc, Nitrogen and Vanadium) that was introduced in smaller quantities as a result of surface preparation was eliminated by RFGDT. RFGDT also reduced the Carbon contamination with a resultant increase in the Oxygen and Titanium concentrations, but had no effect on the Aluminium or Sodium concentrations, in agreement with results reported by Aronsson, *et al*, (1997). The Sodium and Aluminium did not change with RFGDT probably because they are incorporated into the oxide layer by the surface preparation methods employed. The elimination of contaminants by RFGDT indicates that most of the contaminants were present at the outer most surface layer only. According to Aronsson *et al*, (1997) normal contamination layers are typically restricted to one monolayer of atoms or molecules or less. Probably explaining why most of the contaminants disappear after RFGDT.

According to Baun (1982) contaminants in the oxide layer may arise from the bulk composition through heat treatment processes that facilitate diffusion of the lighter elements to the surface and could be the explanation as to why after RFGD treatment only cast samples were detected as having Zirconium. During the recovery of Titanium from its ore a number of zirconium products are used and probably explains its presence (Beder & Ploger, 1959).

Absence of traces of Silicon on any of the samples analyzed can be related to the storing of samples under Argon in polypropylene containers after RFGDT. Silicon contamination is normally observed on samples stored in glass vials but not in polypropylene vials (Esposito *et al*, 1999).

The fact that differences in chemical composition are negligible amongst samples after RFGDT could be due to the high stability of the oxide formed during the RFGDT (Taborelli *et al*, 1997). As in accordance with the criteria for RFGDT (Aronsson *et al*, 1997) the plasma cleaning process did not

introduce any new impurities to the surface. The reoxidation *in situ* in pure oxygen passivates the surface and minimizes the subsequent formation of unintentional surface reaction layers (Aronsson *et al*, 1997).

The oxide layer of cast and machined enhanced samples had similar elements detected that relates to the adopted procedure of grit blasting. Buser *et al* (1991) also found levels of Aluminium and Sodium on their sandblasted samples.

As it is the oxide layer and not the metal itself that determines the chemical properties of the implant, the chemical "quality" of the Titanium oxide superficial layer together with other related factors become the determining factors of bio-acceptability (see 2.6.4, p37). The concentration of the Aluminium and Sodium from cast and enhanced samples could probably be reduced by meticulous post blasting using air to dislodge the loosely adhered particles followed by an ultrasonic bath using distilled water for 30 min. As no significant differences were observed between the different samples with regard to material, fabrication procedure or surface enhancement, it was difficult to relate if bio-acceptability was influenced by the presence of the different elements.

6.2 Surface Roughness

A major consideration in designing implants has been to produce surfaces that promote desirable responses in the cells and tissue contacting the implant. In an attempt to improve cell attachment various forms of dental implants are available with differing surface characteristics that range from relatively smooth machined surfaces to more roughened surfaces created by coatings, blasting by various substances, by acid treatments, or by employing combinations of these mentioned treatments (Cochran, 1999). Currently, virtually all dental

implant manufacturers market implants that feature some form of roughened surface (Cochran, 1999).

Area analysis was determined from a 3D image scan of the surface while Line analysis was determined from a line drawn across a 2D image scan. The importance of each value in the determination of surface topography is discussed separately (see 2.6.2.1, p33-34).

6.2.1 Area Analysis

Both addition and subtraction methods can increase the surface area of an implant, but they do not necessarily make the implant surface rougher (Klokkevold, 2002). The surface topography of samples was introduced by the fabrication procedure adopted and was not related to the material employed. The Ra values of cast samples (0.50µm) were much higher than machined samples (0.11µm). Larsson *et al*, (1997) measured a Ra value of 0.05µm for machined Titanium surfaces and Buser *et al*, (1998) reported a Ra value of 2.0µm for the SLA surface and a Ra of 1.3µm for the Osseotite surface. Zhu *et al*, (2002) reported a Ra value of 0.340 ± 0.01µm before being anodized and after anodizing with a voltage of 391V, surfaces had a Ra value of 0.516 ± 0.037µm that was similar to the observed Ra value of cast samples in this study. Most studies only specify the Ra values as a measure of surface roughness, which is totally inadequate as the Ra value is the average deviation of the profile from the mean line and has no ability to differentiate between peaks and valleys. RMS that is the root mean square of the Ra value is inadequate when reported with the Ra value or alone. It is important therefore to report other area analysis values for proper interpretations to be made (see 2.6.2.1, p33).

