The Effects of Hydroxyapatite Coated Micromachined Substrata on Osteogenesis.

By

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Abstract

Osteogenesis was studied using rat bone cells cultured on hydroxyapatite (HA) or titanium (Ti) coated smooth and grooved substrata. Osteoblast cultures were maintained from 24hrs to 6 weeks in culture medium and supplemented with L-ascorbic acid-2-phosphate and ß-glycerophosphate to promote mineralization. The HA coatings were characterized using X-ray diffraction, surface roughness and scanning electron microscopy (SEM). The ceramic hydroxyapatite coating, was dense and uniform, containing HA crystals and was $\approx 1\mu m$ thick. Time-lapse cinemicrography of osteoblasts locomotion on HA surfaces revealed osteoblasts moved with the direction of the grooves, that is they exhibited contact guidance. Scanning electron microscopic observations revealed osteoblasts were elongated and orientated on both Ti and HA grooved surfaces. Collagen fibers, as assessed by picro-sirius staining and polarized light microscopy, were aligned on both HA and Ti grooved surfaces. Although not quantified, it appeared that nodule formation was greatest under culture conditions that produced aligned collagen. Osteogenesis was measured by counts of tetracycline labelled bone-like nodules and alkaline phosphatase activity. HA coated surfaces produced

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significantly more mineralized nodules than Ti surfaces. Surfaces with grooves and narrow gaps produced the highest number of nodules. All grooved substrata produced significantly more nodules than smooth surfaces. These results are consistent with the concept that substrata that form a microenvironment by restricting diffusion increase bone-like tissue production. A novel finding in this thesis was that there was a statistically significant interaction between topography and chemistry in the formation of mineralized nodules. An excellent correlation (r= 0.958) between alkaline phosphatase at 2 weeks and nodule counts at 6 weeks was observed, suggesting that Alk-P is a good leading indicator of osteogenesis on microfabricated surfaces. Therefore Alk-P might be used to screen surfaces for their nodule production, thus saving time and expense. The results of this study indicated that topography and chemistry can affect osteogenesis on biomaterials, and that interactions between chemistry and topography can occur.

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I. Introduction

1. Overview:

For an implant to be successful it must be non-toxic, cause minimal foreign-body reaction, and integrate with the tissue (Ong *et al*, 1998). Osseointegration has been defined as contact established between bone and an implant surface without the interposition of non-bone or connective tissue, at the light microscopic level (Gross *et al*, 1997). Bone formation on an implant surface depends upon the metabolic and secretory activities of only one cell type: the osteoblast (Davies, 1996).

The success, that is the longevity, of bone-contacting implants depends on the amount and rate of bone formation occurring on their surfaces (Massas *et al*, 1993). The material used plays an important role in the longevity of an implant as it affects the amount of osseointegration (Vercaigne *et al*, 1998). The more osseointegration that an implant has the better the prognosis (Gross *et al*, 1997).

It is widely recognized that surface properties, most particularly topography and chemistry, are of prime importance in determining cell and tissue responses to a biomaterial because it is the surface that forms the interface with the tissue (Brunette and Chehroudi, 1999). Although there is no agreement on what constitutes the ideal topography, there is some agreement on which materials are biocompatible (Burnette, 1988). The two materials studied in this thesis, Ti and hydroxyapatite (HA), have

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been shown to be biocompatible (Massas *et al*, 1993).

The surfaces of commercially available dental implants vary greatly in their topography and chemical compositions (making them hard to compare) (Brunette, 1988; Wennerberg, 1996; Brunette and Chehroudi, 1999; Wieland, 1999). Moreover, there is sparse information on the best surface topography (Wennerberg, 1996), possibly due to the number of surface topographies being used and the lack of a clear understanding of how surfaces interact with tissues (Qu *et al*, 1996). There is thus a need for studies using precisely controlled surfaces. Our laboratory introduced micropatterning and micromachining processes, used in microelectronics to biomaterial applications, and this approach has enabled the investigation of how well defined features of topography affect cell behaviour *in vitro* (Brunette, 1986a,b; Brunette *et al*, 1983; 1991) and *in vivo* (Chehroudi *et al*, 1990; 1992). Of particular interest to this thesis is that titanium coated micromachined substrata have been shown to increase the formation of bone-like tissue adjacent to both *in vitro* (Ratkay, 1995) and *in vivo* implanted surfaces (Brunette *et al*, 1991, Chehroudi *et al*, 1992).

Although titanium implants have had considerable success, investigators have also studied calcium phosphate ceramics, especially hydroxyapatite and tricalcium phosphate, and found that ceramic implants

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were associated with enhanced mineralization (Ong *et al*, 1998; Chang *et al*, 1999). One reason for the interest in hydroxyapatite is that its Ca/P ratio are similar to that of natural bone (Sun *et al*,1997). Moreover, HA is biocompatible and produces very low (if any) inflammatory response. HA implants have also produced direct contact with bony structures (Sun *et al*,1997).

To date, there has not been any work on hydroxyapatite (HA) coated micromachined substrata. Moreover, there is no agreement on the optimal topography of an implant for bone contacting applications and commercially available implants vary in this aspect. As this thesis will examine both titanium and hydroxyapatite surfaces on bone-like tissue formation *in vitro*, a brief introduction of Ti and HA implants will now be given.

2. Titanium and Hydroxyapatite Implants:

In orthopaedics and dentistry, titanium or titanium alloys are among the materials of choice, with cobalt-chromium and stainless steel being used in orthopaedics to some extent (Ahmad *et al*, 1999). The attractiveness of Ti implants lies in their high strength, relatively low elastic modulus, high corrosive resistance and biocompatibility (Thomas *et al*, 1987; Soballe, 1993). Furthermore, Ti implants have been shown to be successful (compared to other metals) in short and long term studies

for mineralized matrix production (Ahmad *et al*, 1999).

Bioactive materials, materials that have been designed to induce specific biological activity such as HA, have been found to result in more bone contact than Ti (Soballe, 1993). Moreover HA implants support more bone ingrowth than Ti (Soballe, 1993).

Hydroxyapatite, a calcium phosphate material, is accepted as osteoconductive, causing bone to grow into areas that it would otherwise not occupy (Jarcho, 1981; Schwartz *et al*, 1993; Gross *et al*, 1997; Davies, 1998; Chang *et al*, 1999). Beckham *et al* (1971) found that there is an intimate relationship between mineralized bone and calcium phosphate ceramic. In 1973, the first indication of a chemical bond between bone tissue and HA was reported (Driskell *et al*, 1973 cited in de Bruijn *et al*, 1991). Moreover HA is the only substance shown to elicit a chemical bond with bone when implanted, causing the implant to be more stable. For these reasons HA is very attractive for clinical applications (de Bruijn *et al*, 1991; Kay, 1993; Manley, 1993; Thomas, 1994; Sun *et al*, 1997; Chang *et al*, 1999). HA was introduced commercially in 1981 for medical applications in granular form for alveolar ridge augmentation and periodontal lesion fillings (Manley, 1993). As development continued, applications have expanded to include blocks and coatings, increasing the options for restorative dental and orthopaedic applications (Manley,

1993).

There are several additional advantages to using HA for implants. HA does not elicit an immune response; moreover, synthetic HA is not readily bioresorbable (it does not readily undergo biological resorption by solution- or cell-mediated processes) and is therefore appropriate for long-term clinical use (Manley, 1993; Thomas, 1994). HA is also known to increase the bone-implant shear attachment strength up to five times that of uncoated metal surfaces, indicating a more rapid tissue adaptation to the implant surfaces (Thomas, 1994). Clinical studies have reported high success rates for implants with HA, nevertheless some controversies exist regarding the effects of HA coatings (Hench et al, 1984; Gross et al, 1997; 1998).

Hydroxyapatite has been used as a coating on metallic substrates (such as Ti-6A1-4V alloy and commercially pure Ti) and as a dental implant material for many years (Chang et al, 1999; Chou et al, 1999). The most popular method of applying HA onto metallic substrates is a plasma spray procedure (Whitehead et al, 1993; Cheang and Khor, 1996). This procedure yields a coating mainly consisting of the basic apatitic structure, but an amorphous phase of HA is also formed (Cheang and Khor, 1996). This amorphous phase of HA is found to have less hydroxyl groups (Whitehead et al, 1993; Cheang and Khor, 1996). Furthermore, other

calcium phosphate phases have been identified in the coatings such as ß-tricalcium phosphate (TCP), α-TCP and oxyhydroxyapatite, as well as non-crystalline calcium phosphate material (Whitehead *et al*, 1993; Cheang and Khor, 1996). These problems occur because the plasma flame reaches a high temperature causing the HA to be thermodynamically unstable (Wolke, 1997). Furthermore the HA ceramic is heated to a molten state causing the HA to be partially dehydroxylated and undergo phase transitions (Cheang and Khor, 1996). Therefore care must be taken to achieve appropriate conditions to yield desirable structure and composition of the HA coating (Cheang and Khor, 1996; Wolke, 1997). A better method is sputter coating, specifically RF Magnetron sputtering, which forms a dense and uniform coating (Lacefield, 1988; Jansen *et al*, 1993; Lacefield, 1998). In addition to the RF magnetron sputtering process, heat treating the HA coated substrate has been found to increase the bond strength between HA and the substrate (Lacefield, 1988; Wolke *et al*, 1997). Other advantages to this method are the high rate of deposition, the ease of sputtering, the production of high-purity films, the extremely high adhesion of films and the excellent coverage of difficult surface geometries (Jansen *et al*, 1993). The deposited HA films are characterized by methods such as scanning electron microscopy (SEM) and x-ray diffraction (XRD), to ensure that the coating has a uniform thickness

and well-crystallized calcium phosphate ceramic (Jansen *et al*, 1993). By evaluating the characteristics of both amorphous and crystalline coatings through cellular responses, it is thought that a HA coating can be designed that will accomplish a specific purpose in the body (Gross *et al*, 1997).

One problem associated with the use of HA coating is that they are sometimes lost *in vitro* and *in vivo* through processes such as delamination, abrasion or dissolution (Bauer, 1993). The dissolution of HA is a function of crystallinity (Morgan *et al*, 1996). One way of testing dissolution of crystalline and amorphous HA coatings is by immersing the HA coated surfaces in Ringer's solution (Gross *et al*, 1997). The dissolution rate is correlated with the pH of the surrounding environment (Chou *et al*, 1999). Furthermore crystalline coatings have been found to be more stable than amorphous coatings, because crystalline HA coatings show no signs of degradation except cracking, which is attributable to release of residual stresses (Gross *et al*, 1997). An advantage of highly crystalline coatings is that they are less soluble (Caulier *et al*, 1995) and do not alter the pH of the surrounding fluids such as culture medium. Changes in pH can occur as a result of dissolution of the ceramic coatings (Chou *et al*, 1999) and would be expected to alter experimental results.

An exception to the correlation between crystallinity and dissolution was found by Ogiso *et al* (1998). They suggest that the

crystallinity may decrease the binding strength between the HA coating and substrate, and not the dissolution. However, Ogiso *et al* (1998) used plasma sprayed implants that were not additionally heat treated, and heat treatment is found to ensure higher crystallinity (Lacefield,1988; Gross *et al* ,1998).

Cellular responses are also influenced by the crystallinity of HA surfaces (Ong *et al*, 1998). For example HA crystallite size influences the expression of osteoblast characteristics (Ong *et al*, 1998). The higher the crystallinity, the higher the rates of cell proliferation and differentiation achieved (Ong *et al*, 1998; Chou *et al*, 1999).

HA supports the proliferation and differentiation of osteoblasts better then Ti (Massas *et al*, 1993; Ong *et al*, 1998). This increase in proliferation and differentiation may be one of the causes of the increased bone formation seen with HA implants (Massas *et al*, 1993). Furthermore HA is generally associated with a higher rate of formation and higher amount of bone tissue in contact with the implant compared to titanium implants (Massas *et al*, 1993; Chang *et al*, 1999).