Cast samples analyzed had a Ra value of 0.50µm and a RMS value of 0.65µm together with an average height of 1.93µm and a Maximum range of 3.86um while machined samples had significant

lower values of Ra (0.11µm), RMS (0.14µm), Average height (0.54µm) and Maximum range (1.03µm). The surfaces of machined samples showed typical features of grooves that were preferentially oriented in the machining direction while cast samples showed a relatively irregular topography of peaks and valleys. The spatial relationship was different for the machined and cast samples and this difference may have contributed to the surface roughness parameters being much higher for cast samples.

The irregularities resulting from grit-blasting treatment typically range from submicron size to approximately 10 microns (Pilliar, 1998). Wennerberg *et al* (1996) concluded that surfaces created by blasting with Al_2O_3 were isotropic and they recommended a surface roughness with a Ra of 1-1.4µm as being suitable for good bone to metal fixation. Samples that were fabricated by casting also had an isotropic surface but with a much lower Ra value than that recommended by Wennerberg *et al*, (1996). Grit blasting and casting are both methods of surface creation that are based on forming processes leading to isotropic surfaces. The machined surfaces also displayed a similar topography because the surfaces were not really produced by turning but by cutting into the discs. Machining of implants normally entails a turning procedure (anisotropic surface) (see 2.6.2, p29). Therefore, though our results show that the machined surfaces and the cast surfaces are similar, it should be remembered that the overall topography could differ.

Grit blasting not only takes away material but also creates depression on the surface. The blasting pressure together with the size of the grit particles used could be a determining factor to the surface roughness of the cast samples. Cast surfaces were observed to have deep depressions in comparison to their high peaks resulting in the increase in surface area. According to Scacchi (2000), the requirements for an oral

implant design include maximum surface area for attachment and primary stability.

The significant increase in surface area for the 20µm scan with RFGDT can be related to the intentional oxidation that is done after removal of contaminants during the RFGDT (Kasemo & Lausmaa, 1988b). Vargas *et al*, (1992) hypothesized that after removal of contaminants the remaining oxide is leveled but this study found a significant build up of oxide especially over the peaks. At 5µm scan only few peaks are present in comparison to the 20µm scan hence oxide needles thus formed from the oxidation process were probably too few to have a significant effect on surface area.

6.2.2 Line Analysis

Taborelli *et al* (1997) found distinct topographies introduced by surface preparation that could be identified by their peak to valley values of micro roughness.

In this study parameters of line analysis were definitely higher for cast samples compared to machined samples. For cast samples the Ra value of 0.31µm was nearly the same as the Rpm values of 0.37µm indicating that the distance of the profile from the mean was actually contributed to by the peaks. The Rt values of 1.66µm were also approximately double the Rp value of 0.77µm for cast samples, implying that the peaks and valleys on the surface were of approximately similar distances from the mean. The Rt and Rp values contributed to the Rtm being nearly double the Rpm. The ratio of Rpm/Rtm for cast samples was 1:2 signifying a surface that is relatively rough as a low ratio normally is preferred for bearing surfaces. Wong *et al* (1995) noted that the presence of many small peaks resulted in more effective fixation of bone than a smaller number of higher peaks. We did not determine the number of peaks or valleys within a line scan and it would have contributed to a better surface topographical analysis, though

it is accepted that reported values are representative (see 2.6.2.1, p33-34).

The Rp value for cast samples was significantly increased by RFGDT and the increase can be explained by the oxide protrusions that occurred over the peaks in agreement with Solar *et al* (1979).

Similar to our findings, Abron *et al*, (2001) in their study reported that the average surface roughness values (Ra) and the average peak to valley distance (Rp-v) were similar regardless of blasting and acid etching or incomplete blasted surfaces, indicating that the deviation from the mean surface plane in the form of a peak or the deviation in the form of a valley were practically the same. As explained (see 2.6.2.1, p33) the Ra value does not differentiate peaks from valleys.

Although the critical level of roughness magnitudes that influence biological response has not yet been established, it is possible that cells tend to respond most actively to structures that approach their size. This was the reason why analysis of 20µm scans for surface topography were performed. Smaller points of attachments were evaluated from the 5µm scans. Determining the number of peaks and valleys within a line scan could be an added indicator of bio-acceptability as it was observed that the presence of peaks is necessary for cell attachment. From this investigation it is indicative that surface topography can be manipulated for better bio-acceptability.

6.3 Depth Profile

Titanium dioxide is indeed the definitive material that governs the interaction between host tissue and the implant surface in contact with the body tissues, allowing the direct apposition of bone to the implant surface, enhancing

osseointegration (Branemark *et al*, 1977; Kasemo, 1983; Branemark *et al*, 1985).

The oxide thickness between cpTi and Ti6Al4V showed no significant differences in this study though Keller *et al* (1994) found that the oxide thickness on Titanium alloy oxidized surface was approximately 2.5 times thicker than the oxides on cpTi. Differences, if any, between the surface oxides formed on the alloy Ti6Al4V and on unalloyed cpTi might play a role in the acceptance (and integration) by the host tissue (Ask *et al*, 1988) (see 2.6.3, p34-35).

After RFGDT the machined control surfaces were found to have the lowest oxide thickness compared to other samples that were either cast or enhanced, suggesting that the fabrication method and surface enhancement were the determining reasons for the significantly higher oxide thickness. Machnee *et al* (1993) did not find any significant differences in oxide thickness between the differently prepared samples they studied while Binon *et al* (1992) found that the oxide thickness of the implants investigated greatly depended on the manufacturing process and conditions. Lausmaa *et al* (1990) and Sittig *et al* (1999) in their studies measured oxide thickness of 2-6nm depending on method of sterilization. Placko *et al* (2000), in their study estimated the thickness of oxides to be about 3nm, similar to that found by Ong *et al* (1993). The reported difference in oxide thickness by authors could be partly attributed to the different equipment and methodologies adopted to measure the Titanium oxide layer.

RFGDT surfaces had a significantly thicker oxide than untreated surfaces. In agreement, Aronsson *et al* (1997) reported that reoxidation of surfaces produces oxide thicknesses of about 2-150nm and RFGDT treatment normally produced a stoichimetric TiO_2 surface oxide of uniform thickness with no traces from earlier treatments.

The ideal oxide thickness that is required for bio-acceptability has not been delineated by this study. In disagreement to Kasemo & Lausmaa (1986) and Albrektsson *et al* (1981) who reported that the oxide layer is assumed to grow over time after implantation, Wennerberg *et al* (1996) found that after implantation and removal of the screws, the 75µm blasted implants had become smoother than before implantation but were still distinguishable from the 25µm implants in terms of roughness. Arys *et al* (1998) also found that the TiO_2 overlay on failed implants was considerably reduced in thickness or absent and speculated it to being stripped away either by mechanical stress, or being chemically modified and dissolved in the microenvironment. Another explanation of the absence of the Titanium oxide layer onto implanted implants is the possibility that osteoclast cells phagocytosed the oxide, as osteoclasts are essential cells in bone remodeling (Davies *et al*, 1989). The importance of a stoichiometric oxide layer that is not prone to the phagocytosis activity of the osteoclast is thus emphasized.

The overall chemical reaction involved in the oxidation process or formation of the oxide layer can be described in terms of three layers: (i) a metal/oxide interface containing suboxides (with the metal in a lower oxidation state), (ii) a uniform crystalline oxide layer, and (iii) a thin outer layer where the metal is in its highest oxidation state. For alloys, the selective oxidation of the alloy components combined with competing ion movement govern the growth and subsequent structure of the oxide layer (Louw, 1997).

Cast samples had a significantly thicker oxide than machined samples and can be related to the fabrication process of the cast samples. The stoichiometric composition of the oxide layer formed by casting could be entirely different from that formed by machining, resulting in a variation between samples. Other characteristics like grain size and crystal orientation

could also be contributory (see 2.1.4, p7). According to Low *et al* (2001) the thicker oxide layer of cast samples is related to the heavy reaction layers formed during the casting process and increased surface area introduced by grit blasting.

Defective oxides that are formed as a result of other elements bonded to Oxygen and the Oxygen chemisorbed on the surface could lead to its breakdown causing failure in bio-acceptability. The type of oxide formed, amorphous in relation to stoichiometric could be another determinant of bio-acceptability as the surface charge at the peak may be dependent on the type of crystalline structure.