A brief introduction to the components of bone, the tissue of primary interest in this thesis follows.

3. Bone Matrix:

Bone consists of inorganic and organic components. Calcified bone

was found to be composed of 76 to 77 percent (dry weight) inorganic bone substance, the balance is organic (Ham, 1974).

The organic matrix is formed mostly of collagen, with collagen type I, being the major constituent (Ham, 1974; Satomura and Nagayama, 1991). Collagen type I in the form of a fibrous network provides the structural integrity of many connective tissues, such as bone and dentin (Watt, 1986; Uitto and Larjava, 1991). Moreover collagen type I directly supports mineralization by serving as a site of nucleation for the induction of mineral components such as hydroxyapatite crystals (Bellows *et al*, 1986; Glimcher, 1989). There are several other minor collagenous components in bone: types VI (cell to collagen binding), V, X, XI, and XII collagens (Uitto and Larjava, 1991). Although type V and III collagens are also found in bone, they are not present in sufficient quantities to be measured accurately (Bellows *et al*, 1986; Uitto and Larjava, 1991; Veis, 1993). Moreover there is also evidence of the presence of collagen type XII; that is associated with type I collagen and forms cross bridges between collagen fibrils as well as mediating the binding of other connective tissue components such as proteoglygans (Uitto and Larjava, 1991).

Proteoglycans exist in bone and are also found in the extracellular matrix (Watt, 1986). The main dermatan sulfate proteoglycans found in bone and cartilage are *decorin* and biglycan, which share significant

sequence homology (Uitto and Larjava, 1991). *Decorin* expression is exclusive to matrix-centred functions, such as regulating cell growth and collagen fibril formation (Bianco *et al*, 1990; Yamada *et al*, 1999). Biglycan, very similar to *decorin*, is expressed in a range of specialized cell types, including connective tissue (skeletal myofibers, bone) and is directly involved in cell regulatory functions including cell proliferation and differentiation (Bianco *et al*, 1990; Inoue *et al*, 1999; Yamada *et al*, 1999). Furthermore biglycan is found in developing bone and possible plays a role in bone development (Inoue *et al*, 1999).

There are also families of non-collagenous proteins present in bone that play a key role in differentiation, activation of bone cells, maturation and mineralization of bone matrix (Nefussi *et al*, 1997). One glycoprotein that is used as a marker of mineralization, is osteonectin. Osteonectin has affinity to both collagen type I and HA (Romberg *et al,* 1985) and appears to mediate the *in vitro* mineralization of collagen type I (Romberg *et al,* 1985). Osteonectin synthesis may be increased by active osteoblastic cells in a nodule, or it may be that its accumulation within the nodule is related to its association with other matrix components (Bellows *et al*, 1986; Nefussi *et al*, 1997).

Another marker for mineralization is osteocalcin (bone GLA protein) a bone and dentin specific protein. Osteocalcin is primarily restricted to

mineralized tissues, in particular to mineralization fronts (Nefussi *et al*, 1997). Moreover osteocalcin functions as an osteoclast recruiter (Davies, 1996). Osteocalcin is synthesized almost exclusively by osteoblasts (Beresford *et al*, 1993; Davies, 1996; Okumura *et al*, 1997).

Another family of non-collagenous proteins related to bone include the sialo (phospho) proteins: osteopontin (secreted phosphoprotein (SPP-1) or BSP-I) and bone sialoprotein (BSP-II) (Veis, 1993). Osteopontin is not specific to bone or dentin and is multi-functional (Pinero *et al*, 1995; Davies, 1996). It is involved in early bone development, as it influences the attachment of osteoblasts to sites of bone growth (Pinero *et al*, 1995). It has also been suggested that during bone remodelling osteopontin may aid the attachment of osteoclasts to bone (Pinero *et al*, 1995). Both osteopontin and bone sialoprotein appear to be distributed in osteoids, mineralized matrices and mineralization fronts (Pinero *et al*, 1995).

Osteoblasts secrete bone sialoprotein (Pinero *et al*, 1995). The association of amorphous, electron-dense, granular extra-fibrillar materials with BSP-II acts as a nucleation structure involved in the mineralization process (Nefussi *et al*, 1997). Moreover BSP-II is located at sites of early mineralization specifically restricted to areas at which crystallites are present (Pinero *et al*, 1995; Nefussi *et al*, 1997).

There are numerous other enzymes and growth factors found in bone that appear to have important roles in mineralization. Some growth factors associated with bone matrix formation include transforming factor (TGF)-β_1 and -β_2 (Yamada *et al*, 1999), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), insulin-like growth factors (IGF) I and II, bone morphogenic proteins (BMP's) and osteo-inductive factor (Hollinger and Leong, 1996; Bostrom, *et al*, 1999; Strayhorn *et al*, 1999). However, there is not just one growth factor but numerous growth factors that are working in bone maintenance and repair (Hollinger and Leong, 1996; Bostrom, *et al*, 1999). Bone matrix associated enzymes include collagenases, proteinases, cathepsins and alkaline phosphatase (Golub, 1996). Alkaline phosphatase activity is a major marker of the osteoblastic phenotype and sites of bone mineralization activity (Ballanti *et al*, 1995).

Bone also contains albumin, which is synthesized in the liver and has widespread distribution (Peel, 1995). Albumin's suggested function in bone is as a regulating carrier for small molecules and ions involved in mineralization (Peel, 1995; Davies, 1996).

The other major component of bone is inorganic. The inorganic portion of bone matrix is primarily hydroxyapatite ($Ca_{10}(PO_4)_6(OH_2)$), which gives bone its hardness and rigidity (Ham,1974). The precise mechanism

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of mineralization, in which the organic matrix becomes calcified by inorganic HA is still under investigation. One suggestion of the underlying physicochemical basis and mechanism for the formation of calcium phosphate crystals in bone and other tissues, and in biological mineralization is a heterogeneous nucleation by one or more of the organic constituents (Glimcher, 1989).

Bone matrix vesicles play a role in initial hydroxyapatite formation (Schwartz and Boyan, 1994). They are associated with primary calcification and mineralization fronts in a number of tissues (Boyan *et al*, 1993). These bone matrix vesicles are secreted by osteoblasts and contain non-collagenous proteins, alkaline phosphatase, ATP-ase. In time HA crystals form along the inner portion of the matrix vesicle membrane (Boyan *et al*, 1993). Furthermore bone matrix vesicles are thought to be the vehicles used to transport media for triggering nucleation of mineral crystals at distant locations (Mikuni-Takagaki *et al*, 1995). After the secretion of specific and non-specific bone proteins contained in the bone matrix vesicles, mineralization of the matrix is initiated by expression of the membrane glycoprotein alkaline phosphatase (McComb *et al*, 1979).

4. Effects of Surface Topography on Cell Behaviour:

It is known that surface topography influences cell behaviour, *in vivo* and *in vitro* (Brunette, 1988; Brunette *et al*, 1991; Brunette and

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Chehroudi, 1999; Chehroudi *et al*, 1992; Chehroudi and Brunette, 1995; Qu *et al*, 1996; Chang *et al*, 1999). More specifically surface topography has affected osteoblast behaviour resulting in enhanced mineralized tissue formation *in vivo* and *in vitro* (Brunette, 1988; Brunette *et al*, 1991; Brunette and Chehroudi, 1999; Chehroudi *et al*, 1992; Chehroudi and Brunette, 1995; Qu *et al*, 1996). One aspect in which surface topography influences cell behaviour is by altering cell shape (Brunette 1988). Cell shape in turn influences many aspects of cell behaviour including cell adhesion, locomotion, and gene expression (Watt, 1986; Brunette and Chehroudi, 1999).

For cells to adhere to a surface cells must contact, attach and spread onto the surface (Weiss, 1975). Several surface properties influence cell attachment, for example, wettability (Baier, 1986), surface area (Brunette, 1988) and composition (Chang *et al*, 1999). Wettability is measured by the critical contact angle formed by liquid droplets spreading on a surface (Baier, 1986). A way to increase the wettability is by glow-discharging the surface so that a high-energy surface is formed (Baier, 1986). Surface area is also a factor affecting cell attachment. At the simplest level a surface with a greater surface area, for example a grooved versus a smooth surface, provides more opportunity for cell attachment (Brunette, 1988). The composition of the surface affects

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attachment. Osteoblasts have been shown to attach and adhere better to HA than Ti implants (Chang *et al*, 1999), perhaps because osteoblasts produce more extracellular matrix and expand cytoplasmic extensions (both associated with cell adhesion) over the entire HA surface compared to osteoblasts on Ti surfaces (Wilke *et al*, 1998).

Another cellular function of bone cells affected by surface topography is cell locomotion (Chesmel *et al*, 1995). Contact (topographic) guidance, which refers to the tendency of a cell to be oriented and guided in its direction of motion by the shape of the surface with which it is in contact. This behaviour was observed in some of the earlier studies of cells in culture (Harrison, 1914). Using time-lapse cinemicrography (Brunette *et al*, 1991) it was found that osteoblast-like cells initially elongate and orient themselves on Ti grooved substrata, and became progressively more oriented to the grooves over time (Qu *et al*, 1996).

Gene expression is also affected by cell shape which in turn is affected by surface topography. The shape of a cell on a surface will affect it's proliferation and differentiation (Folkman and Moscona, 1978; Watt, 1986; Baslé *et al*, 1998). Cells in a rounded configuration will have their proliferation restricted, which is shown by a reduction in the proportion of cells synthesizing DNA. Furthermore changing cell shape by

stretching cells by mechanical means stimulates proliferation (Brunette 1984) as can changing substratum adhesiveness (Folkman and Moscona, 1978). Moreover the differentiation of cells is affected by cell shape, for example erythroleukemic cell-fibroblast hybrids that grow in suspension can express haemoglobin, whereas adherent clones of the cells can not (Allan and Harrison, 1980; Watt, 1986).

A brief discussion on proposed mechanisms of osteogenesis on microfabricated substrata follows.

5. Proposed Mechanisms of Enhanced Osteogenesis on Micromachined Surfaces:

Attachment, proliferation, metabolism, matrix synthesis and differentiation of osteoblast-like cell lines *in vitro* are affected by one or more of the four interrelated properties of biomaterials: chemical composition, surface energy, surface roughness and surface topography (Schwartz and Boyan 1994). One biomaterial, such as Ti, used by Chehroudi *et al* (1992) on grooved substrata proved more effective than smooth surfaces in enhancing mineralization *in vivo*. The process by which mineralization is initiated and regulated by topography *in vivo* and *in vitro* is not well understood but Chehroudi *et al* (1992) suggest three mechanisms. One possibility is that topography can produce a bone-inductive microenvironment. For example grooved surfaces could produce

a confined environment in which growth factors can achieve the concentrations required for bone production.

A second possibility is that the organization of collagen bundles may be significant in the initiation of mineralization (Chehroudi *et al*, 1992). This suggestion arises from consideration of the work of Veis (1993) who found that the collagen fibrils in association with phosphoproteins initiate the nucleation of apatite crystals. *In vivo* the development of intra-membranous bone tissue follows a sequence in which the first step is characterized by a random, felt-like orientation of collagen fibrils which is considered as a primary scaffold *in vivo* (Schenk and Buser, 1998). In the second month collagen fibrils are packaged into parallel arrays with alternating courses (such as plywood) and this organization gives bone the highest structural strength (Schenk and Buser, 1998). Furthermore Davies and Matsuda (1988b) found that collagen fibrils were morphologically more organized into linear bundles on some substrata, for example bioactive glass, that enhanced mineralization. Therefore it is conceivable that micromachined grooved surfaces may encourage the orientation and arrangement of collagen fibrils that promote mineralized-tissue production (Chehroudi *et al*, 1992). Orientation and spatial distribution of collagen can be examined using a histochemical stain that enhances the birefringence of collagen (Dziedzic-Goclawska *et al*, 1982;

Pierard, 1989; Montes and Junqueira, 1991; Canham *et al*, 1999).

A third possibility is that cell shape and polarity can influence the cell's gene expression, differentiation and function (Watt, 1986). Overall it appears that micromachined grooved surfaces could therefore promote mineralization through diverse mechanisms by their known property of orienting cells (Chehroudi *et al*, 1992), extracellular matrix (Qu *et al*, 1996), and altering cell shape and gene expression (Chou *et al*, 1999).

A brief discussion on conditions promoting mineralization follows.

6. Conditions Promoting Mineralization:

Osteoblasts are the only cells that have the ability to form bone on material surfaces (Davies, 1996). The mineralization process is still under investigation, but some supplements are known to favour mineralization (Bellows *et al*, 1991; 1992). One important supplement is ascorbic acid (Vitamin C) (Bellows *et al*, 1991). Ascorbic acid stimulates the formation and hydroxylation of collagen, permitting sufficient amounts of collagenous matrix to be deposited for nodule production (Bellows *et al*, 1986). Ascorbic acid is necessary for mineralization, but the mineralized tissue produced in the presence of ascorbic acid without organic phosphate is not morphologically identical to tissue mineralized *in vivo* (Tenenbaum and Heersche, 1982; Bellows *et al*, 1986; 1991). For mineralization to mimic physiological bone, *in vitro* systems must often

be supplemented with a source of organic phosphate (Tenenbaum and Heersche, 1982; Bellows *et al*, 1986). The rationale for using organic phosphate is that it provides an additional source of phosphate ions that are made available in places where alkaline phosphatase is present (Tenenbaum and Heersche, 1982). Moreover it has been suggested that organic phosphates may be more important for mineralization than circulating inorganic phosphate, both as a source of phosphate ions and as a cellular regulator of mineralization (Tenenbaum and Heersche, 1982). Although circulating levels of inorganic phosphate *in vivo* are abundant in serum and bone they play a relatively small role in the initiation of mineralization; nevertheless, for hydroxyapatite crystals to form some inorganic phosphate is required (Tenenbaum and Heersche, 1982).

For *in vitro* systems to mimic physiological bone it is necessary that osteoblasts produce a multi-layered structure surrounded by a mineralized matrix (Davies, 1988a), to achieve a three-dimensional structure of mineralized tissue housing osteocytes (Bellows *et al*, 1986).

Since L-ascorbic acid has a very short half life of about 1.5 hours in culture (Feng *et al*, 1977), many investigators adopted a protocol that entailed refreshing the medium at least three times a week. Within the past decade a new longer acting ascorbate analogue, L-ascorbic acid phosphate (Asc-P), has been developed (Hitomi *et al*, 1992). This

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ascorbate is more stable and lasts longer than natural L-ascorbic acid (Hitomi *et al*, 1992; Jaiswal *et al*, 1997). Refreshing the medium twice a week with the ascorbate analogue yields comparable results to L-ascorbic acid (Beresford *et al*, 1993; Jaiswal *et al*, 1997). In addition L-ascorbic acid phosphate supplementation can produce mineralized nodules even in the absence of ß-glycerophosphate (Beresford *et al*, 1993). Asc-P also is capable of inducing rapid osteogenesis as defined by the appearance of osteoblastic cell morphology, increased expression of alkaline phosphatase and the formation of a mineralized extracellular matrix containing hydroxyapatite (Jaiswal *et al*, 1997).

A brief introduction of some markers used to identify osteogenic tissue follows.

7. Techniques used to Identify Osteogenic Tissue:

Some simple criteria that have been used to identify osteogenesis *in vitro* include a) morphology: an aggregation of cells with osteoblastic morphology; b) collagen deposition; and c) mineralization as assessed by varies stains (see for example Ratkay, 1995). Based on previous reports as well as preliminary studies, this thesis employed these markers as follows a) morphology as assessed by reflected light differential interference contrast and scanning electron microscopy; b) collagen as visualized by staining with picro-sirius red and polarized light

microscopy; and c) mineralization using tetracycline labelling and alkaline phosphatase activity using naphthol AS-MX phosphate substrate. More detailed information on some of these techniques follows.

Polarization microscopy is usually used for unstained tissues (Puchtler *et al*, 1973). There are several dyes that stain collagen and will show birefringence but the most specific is picro-sirius red F3BA. This stain has been used extensively for staining collagen-containing structures and was used in this thesis (Puchtler *et al*, 1973; Canham *et al*, 1999).

Sirius red F3BA is a long and fairly linear azo dye that has greater refractivity for light vibrating along its long axis than across it, thus this and similar dyes are know as intrinsically anisotropic dyes (Puchtler *et al*, 1973). Puchtler *et al* (1973) also found that the intensity and colour in polarized light are related to the concentration of the dye and the thickness of the collagen. As more information on this dye became known, Junqueira *et al* (1979) realized that all structures stained with Sirius red had enhanced birefringency. These areas were known to contain collagen; however, the stain by itself is not specific for collagen (Junqueira *et al*, 1979). It is the combination of aqueous picric acid solution and Sirius red that appears to lead to selective staining of collagen (Junqueira *et al*, 1979). Since collagen is a basic protein, the sulphonic groups of Sirius

red interact at low pH with the amino groups of lysine and hydroxylysine, and the guanidine groups of arginine in collagen (Junqueira et al, 1979). The picro-sirius staining increases the birefringent nature of collagen at least 700% in light intensity (Junqueira et al, 1979). The increase in the birefringence is because the sirius red molecules attach to the collagen fibrils in such a way that their long axes are parallel with the collagen fibrils that are themselves intrinsically birefringent (Junqueira et al, 1982).

One way of observing the increase in birefringence is linear polarized light microscopy (Wolman, 1970). Using linearly polarized light, collagen orientation can be demonstrated by comparing two views of the same specimen rotated 45° to each other so that only the collagen that is parallel to the polarizer will be brightly seen (Wolman, 1970; Boyde et al, 1984). Polarizing microscopes, however, do not detect birefringent material if its optic axis is perpendicular to the plane of focus (Frohlich, 1986).

It has been suggested that polarized light can be used to distinguish different types of collagen (Junqueira et al, 1982; Montes and Junqueira, 1991). It was observed that picro-sirius staining causes different types of fibres to appear in different colours when seen in polarized light (Montes and Junqueira, 1991). Collagen fibres show up in the form of

thick, brilliant (strongly birefringent) yellow or red fibres against a dark background when studied by the picro-sirius-polarization method, whereas reticulin fibres display a weak birefringence and are characterized by their thinness and greenish colour (Montes and Junqueira, 1991). Moreover differences between collagen types I, II and III are observed but it was subsequently realized that the differences are the result of the thickness of the fibres and the packing of these different collagens (Dayan et al, 1989; Andrade et al, 1997).

However, some caution in the interpretation of collagen types by picro-sirius red staining must be made. Pierard (1989) states that identifying collagen type by this colour method is erroneous. This is because the colour of collagen fibers are unrelated to the fiber's molecular nature and staining it may vary according to the orientation of the slide on the stage of the microscope (Pierard, 1989). He suggests that picro-sirius staining should be limited to the orientation and density of collagen.

Overall it is believed that the picro-sirius polarization method is an extremely simple, reliable, relatively specific, sensitive and cheap method which provides an easy and precise method of qualitative or quantitative analysis of collagen (Junqueira et al, 1979; Montes and Junqueira, 1991; Andrade et al, 1997).

The picro-sirius staining method is a reliable method and it has found applications in pathology in the diagnosis of collagenous diseases (Dziedzic-Goclawska *et al*, 1982; Montes and Junqueira, 1991; Trau *et al*, 1991; Rabau and Dayan, 1994). However for identifying mineralization different markers, such as tetracycline and Alk-P activity, are required.

Tetracycline is a widely used antibiotic in clinical medicine and dentistry (van der Bijl and Pitigoi-Aron, 1995). It has been found that tetracyclines are taken up and accumulate in newly-formed mineralized tissues more readily than in old bone (Ibsen and Urist, 1964; Frost, 1968; McClure, 1982; Misra, 1991; van der Bijl and Pitigoi-Aron, 1995). Furthermore because tetracycline does not bind to old bone as readily as newly-formed bone, tetracycline-stained old bone normally does not fluoresce under ultraviolet or blue light (Ibsen and Urist, 1964; Perrin, 1965; Frost, 1968; Davies *et al*, 1985; Misra, 1991). Moreover since tetracycline fluoresces, the binding of tetracycline and calcium has been successfully used as fluorescent labels for studying bone metabolism (Ibsen and Urist, 1964; van der Bijl and Pitigoi-Aron, 1995). Tetracycline incorporates preferentially with new bone calcium because (1) *in vivo* sites of new bone formation are closer to the blood supply than is old bone, therefore new bone is richer in tetracycline; (2) the bone crystals are smaller, and therefore present a greater surface area for

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incorporation; (3) the diffusion rate of tetracyclines through matrix and minerals in older, denser bone is slower, since tetracyclines are relatively large molecules ($\approx$5x bigger than a water molecule) (Ibsen and Urist, 1964). Another advantage of tetracycline is that it can be used *in vivo* and *in vitro* to observe bone cell behavioral rates such as bone resorption and formation, and population dynamics (Frost, 1968).

The effects of tetracycline on cell cultures can be either beneficial or inhibitory depending on concentrations (Vernillo and Rifkin, 1998). Inhibition of bone growth occurs with high concentrations (50μg/ml) of tetracycline but lower concentrations (20μg/ml) do not impede mineralization (Gruber,1993). Moreover the use of 5μg/ml of tetracycline gives a signal that is readily detectable under UV fluorescence microscopy with little effect on mineralization (Gruber, 1993).

A beneficial effect of tetracycline for some clinical conditions is that it can slow osteoclast-mediated bone resorption directly by reducing the bone-binding capacity of osteoclasts (Keller and Carano, 1995; Vernillo and Rifkin, 1998). Tetracycline inhibits bone resorption *in vitro* by, in part, reducing collagenase activity (Ramamurthy *et al*, 1990; Vernillo and Rifkin, 1998).

Another marker used to detect mineralization activity is alkaline phosphatase (Alk-P) which is associated with cell membranes and occurs

in many cell types, including osteoblasts (Kiernan, 1981). Alk-P is believed to be involved in bone formation, and is a characteristic of bone cells producing mineralized matrix (Farley and Baylink, 1986; Mikuni-Takagaki *et al*, 1995). Alk-P activity is not expressed in any significant amount in osteocytes (Mikuni-Takagaki *et al*, 1995). Furthermore the presence of Alk-P hinders the extracellular phosphorylation of bone proteins by osteocytes possibly through a phosphoprotein kinase pathway (Mikuni-Takagaki *et al*, 1995). Overall Alk-P activity is thought to be an excellent differentiation marker for osteogenic cells involved in mineralization (Ballanti *et al*, 1995). Moreover use of Alk-P as an indicator of mineralization activity correlates well with tetracycline labelling in studies of mineralization of osteoblastic cell lines (Ballanti *et al*, 1993; 1995).

Measurements can be made of Alk-P activity using an enzyme-substrate reaction end product (Sabokbar *et al*, 1994). The substrate is a monobasic sodium salt of α-naphthyl phosphate, the monoester of α-naphthol, and phosphoric acid (Kiernan, 1981). The α-naphthol freed by the hydrolysis is a phenolic compound and can be coupled with a diazonium salt (Kiernan, 1981). Furthermore naphthol AS-MX phosphate, a substrate, has been found to be the most satisfactory for the detection of Alk-P activity compared to other naphthol substrates (Ackerman, 1962).

Although naphthol AS-MX forms a more intense colour then does α-naphthol, its rate of coupling with diazonium salt is slower (Kiernan, 1981). The reaction between the substrate and Alk-P occurs in an alkaline pH and an insoluble coloured azoic dye is produced, since the dye is added to the reaction medium (Kiernan, 1981). The diazonium salt used is Fast Blue RR, thought to be one of the best available for this purpose (Ackerman, 1962; Kiernan, 1981; Ballanti et al, 1995).