6.4 Cell Culturing

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. Using the direct contact method, cells are cultured directly onto the material under investigation and observed over a period of time relative to their morphologic and functional features (see 2.7, p41).

Fibroblasts were obtained from human gingival biopsies, according to the method by Kononen *et al* (1992) and Botha (1995) while the osteoblasts-like cells used in the study were from a human osteosarcoma cell line. Since human cells are more reliable as test systems than other tissues and species in culture the use of human cells was motivated.

Viable cells were counted at predetermined times using a Neubauer haemocytometer and the Trypan-blue exclusion method (Botha, 1995). The percent attachment and proliferation was then determined from the counts. While some researchers counted the unattached cells using a Coulter Counter (Bowers *et al*, 1992), others counted the attached cells (Morra &

Cassinelli, 1997). None of these methods distinguished viable from non-viable cells. In this investigation it was regarded as an important consideration to distinguish between viable and non viable cells (see 2.7.5, p48).

For comparison of data a base-line control is necessary and tissue culture plastic was used as a control because of its suitable characteristics. Keller *et al* (1990) demonstrated that after one hour, more than 90% of the inoculated cell population had attached to the tissue culture plastic. Tissue culture plastic is plasma treated to promote cell adhesion and growth (Amstein & Hartman, 1975).

The percent attachment efficiency and proliferation of both fibroblasts and osteoblasts-like cells displayed a significant difference with time. They both reached their peak proliferation by day 14, with osteoblasts-like cells reaching a maximum of 180% and fibroblasts a maximum of 120%. At day 28, the fibroblasts on the control surface showed a significant rise in % attachment efficiency and proliferation while the osteoblasts-like cells showed a significant decline. The % attachment efficiency and proliferation of fibroblasts on the surfaces of machined cpTi and Ti6Al4V control samples was observed to be similar to the control unlike the cast and enhanced samples that showed a decline in relation to the control. This observation was probably related to the type of surface topography (see2.6.2, p30-31). Over time, the % attachment efficiency and proliferation for osteoblasts-like cells was observed to decline for most samples similar to the control, with the exception of the cast Ti6Al4V control sample that displayed an increase in % attachment efficiency and proliferation as from day 2 to day 28 and machined cpTi SI sample that maintained a steady % attachment efficiency and proliferation. Cast Ti6Al4V ES sample and machined Ti6Al4V SI sample showed a very slight decline in % attachment efficiency and proliferation of fibroblasts from day 14 to 28. The array

in the display of results could not be explained from either the chemical or topographical analysis but is probably related to the phenomena of attachment and spreading. From this investigation it appears that spreading and attachment are two different phenomena of cells that are determined by different factors.

There were no significant differences in the % attachment efficiency and proliferation of fibroblasts or osteoblasts-like cells between the different materials used (cpTi and Ti6Al4V), fabrication employed (machined and cast) or surface enhancement procedures (control, SI and ES) and the control. This observation could have been contributed to by the limitations of funds that permitted only two readings of cell counts per sample. Statistical analysis thus performed was not able to detect differences and deductions made from these results should be interpreted as possible trends.

The % attachment efficiency and proliferation of fibroblasts did not show any statistical difference between materials used. Machined control samples were similar to the control while cast and enhanced samples had a different % attachment efficiency and proliferation that was much lower than the control. Mustafa *et al* (1998) showed that smooth surfaces favour fibroblast attachment and least attachment occurs on rough surfaces (see 2.6.2, p30). The % attachment efficiency and proliferation of fibroblasts on all SI samples and machined Ti6Al4V ES and cast cpTi ES was much lower than the control while the machined cpTi ES and cast Ti6Al4V ES samples nearly matched the control. These results imply a possible trend that the SI surfaces are not compatible for the proliferation of fibroblasts. It is possible that the reduced % attachment efficiency and proliferation of fibroblasts was related to the delay in finding attachment points, as the surface was probably smoother than other samples.