By definition the pH of the medium used in the enzymatic reaction must be alkaline to detect Alk-P activity (Yabe, 1985). The optimum pH is in the range of 9.6-10.0, with a pH of 9.6 being best for cells in contact with HA coatings (Ericson, 1969; Yabe,1985). The optimum temperature, determined for the detection of human Alk-P was 25°C, thus the Alk-P reaction can be carried out at room temperature (Copeland et al, 1985).

8. Objective of the Thesis:

Since micromachining can produce precise and controlled topographies, it can be used to investigate the role of substratum topography in mineralization. Hydroxyapatite is also thought to increase bone formation, but its interaction, if any, with topography is not known. The aims of this thesis are:

A. To confirm that micromachined substrata in the form of grooves of varying depths increase mineralized tissue formation.

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B. To investigate the effects of smooth, dense HA coatings on bone-like tissue formation.

C. To determine whether the combined effects of HA coatings and topography are additive, subtractive or interactive.

D. To determine the contribution of collagen orientation and microenvironment to the increased bone formation seen on micromachined substrata.

II. Materials & Methods

1. Substrates:

A. Micromachining:

Micromachining is a technique that produces topographic features with precise dimensions in silicon wafers. The particular micromachining techniques used in this study were those developed at the University of British Columbia by Camporese *et al* (1981) for the fabrication of high quality photomasks for solar cells.

Micromachining begins with the production of a master pattern which is decreased to the appropriate dimensions by a step-and-repeat photographic process to produce photomasks, glass plates on which a metallic master pattern is placed. Then the master pattern is transferred onto a silicon wafer by photolithography. A thin silicon oxide layer is produced on the surface of the wafer, which is then covered with a UV sensitive organic polymer, called photoresist. Then the wafer is exposed to UV-radiation through the photomask. After exposure the photoresist is removed chemically, creating a negative copy of the master pattern. Next a chemical reaction removes the silicon oxide from the areas that do not have photoresist and finally the rest of the polymer is removed. To create the desired depth for the silicon oxide pattern it is chemically etched by anisotropic etchants which can create well defined shapes with sharp

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edges and corners. The result is a positive copy of the master pattern made out of silicon oxide on the surface of the silicon wafer.

B. Surface Pattern:

The pattern used in this study was developed by Khakbaznejad (2000). The pattern was etched into a silicon wafer, as described earlier, in Dr. N.A.F. Jaeger's Laboratory, Department of Electrical Engineering, UBC. The over all dimensions were 1cm by 1.5cm and within this grooves were etched out. The angle of the grooves tapered at 55°. The grooves were etched to three depths (3μm, 10μm and 30μm) each having a pitch of 47μm and a ridge of 5μm. (Figure 1). In the same surfaces, within the grooves, there were smooth gaps etched (interrupting the grooves). There were four different smooth gaps all having the same area of 90000μm^2 but differing widths of 50μm, 100μm, 150μm and 200μm. There are ten smooth gaps of each width on each surface depth. (Figure 2).

C. Coatings:

The wafers were coated with titanium or hydroxyapatite. For titanium wafers they were sputter-coated with 50nm of titanium in Dr. N.A.F. Jaeger's laboratory. For HA coating silicon wafers were sent to Dr. W.R. Lacefield at the University of Alabama School of Dentistry. There the wafers were cleaned with substrate bias of 100W for 5 minutes prior to sputtering. They were then R.F. Magnetron sputtered Ca-P coated at 400W,

A) 3μm deep grooves

Pitch 47μm

Ridge 5μm

3μm

Groove Depth

B) 10μm deep grooves

Pitch 47μm

Ridge 5μm

10μm

Groove Depth

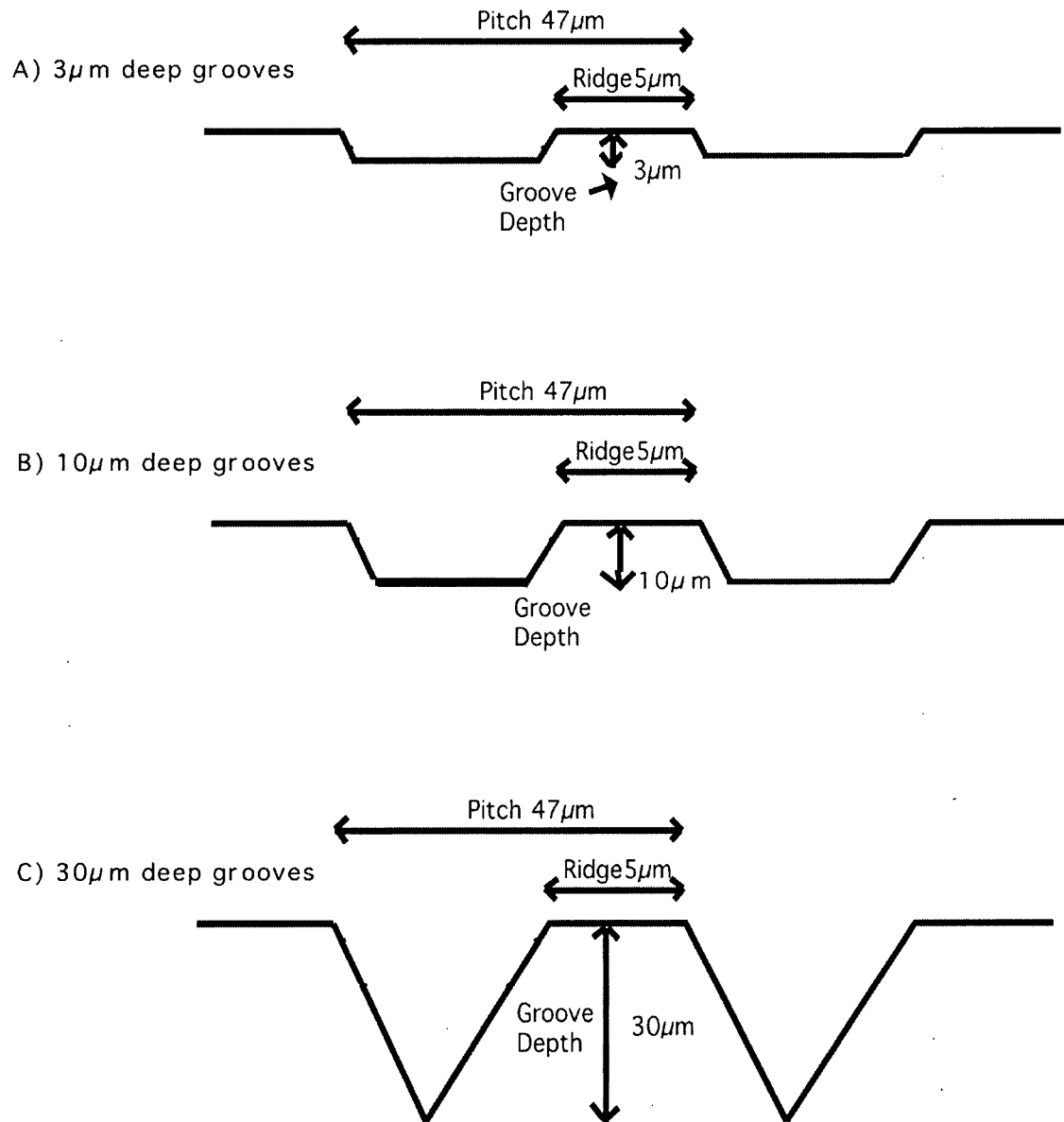C) 30μm deep grooves

Pitch 47μm

Ridge 5μm

Groove Depth 30μm

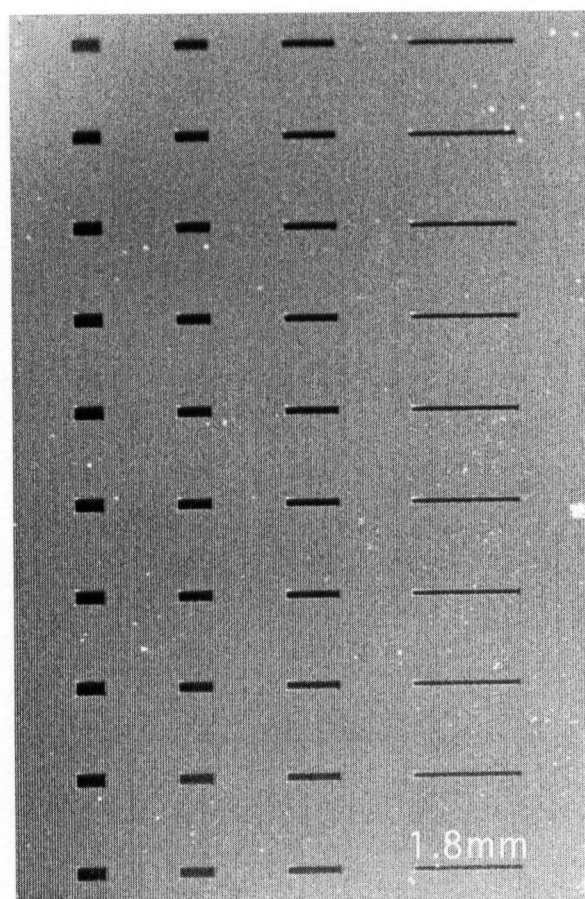Figure 1. **Schematic cross-sections of grooved surfaces**. The three used in this thesis.

Figure 2. **A macro-image of the pattern**. This pattern is seen on the substrata used in this study. Notice the different gaps sizes interrupting the grooves.

with 5×10^{-3} mbar working pressure and sputtering rate of 100-150 nm/min for 1 hour (thus $\approx$ 1μm thick). Following the coating the wafers were heat treated for 2 hours at 500°C and analyzed by a Thin Film X-ray Diffractometer (Philips X'Pert, Multi Purpose Diffractometer) to characterize the coating of HA. A copper target is used as an x-ray source (wavelength= 0.15406nm) and the incident angle omega was fixed to 1 degree and 2-theta angle was scanned for the thin film x-ray diffraction measurments. This method is very similar to the one used by Wolke *et al* (1998).

Surface roughness was measured using a profilometer (Alpha-step 200, Tencor Instruments, Mountain View, CA, USA). Six samples for each surface type were measured and the average roughness (R_a) was quantified.

D. Preparations for cell culture experiments:

The wafers after being coated were cut with a diamond pen so that the overall pattern (1 by 1.5 cm) and smooth pieces were used. In order to retain maximum overall HA coating the wafers were not washed and to ensure that the wafers were sterile they were glow-discharged (Baier, 1986) for 3 minutes in an argon-gas chamber developed by Aebi and Pollard, (1987). The glow-discharge treatment also produces a high surface energy surface that facilitates cell attachment.

2. Culture of osteogenic cells derived from rat calvaria:

To test the effect of topography on bone formation between HA and Ti coated surfaces newborn rat calvaria osteogenic cell cultures were used. These cultures have been shown to produce mineralized tissues on standard culture dishes (Rao *et al*, 1977; Bellows *et al*, 1986; Bhargava *et al*, 1988; Qu, *et al*, 1996; Dubois *et al*, 1998).

The method used for osteoblast isolation was similar to that described by Hasegawa *et al* (1986) and Chehroudi *et al* (1992). Briefly, frontal, parietal and occipital bones (calvaria) of newborn (24-36 hours old) Sprague-Dawley rats were carefully dissected, rinsed in plentiful amounts of sterile phosphate buffered saline (PBS) and placed in tissue culture medium (α-minimum essential medium, α-MEM) with 15% fetal calf serum (FCS) and antibiotics (100μg/ml Penicillin G, 50μg/ml Gentamycin sulphate and .3μg/ml Fungizone) added. Eighteen to twenty calvariae were dissected and minced into pieces $\approx$ 1mm^3. The minced tissue was then incubated in 5ml of a digestion mixture containing 180U/ml of clostridial collagenase (type Ia, Sigma, St. Louis, MO, USA) and .5mg/ml trypsin (Gibco, Burlington, Ontario, Canada) in PBS. The suspension was digested for 2x-20 minutes at 37°C with stirring in a Pierce "Reacti-vial" (Pierce Chemical Company, Rockford, IL, USA) and the supernatant discarded. After a further 20 minutes incubation the

supernatant was kept and mixed with an equal volume of cold fetal calf serum and centrifuged at 1500rpm. Following the centrifugation the supernatant was discarded and the cells were resuspended in α-MEM containing 15% FCS and antibiotics. This population of cells was designated sub-culture O. The cells were placed in a 75cm^2 tissue culture flask (Falcon, Bector Dickinson Labware, Franklin Lakes, NJ, USA).

3. Experimental Design:

The design of the experiments in this thesis is schematically illustrated in figure 3 and the details of the procedures follow.

A. Culture:

Osteoblasts cells were removed from the culture flask by using a trypsin (Gibco) solution (0.25% trypsin, 0.1% glucose, citrate-saline buffer, pH 7.8) when required. Subcultures I-III were used only. Calvarial cells were electronically counted (Coulter Cell Counter) and plated in six-well culture plates (35mm diameter each well; Falcon) at a population density of 1.0×10^5 cells/cm^2. Cultures were incubated in a humid atmosphere of 95% air and 5% CO_2 at 37°C. When the cells became confluent 7-14 days after plating the medium was further supplemented with 58μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate (Wako
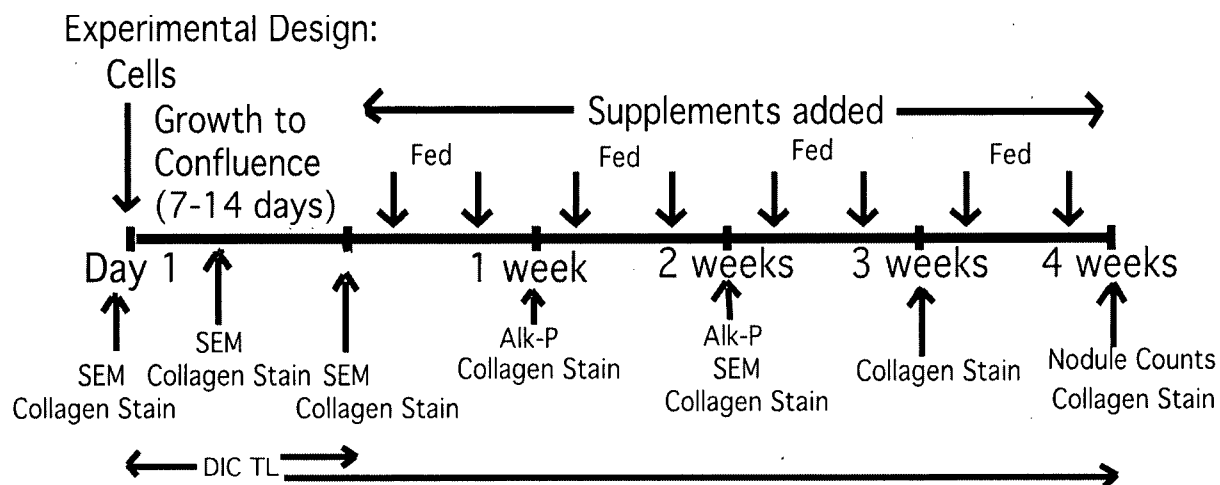
Figure 3. **A schematic diagram of the experimental design.** SEM, (scanning electron microscopy). Collagen stain, (Picro-sirius red F3B stain). Alk-P, (Alkaline phosphatase activity). Nodule counts. Tetracycline was added along with the supplements for ,four weeks and the nodules counted using fluorescence microscopy. DIC TL, (Differential Interference Contrast Time-Lapse movies).

Pure Chemical Industries Ltd., Richmond, VA, USA) and 3.15mg/ml Na-ß-Glycerol-phosphate (Sigma, St. Louis, MO, USA) over the next 4 weeks. The medium was changed twice a week. At the end of the culture period the tissue cultures were fixed and processed for microscopy. Fixative used for most cultures was 10% buffered formalin phosphate (Fisher Scientific Co, Fair Lawn, NJ, USA) for 1hr, for scanning electron microscopy was 2.5% Glutaraldehyde (JBS-Chem, Dorval, QUE) for 30 minutes and for alkaline phosphatase activity was citrate acetone (Sigma) for 30 seconds.

B. Scanning electron microscopy (SEM):

To observe the morphology of osteogenic cells and mineralized nodules, some cultures were prepared for scanning electron microscopy (SEM). Fixed cultures (24hours,1,2 and 4 weeks old) were post-fixed in 2% buffered osmium (Canemco Inc, Lachine, QUE) for 30 minutes. After rinsing with .1M phosphate buffer, pH 7.4 followed by distilled H_2O, 2% tannic acid (Fisher Scientific Co) was added as this enhances osmium fixation making it more electron dense. The specimens were then put back into 2% buffered osmium for 30 minutes. After fixation they were dehydrated with graded alcohol (50%-100%, 1hr total), critical-point dried with CO_2(LADD Research Ind, Burlington, VT, USA), sputter-coated (Hummer VI Sputtering System, Technics, Alexandria, VA, USA) with 10nm of gold and observed in a scanning electron microscope (Cambridge

Stereoscan 100, Leica, Canada). To observe structures underlying the nodule (4 week cultured surfaces were used) cellophane tape was used to lift the top of the surface, then both tape and the underlying area of the wafer were re-coated with 10nm of gold and re-examined under SEM.

C. Polarization microscopy:

To observe collagen and its orientation, picro-sirius red staining and polarized light microscopy were used. The bireference of collagen is enhanced with this stain. The method used is similar to the one used by Pearse (1985). Cultures from 24hrs and 1 to 6 weeks were used. At the end of each period they were fixed in 10% buffered formalin for 1 hour. After rinsing in distilled water followed by 95% ethanol the cultures were treated for 10 minutes in alkaline alcohol at 60°C (alkaline alcohol: 95% ethanol + ammonium hydroxide (Fisher Scientific Co.); pH > 8.0). Following this treatment they were rinsed with distilled water and put in with picro-sirius red (100ml Saturated aqueous picric acid + 0.1g sirius red F3B (Gurr, BDH Laboratory Supplies, Poole, England)) for 1 hr at room temperature. Surfaces were rinsed in 1% acetic acid until acid was clear, dehydrated quickly (75%-100% in repeated stages), cleared in xylene and mounted in Entellan (EM Science, Gibbstown, NJ, USA). The surfaces were examined under a polarising microscope (CarlZeiss Jenapol) at 20x magnification and photographs using Kodak Ektachrome 160T or

Fujicolor Professional 400NPH film.

D. Time-lapse cinemicrography:

HA-coated surfaces smooth, 3, 10, 30µm depths and 5µm depth-100µm pitch were used. Individual surfaces were mounted on glass slides and placed in a Pentz chamber (Bachofer, Reutlingen, W. Germany) which was placed in a stage incubator (Bachofer)at 37°C, with perfused 95% air, 5% CO_2, and viewed with reflected-light differential-interference-contrast optics. A pentium I PC computer with Northern Eclipse software (Empix, Mississauga, ON) was used to capture images from a colour video camera (Sony powerHAD 3CCD) mounted on a Reichert microscope. A PowerTower Pro 180 computer with Scion Image software (1.62) was also used to capture images from a Hamamatsu video camera (model C2400-07). Both sets of images were edited by Scion Image software (1.62) on the PowerTower and made into Quicktime supplier movies.

For the movie following nodule development a 5µm depth-100µm pitch surface was used, following four weeks culture with mineralization supplements, tetracycline was added for an additional week. The tetracycline becomes incorporated into the nodule and images could be taken of nodules formed on the surface using the Princeton PentaMax 12 bit CCD camera at 20x magnification with UV light (488nm).

E. Tetracycline Incorporation:

To quantify mineralized tissue 5μg/ml of tetracycline was added to the media supplements of L-ascorbic acid phosphate and Na-ß-glycerol-phosphate.   At the end of four weeks the surfaces were fixed with 10% buffered formalin for 1hr at room temperature.   After rinsing with distilled water the surfaces were dehydrated (75%-100), cleared in xylene and mounted in Entellan (EM Science).   Mineralized tissue was observed and nodules counted under UV light (488nm) at 20x magnification on a Zeiss ASIO-2-Motorized Stage microscope with a Princeton PentaMax 12 bit CCD camera.   The nodules counted were an aggregation of cells, producing a multi-layer that were at least 35μm in diameter and fluoresced.   Pictures were also taken of smooth and grooved surfaces and all smooth gaps.   In addition the pictures were used to determine the percentage of a nodule's area located on surface features, using NIH Image software.

F. Alkaline Phosphatase:

Alkaline phosphatase activity was measured, using a diazonium coupling reaction (Figure 4), for 2 and 3 weeks only, since Alk-P is highest at those times (Jaiswal *et al*, 1997).   Procedures were those recommended by Sigma diagnostics using Fast Blue RR Salt (Sigma
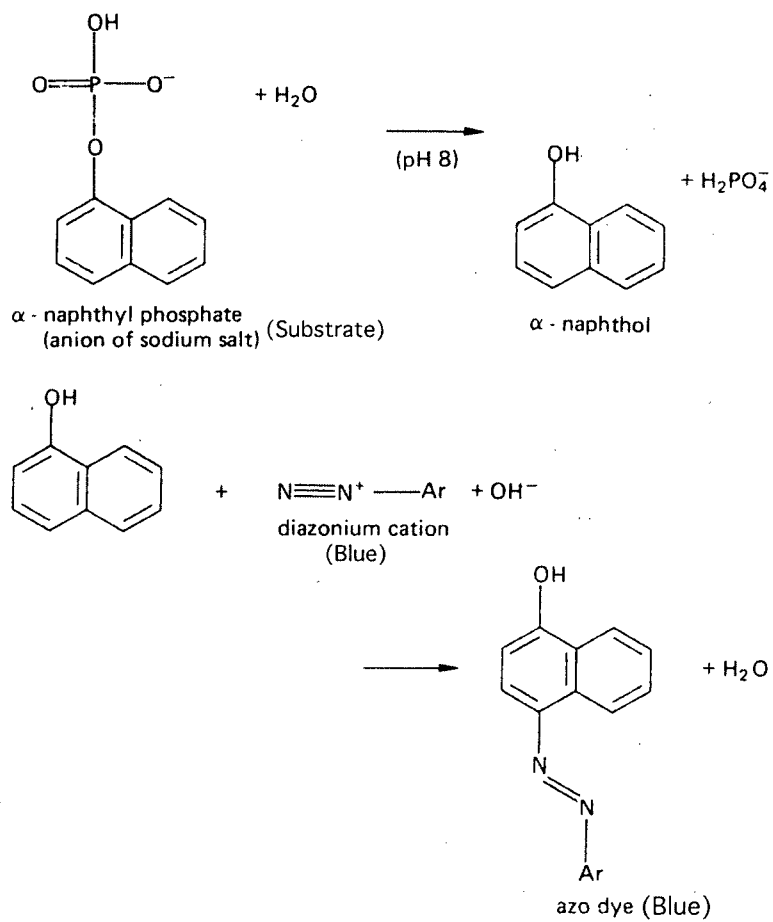
Figure 4. **Schematic diagram of Alk-P reaction**. The reaction between substrate and diazonium cation (Fast Blue RR Salt) (Kiernan, 1981).

diagnostics, St. Louis, MO, USA) and Naphthol AS-MX phosphate alkaline solution was used as the substrate solution from Sigma alkaline phosphatase kit (No. 85L-2). No counter stain was used. The alkaline-dye mixture was adjusted to pH 9.6 which was found to be optimum for this study. Pictures were taken on a CEI pentium computer with Sony powerHAD 3CCD colour video camera at 16x magnification with Northern eclipse software (Empix) and the areas that reacted with the dye were quantified on a Powertower Pro 180 with Scion Image software.