The % attachment efficiency and proliferation for osteoblasts-like cells did not show any significant differences between samples. Machined SI samples were observed to have a higher % attachment efficiency and proliferation of osteoblasts-like cells compared to other machined samples and the control. This observation was probably related to the way the machined SI samples were manufactured and the topography they displayed. The cast samples had a similar presentation to the controls with the exception of cast Ti6Al4V control and cast cpTi ES samples that had a much higher % attachment efficiency and proliferation ($p>0.05$). It is possible that these surfaces were smoother than the other cast surfaces but it could also be related to the hardness of the cast alloy material (see 2.1.4, p6) and the blasting procedure that produced a different surface topography conducive to osteoblasts-like cell proliferation. Numerous studies have associated increased surface roughness with greater osteoblastic activity (Michaels *et al*, 1989; Bowers *et al*, 1992; Keller *et al*, 1994; Walivaara *et al*, 1994; Trisi *et al*, 1999). Though other studies (Mustafa *et al*, 1998; Larsson *et al*, 1997) found that bone derived cells behave differently to fibroblast cells we found a similarity between the behaviour of fibroblast and osteoblasts-like cells. Fibroblast and osteoblasts-like cells are anchorage dependant cells (Brunette, 1988; Zreiqat *et al*, 1996) and therefore it can be assumed that their behaviour would be similar.

The tendency of cells to proliferate on a surface appears to be dependent on the surface topography (see 2.6.2, p29). From this investigation smoother surfaces have been observed to enhance proliferation and spreading probably because of the presence of focal contacts while on rougher surfaces % proliferation and attachment was related to the presence of filopodia.

6.5 Scanning Electron Microscopy

Scanning Electron Microscopy of samples revealed that the fibroblast and osteoblasts-like cells had a similar mechanism of attachment and proliferation depending on the surface encountered. On surfaces with smoother topography cells were observed to be in close contact with each other and formed very close contact with the surface adapting to the underlying topography. On surfaces with a rougher topography the spread or suspended cells exhibited irregular polygonal shapes with gaps or spaces where there was no area of contact. As the adhered cells formed a layer of differently shaped suspended cells over the surface it was difficult to determine the underlying topography. On surfaces that were observed to have a lower % proliferation scanning electron micrographs revealed that there were a lot of empty spaces between the attached cells (see Fig 5-58, p126).

After inoculation, cells settled on the surface of samples by gravitation. Though we did not view the different stages of cell adhesion, it can be hypothesized that once cells settle they react to the encountered surface by the production of filopodia. Extensions of filopodia are indicative of cell spreading (Keller *et al*, 1994). Possibly the filopodia that are produced by the cell have specific functions, namely for exploratory purposes and attachment purposes. The attachment filopodia are presumably shorter in length and used for suspending the cell once it has attached to a point while the exploratory filopodia are normally longer and thicker. The length of the exploratory filopodia is probably determined by the absence of attachment sites within the vicinity of the cell stimulating the cell to continue the search for attachment sites. It is possible that along the path that the exploratory filopodia takes, attachment filopodia are formed when contact points are met. Bagambisa & Joos (1990) have

reported filopodia of as long as 120µm over the surfaces they studied (regular surface).

On smooth surfaces it is assumed that filopodial extension of a cell move in all directions in search of points to adhere. After a failed attempt to find points of attachment on the smooth surface the filopodia are seen to coalesce together by the spread of the cytoplasm to the filopodia causing the cell to flatten maintaining a circular shape. The circular shape of the cell is probably a result of absence of points of tension within the cell that cause the cytoplasm to move in different directions. After the cell has flattened there are still few filopodia that emerge and probably still in search for a point of attachment to which it can attach itself. According to Brunette (1986b) filopodia were found to increase in length with time and have been found to play an important role in cell spreading and that contact however was not required for cell spreading as most isolated cells became fully spread. As the number of spread cells increased it was difficult to differentiate one cell from another as cells on the surface were viewed as a unit of cytoplasm that was in close contact to the underlying topography of the surface. Shelton *et al* (1988) also observed that cells on smooth surfaces eventually formed very close contact with the surface adapting to the underlying topography. Since cells are not attached but just spread over the underlying surface, proliferation is probably an easy task as the cells do not have to detach before proliferation, explaining why the smoother surfaces had a higher % of proliferated cells.

On irregular surfaces that consisted of peaks and valleys filopodia were observed around the entire surface of the cells and extending in all directions (see Fig 5-66, p133). It is presumed that for a cell to adhere to a surface there must be areas (points) to which the filopodia can attach and these points are assumed to be the peaks. Filopodia observed were of

different types, some were short and attached to nearby peaks while others were tapered and some even extended over depression and valleys in search of attachment sites. Ohara & Buck (1979) found that cells did not follow the finer surface contours but bridged over the grooved areas. Where there was no area of contact the cytoplasmic extensions continued over the surface until they found a place to attach. When cells that had settled encountered a surface that had surrounding peaks most of its filopodia were observed to attach to these peaks. After attachment of the filopodia to the peaks the cell cytoplasm was observed to lift off the surface and spread between its filopodia. Depending on the number of attachment points that also acted as tension points the cell was observed to acquire its shape. Using these attached filopodia the cell was then able to suspend itself to the surface creating a gap or space beneath. It is probably the tension that is created within the cell that causes the cell to lift off the surface.