H.   Correlation of Tetracycline and Alk-P activity.

Using the method for quantifying mineralized nodules, 10μm and smooth, HA and Ti coated micromachining substrata were cultured for 3 weeks with mineralization supplements. At the end of 3 weeks the cultures were stained using the Alk-P activity method and mounted with glycerol. Pictures were taken using U.V. light (488nm) on a confocal laser scanning microscopy (MC80, Zeiss) with Fujicolor Professional 400NPH film at 20x magnification.

3. Statistics:

SPSS statistical software was used for the analysis of all data. Analysis of variance (ANOVA) and Student-Newman-Keuls test to identify conditions that differed significantly ($p \leq 0.05$).

III.   Results

In the following sections of this thesis the results are presented in the sequence: the characterization of surfaces, responses of osteogenic cells to micromachined surfaces in terms of cell morphology, collagen organization, bone-like nodule production, alkaline phosphatase activity, and the correlation between tetracycline incorporation and Alk-P activity.

1. Characterization of Sputter coated HA surfaces:

A. X-Ray diffraction:

X-ray diffraction analysis provided by Dr. WR Lacefield using techniques for the calcium phosphate coated substrata indicated that in fact a HA structure was present on the coatings. (Figure 5). Peaks were observed at 24°, 32°, and 33° at 2ø that are representative of HA.

B. Surface roughness:

A profilometer was used to determine roughness of the HA and Ti coated "smooth" surfaces. The mean roughness (R_a) values obtained were HA=2.8nm and Ti=1.3nm. However these values were lower than the accepted resolution of the profilometer (5nm, Wieland personal communication) and thus can be considered as smooth at least as assessed by the usual standards applicable in implantology. Figure 6 shows profilometer printouts of a 10μm deep HA and Ti coated surfaces. From the profiles of the grooves the depth and pitch can be observed and were determined to be ≈10μm deep with ≈47μm pitch, confirming the design
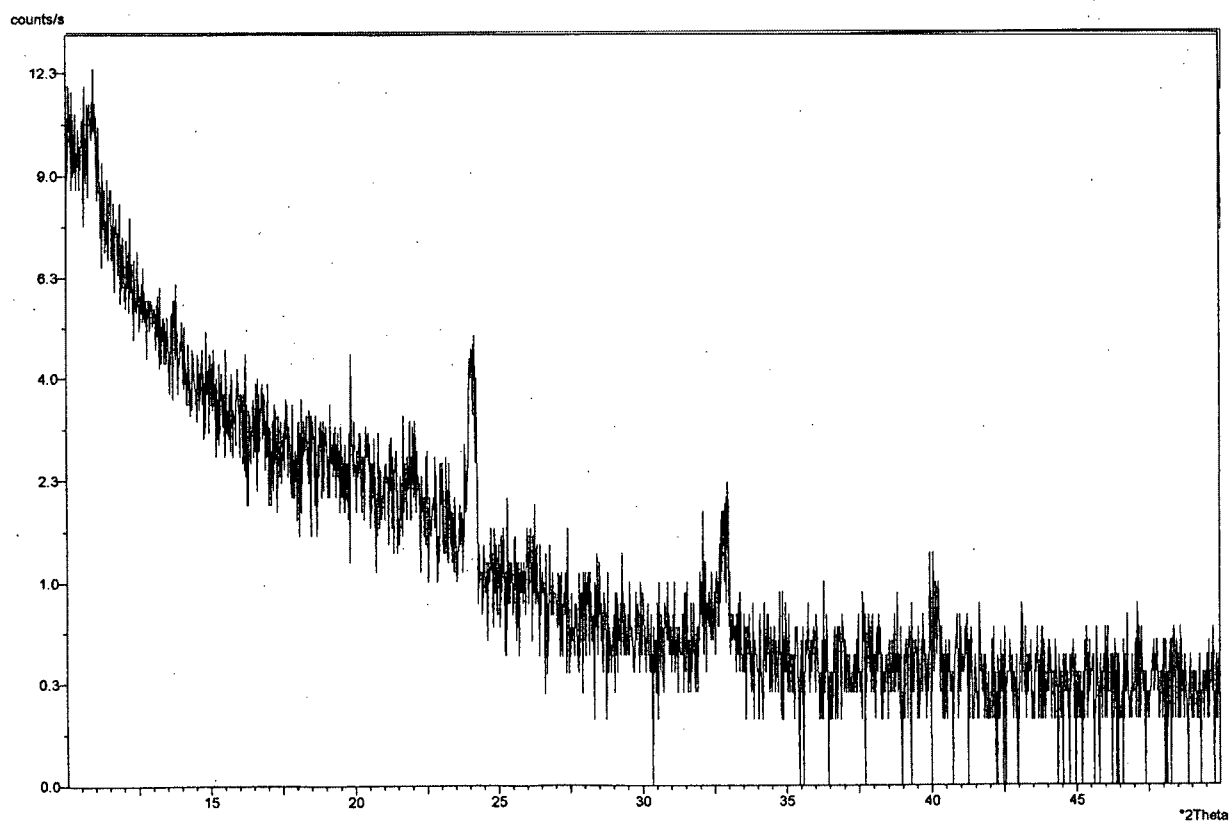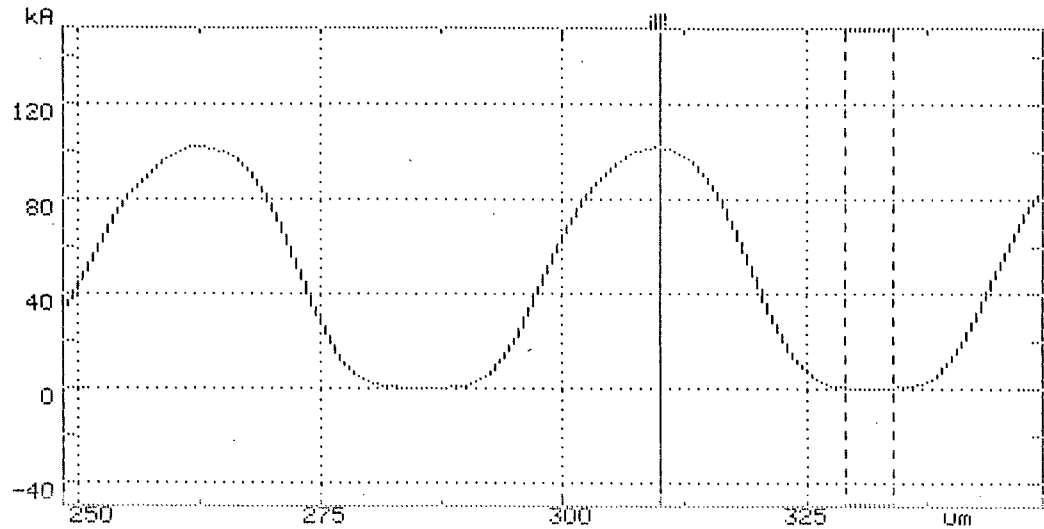
Figure 5. **X-Ray diffraction of HA coated micromachined substrata**. The peaks at 24°, 32°, and 33° degrees area representative of HA. (Degrees 2ø).

A

04/14 02:28

ID #

VERT 200kA

L 101.4kA

R 101.4kR

dAV 101.2kR

Avg 101.4kR

TIR 0. A

Ra 0. A

HORIZ 1000m

L 310.0um

R 310.0um

0.000m

Area 0.0000

SCAN MENU 1

um s/um

2000 .2 1

400 1 5

80 5 25

SCAN t= 8sec

DIR.<—

STYLUS 9mg

0 380um LEVEL

B

05/01 11:04

ID #

VERT 200kR

L 101.3kR

R 101.3kR

dAV 101.3kR

Avg 101.3kR

TIR 0. A

Ra 0. A

HORIZ 1000m

L 243.0um

R 243.0um

0.000m

Area 0.0000

SCAN MENU 1

um s/um

2000 .2 1

400 1 5

80 5 25

SCAN t= 8sec

DIR.<—

STYLUS 9mg

0 164um LEVEL
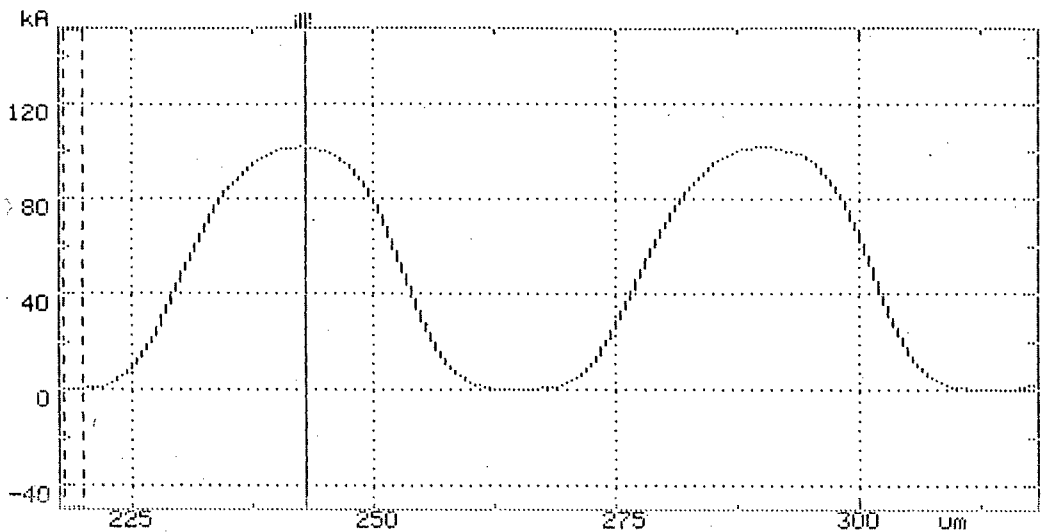
Figure 6. **Surface profiles of substrata**. Profilometer printouts of a) HA and b) Ti coated micromachined substratum. The large peaks are profiles of the 10μm deep grooves coated with HA and Ti. Depth ≈10μm and pitch ≈47μm. Note that the shapes of the profiles do not conform to the topography because of the effect of stylus size (12.5μm) which smooths out the record of the topography at the groove edges.

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specifications. Furthermore both Ti and HA had the same topography, thus the HA coating was uniform and had little affect on the pitch or depth of these substrata.

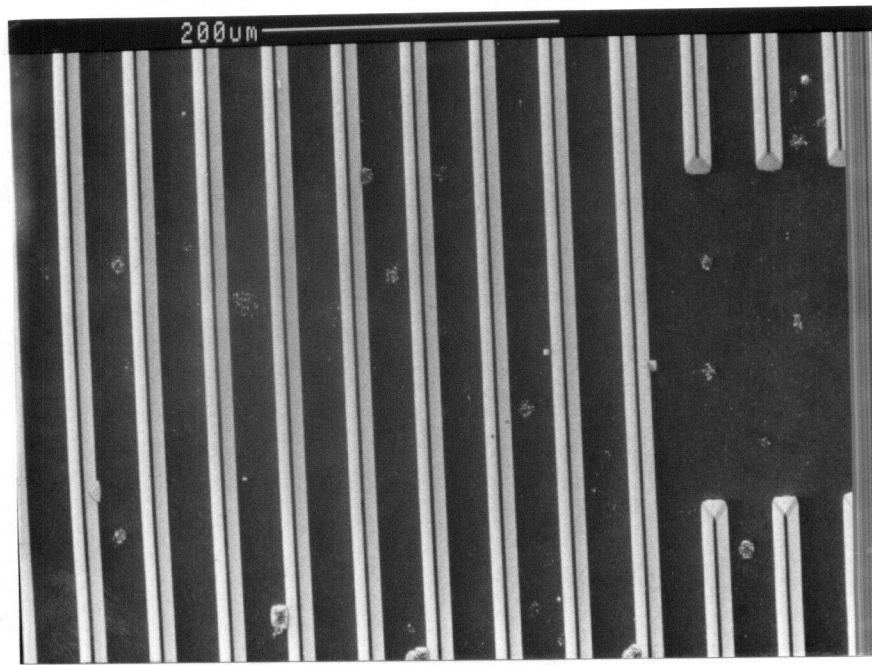### C. Scanning Electron Microscopy (SEM):

SEM observations of the substrata are given in figure 7. A small difference was noticed between the HA and Ti surfaces in ridge width. As a result of the HA coating the ridges of the HA substrata were 4μm wide, whereas the Ti surfaces had ridges of 5μm width. A cross sectional view of the substrata was also obtained (figure 8) and the HA coating was observed to be uniform, dense and approximately 1μm thick.

2. Osteogenic cell cultures:

A. SEM:

After 24hrs of culture, osteoblasts on smooth HA exhibited no preferred orientation. (Figure 9). In contrast osteoblasts on grooves were found to be aligned with the grooves. At 1 week and 2 weeks of culture it was difficult to determine cell orientation because the cells had become densely confluent and cell outlines were not clear. Moreover in 2 week cultures aggregations of cells and globular structures were seen (figure 10), indicating possible areas of nodule formation. Similar results were observed on Ti substrata.

Osteoblasts cultured for four weeks were examined for nodule

A
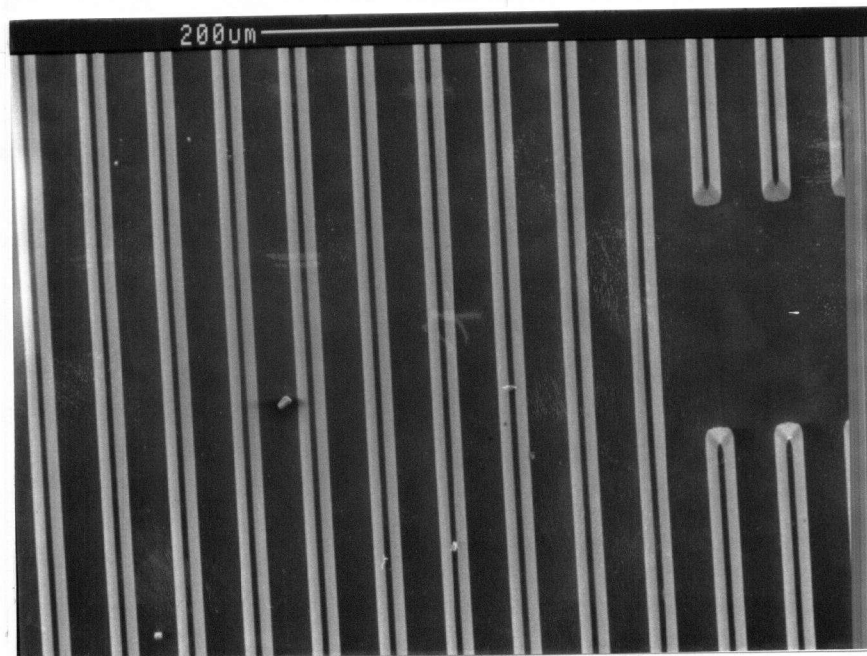
B

Figure 7. A,B) **Scanning electron micrographs of substrata**.   Micrographs prior to being used in culture. A) HA surface. B) Ti surface, notice the small but detectable difference in ridge thickness.
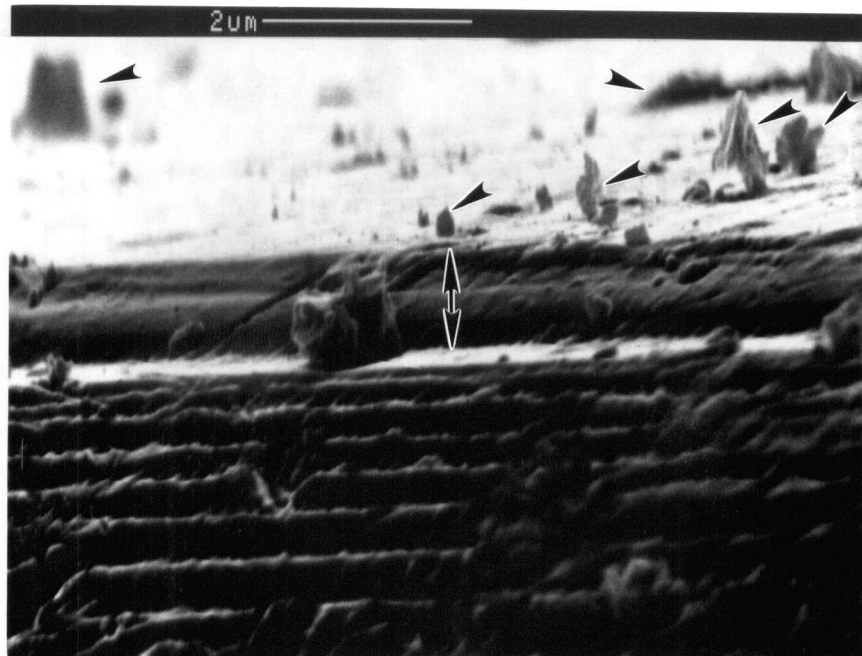
Figure 8. **Scanning electron micrograph of a sputtered HA coating** (<->). Debris presumably formed during fracture of the surface (<-).
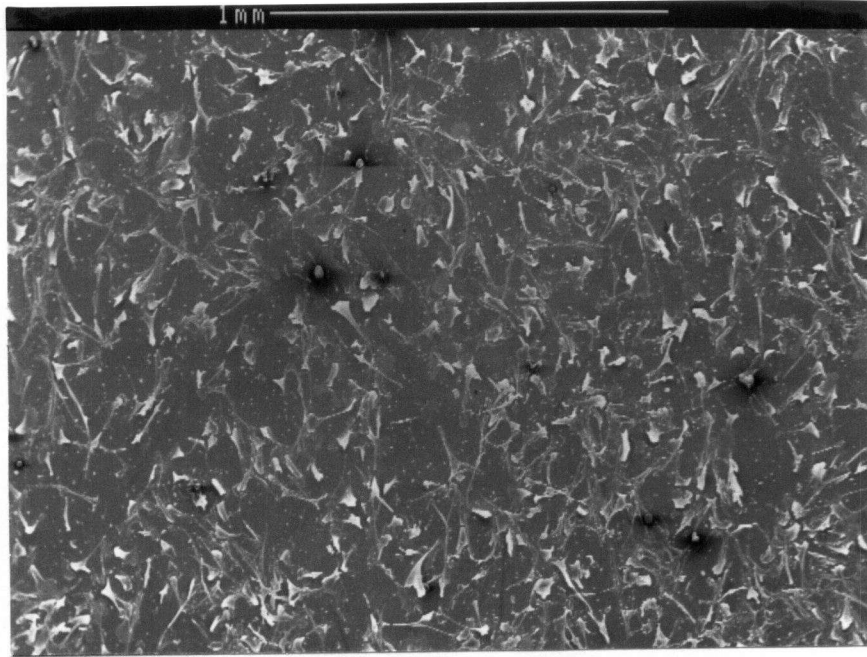
A
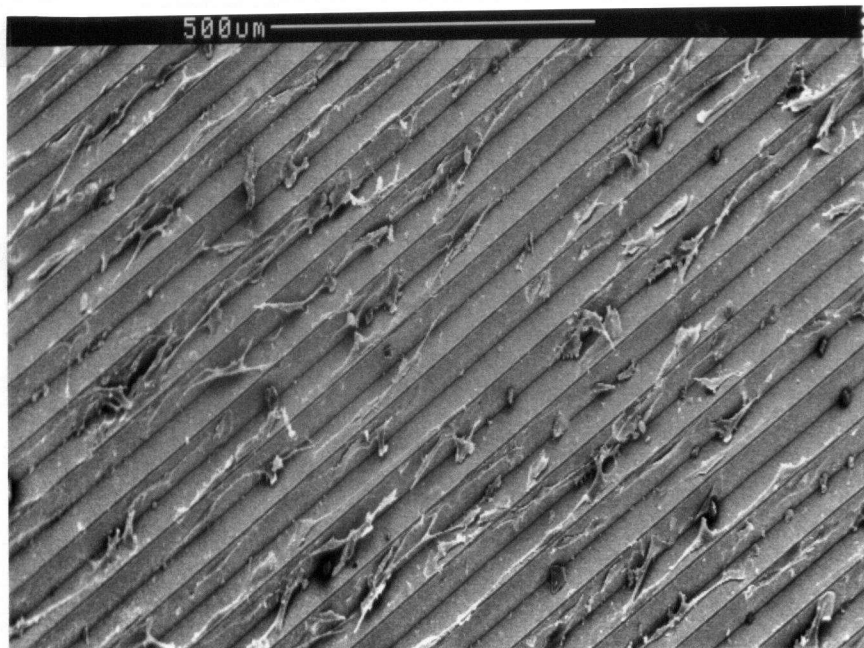
B

Figure 9. A,B) **Osteoblasts cultured for 24hours on HA coated substrata**. A) On the smooth surface, osteoblasts were arranged randomly (x47). B) 30μm deep surface, osteoblasts demonstrated alignment with grooves and cells were elongated (x77). Similar results were observed on Ti substrata.
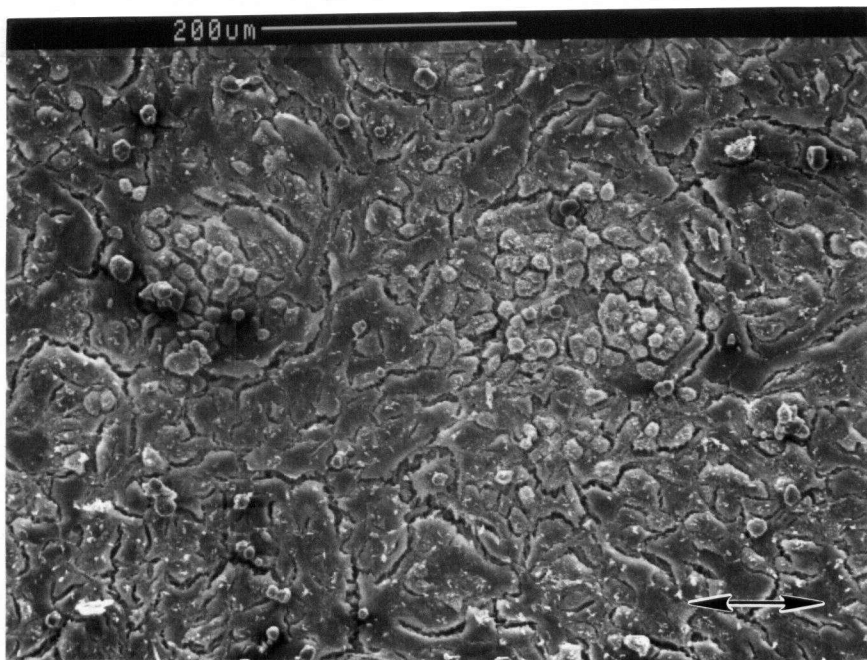
Figure 10. **Scanning electron micrograph of two week culture**. Osteoblasts cultured on a 3μm deep HA coated micromachined substratum. Globular structures are evident in aggregates, possibly indicating areas where nodule formation is beginning. (x153). Similar results were observed on deeper substrata and on Ti coated substrata. (<->, represents groove direction).

production. A representative nodule seen on HA and Ti substrata is shown in figure 11A. Globular structures, similar to those reported in the literature to be mineralized (Davies *et al*, 1988b; Chehroudi *et al*, 1992; Okumura *et al*, 1997) were observed and some osteoblasts were attached to these globular structures. (Figure 11A).

To observe the under structure of the nodules cellophane tape was attached to the nodule and then stripped from the surface. In the area underneath the removed nodule, globular structures (presumably mineralized accretions) were found, and globules interdigitated with fibres that were most likely collagen. (Figure 11B).

3. Collagen Organization:

A. Picro-sirius Red Staining:

As the substrata were complex and had the possibility of producing artificial optical effects, picro-sirius red staining was done on the surfaces in the absence of cells. No stain was observed on either HA or Ti substrata, but the ridges did show some brightness resulting from reflected light. (Figure 12).