Since most of man's functions are determined by impulses due to different charges generated, it is also assumed that for an attraction to occur between two surfaces they must be of different charge. It is possible that the filopodia are of a different charge to the peaks that they seek. According to Tengvall & Lundstrom (1992) Titanium is covered by a surface layer of oxide which is only weakly negatively charged under physiological conditions. It also appears that the filopodia are rigid enough to bridge over surfaces in search of the opposite charge. When a flat or smooth surface is encountered there probably is no charge on the surface explaining why the cell spreads but does not attach. According to Weiss (1978) most biosurfaces carry a net negative surface charge and net positively charged surfaces are not encountered in mammals.

Many researchers (Thomas *et al*, 1987; Mustafa *et al*, 1998; Ellingsen, 1998; Lazzara *et al*, 1999; Abron *et al*, 2001) have reported higher bone-implant-contact for the rougher surfaces

compared to the smoother surfaces and the probable reason could be the many adhesion points over a rough surface in comparison to the smooth surface where cells were spread more but with no contact.

The data from this study indicates that numerous parameters can be measured or identified as an indicator for potential bio-acceptability. Since there were no significant differences in the bio-acceptability between samples it was difficult to relate the effects of chemical analysis of samples investigated. Probably the time interval of the study was not adequate enough to determine the effects of the different elements found on the surfaces of samples with emphasis on Sodium and Aluminium that was introduced by the process of grit blasting (see 6.1, p140). The immediate effect of the oxide thickness was also not obvious but the importance of an oxide of adequate thickness and stoichiometric make up is emphasized, as it will prevent the dissolution of elements (see 2.1.5, p10). It is also probably the oxide crystals that determine the surface charge of the peaks that is necessary for cell attachment. Surface topography was able to display its immediate effects from the % proliferation and attachment of cells.

6.6 Bio-acceptability

The bio-acceptability of machined and cast cpTi and Ti6Al4V from results of this investigation showed no significant differences in terms of % attachment efficiency and proliferation. Surface enhancement that was used as a tool to increase the surface topography also was not able to display any significant differences between the control surfaces and the enhanced surfaces in terms of % attachment efficiency and proliferation. Scanning electron micrographs were able to emphasize the difference in cell morphology as seen on smooth and rough surfaces. Cells (as determined by surface analysis)

were found to attach rather than to spread on the rougher surfaces investigated. The chemical analysis of cast and enhanced samples was similar, probably because the grit blasting process influenced the outer surface of these samples. Grit blasting was used as a method of surface enhancement and for the devesting of cast samples. The oxide layer was significantly thicker for the cast and enhanced surfaces than for the machined control surfaces but no differences were observed in terms of bio-acceptability. RFGDT was significantly able to reduce all contaminants that were introduced from the different sample processing methods with the exception of Sodium and Aluminium that were not affected. The effect of Sodium and Aluminium concentration on bio-acceptability was not noted probably due to the time interval of the study.

Using the results from this investigation that has confirmed the bio-acceptability of cast surfaces, the fabrication of cost effective implants using Titanium alloy is possible. The desired strength that the alloy possesses together with its ease in availability and affordable custom made appliances prepared by casting would have the same results in bio-acceptability. The bio-acceptability of the fabricated cast Titanium alloy implants could also be enhanced employing grit blasting of specific size to ensure the desired surface topography that will determine the peaks and valleys. RFGDT could be utilized as a method of producing a stoichiometric oxide for even better bio-acceptability.

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

From this study that investigated the surface characteristics and *in vitro* bio-acceptability of Titanium and Titanium alloy the following can be concluded:

1. Chemical Analysis:

   a) Chemical and elemental composition of cpTi and Ti6Al4V surfaces showed no significant differences when analysed for their atomic % concentration and the curve fitting for the different elements detected on their surface.

   b) Casting of samples introduced Sodium and Aluminium to the surface in comparison to the machining of samples that did not have these elements on their surfaces. The Oxygen peak of cast samples showed more sub peaks from peak fitting compared to the machined samples and the Titanium peak indicated mostly TiO_2.

   c) Surface enhancement of samples regardless of the size of grit particles used introduced the presence of Sodium and Aluminium onto the surface of the samples.

2. Surface topography

   a) Materials used (cpTi and Ti6Al4V) in the fabrication of samples were of no significance in determining the surface topography of the samples.

   b) Surface topography was determined by the fabrication procedure and the cast samples were found to have a

significantly rougher surface topography compared to machined samples.

3. Depth profiles

a) The oxide thickness of the different materials used (cpTi and Ti6Al4V) for the preparation of samples was not statistically different.

b) The cast samples had a significantly higher oxide thickness than the machined samples.

c) Surface enhancement significantly increased the oxide thickness.

4. Radio Frequency Glow Discharge Treatment (RFGDT)

a) There was a significant reduction in the surface Atomic % concentration of Carbon and a resultant significant increase in Oxygen and Titanium with RFGDT. Aluminium and Sodium were not affected by RFGDT but most of the smaller signals from Calcium, Zinc, Lead, Nitrogen, etc were removed from surfaces by RFGDT.

b) Surface area of samples and the Rp value of the 20µm scan were significantly increased by RFGDT.

c) The oxide thickness was significantly increased by RFGDT.

5. Bio-acceptability

a) Fibroblasts were observed to increase their % attachment efficiency and proliferation (%AEP) with time while osteoblasts-like cells were observed to decrease their %AEP with time.

b) The reaction of fibroblasts and osteoblasts-like cells to cpTi and Ti6Al4V showed no significant differences between the materials.

c) Though no significant differences were found between the machined and cast samples in terms of %AEP of fibroblasts and osteoblasts-like cells, machined samples were observed to have a higher %AEP than cast samples.

d) Surface enhancement of samples showed no significant differences in the %AEP of fibroblasts and osteoblasts-like cells. Machined cpTi ES enhanced samples had a similar %AEP of fibroblasts to the control, unlike the other enhanced samples that had a much lower %AEP. Machined Ti6Al4V SI and cast Ti6Al4V control samples had relatively higher %AEP for osteoblasts-like cells than the other samples.

e) Scanning Electron Micrographs revealed that cells over the machined control samples had spread and proliferated more than the other samples showing the underlying topography of the sample. Cells on the cast and enhanced surfaces were observed to have attached to the peaks and were not in contact with the underlying surface but suspended over the surface and of irregular shapes.

f) After different surface characterization processes, cast cpTi and Ti6Al4V samples showed similar results in terms of bio-acceptability when compared to the proprietary innovative characterized implant surfaces.

7.2 Recommendations

The results from this study identified numerous opportunities for future research:

1) Further development and testing of the cast surfaces are needed to optimise the blasting particle size for the devesting of cast samples and to determine the degree of roughness desired for better osseointegration.

2) The long-term effect of the blasting particles (Aluminium) that get embedded onto the surface should be analysed for their effect on bio-acceptability.

3) Alternative methods of devesting of cast samples should be evaluated.

4) The optimal oxide thickness and the stoichiometry of the cast samples should be defined and evaluated.

5) Increased used of cast Titanium in the dental fraternity will depend on clinical trials to compare the effectiveness of cast Titanium as an equivalent or superior design for osseointegration.

6) As casting changes the crystalline structure, further investigations should link the relationship of the oxide formed to the underlying crystalline structure.

7) Further study of cellular and tissue responses, particularly long-term studies of expression of cell phenotype, would help explicate the importance of surface micro-morphology on biological response.

8) The difference between cell attachment and spreading should be defined, as there may be a difference between contact, initial adhesion, later adhesion and detachment before mitosis.

9) The optimal RFGDT of cast samples for better bio-acceptability should be elucidated.

10) Histomorphometric analysis of cast samples following defined surface topography.

11) Further studies are warranted to determine the effect of surface roughness on osteoblasts attachment and proliferation.

12) Comparative biological evaluation of cast samples in relation to machined samples using epithelial cells will indicate its bio-acceptability in terms of the cast surface.

13) Defining the peak and valley heights and distance on the surface of cast samples will help to determine the optimal deviation for improved attachment of cells.