Cultures form 24hrs to six weeks were analyzed and found to show an increase in collagen production indicated by the amount of birefringent red stain. Figure 13 shows this increased amount in collagen after 2 weeks and 6 weeks culture on HA substrata. As depth increased
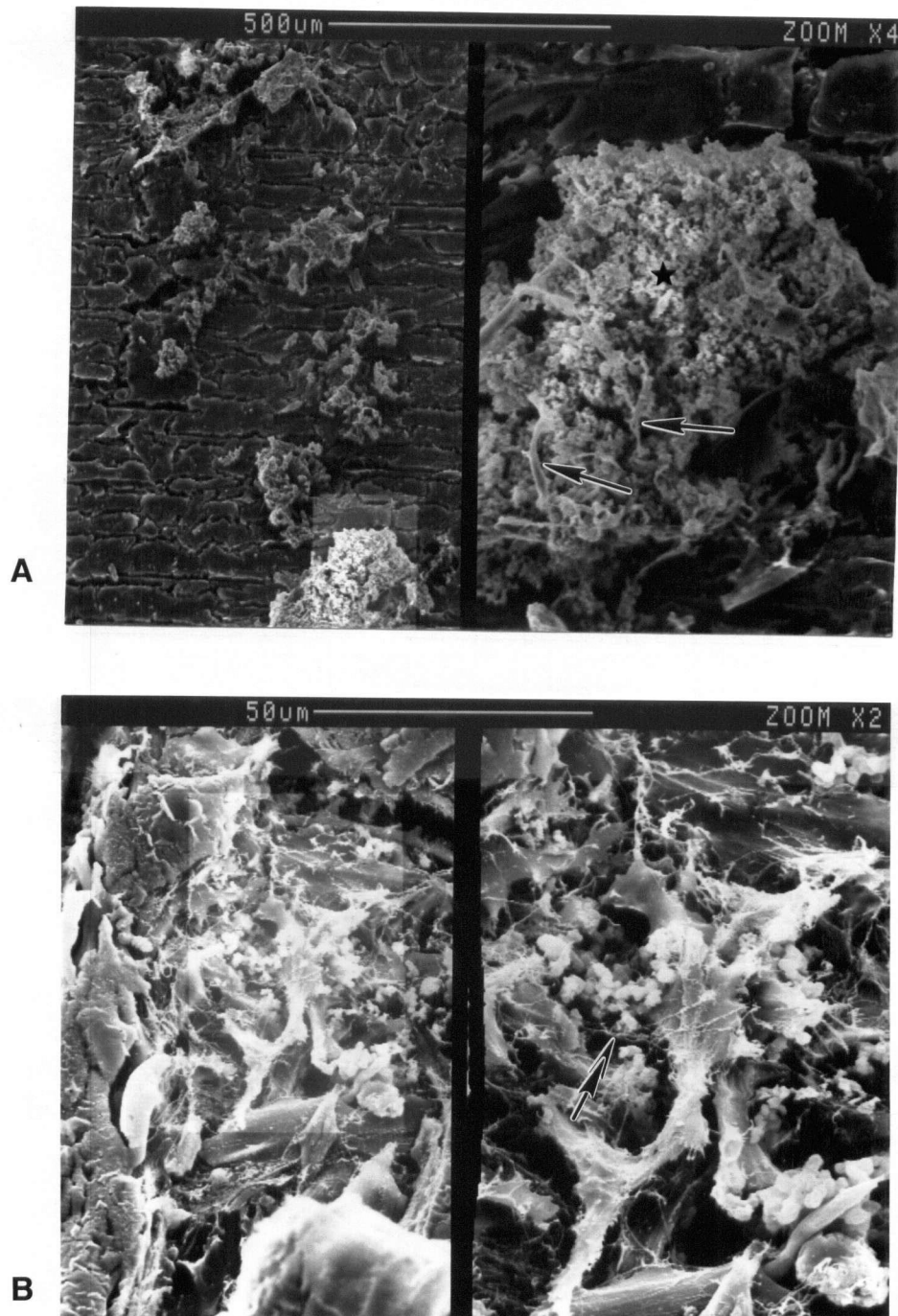
Figure 11. **Nodules on micromachined substrata**. A,B) Osteoblasts cultured for 4 weeks on HA coated micromachined substrata. A) A nodule formed on the 30μm deep substratum. Mineralized globules (*) and osteoblasts (<-) are evident (x70). B) An area of culture where part of a nodule has been stripped off using cellophane tape. Mineralized globules interdigitated with presumptive collagen fibres (<<-) found at the base of a nodule (x696). Similar results were observed on all depths and surfaces.
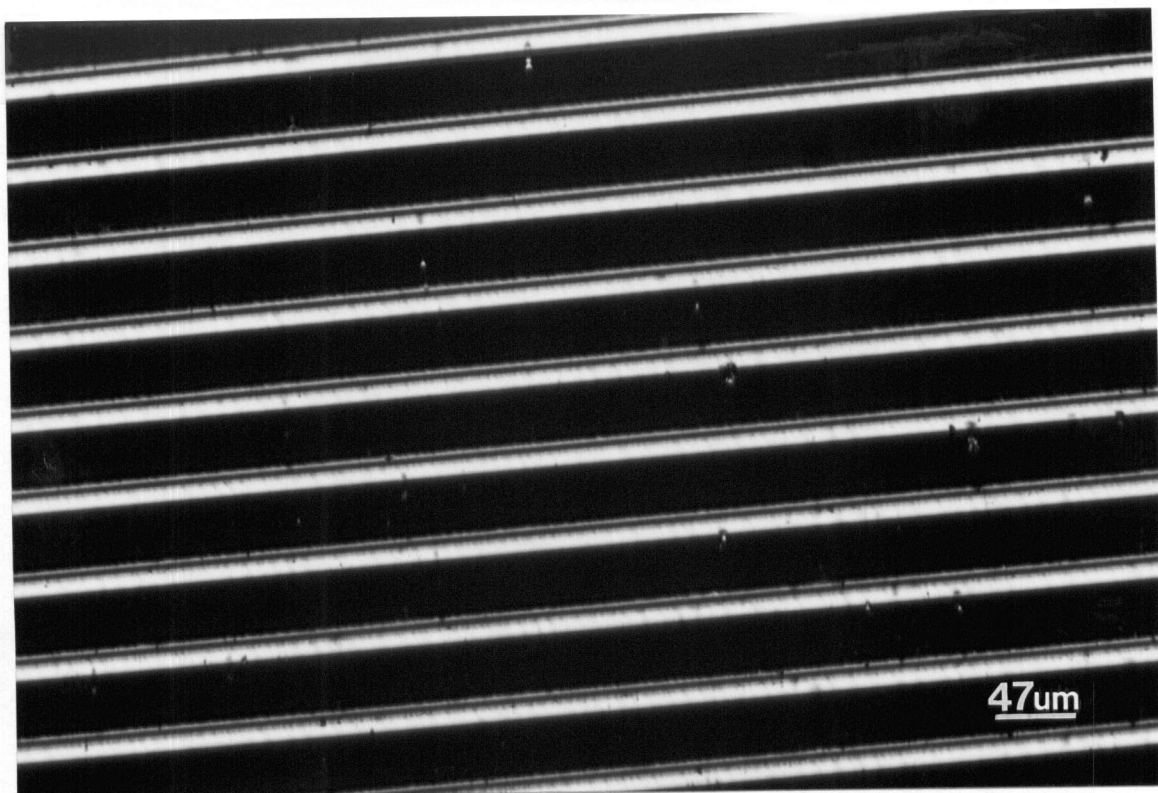
Figure 12. **Reflected polarized light micrograph of HA surface stained with picro-sirius red**. HA coated10μm deep micromachined substratum used as the control. No cells were present but the substratum was incubated for 1.5 weeks with culture medium. No stain was observed on this HA control surface nor was there stain on a Ti surface treated identically. (x20)
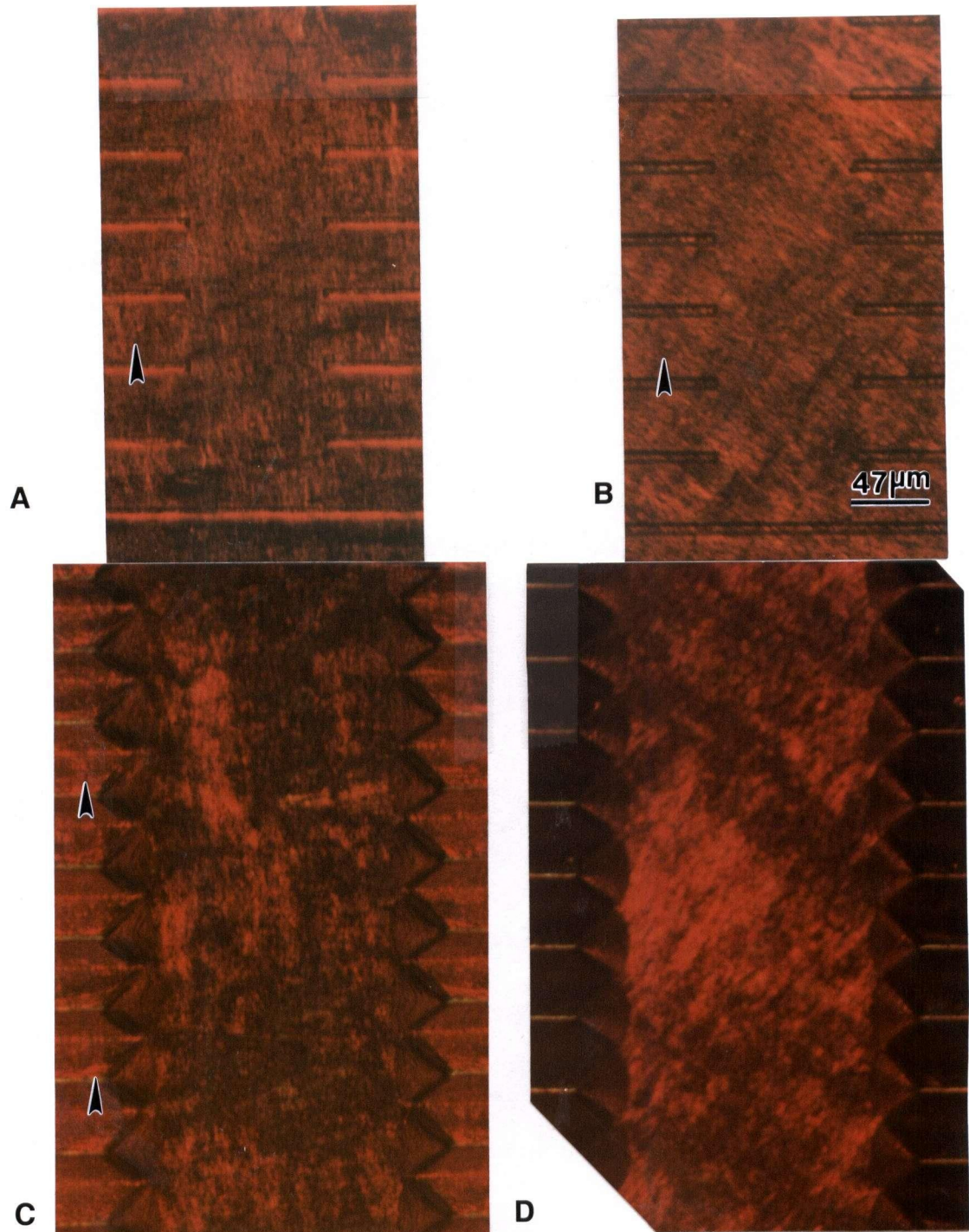
Figure 13. **Orientation of collagen on grooved substrata**. Picro-sirius staining of osteogenic cells cultured on HA substrata. A&B) 2 week culture, 3μm deep, showing less alignment in grooves and less collagen than C&D. A) 0° degree. B) 45° degree rotation of same area as A. C&D) 6 week culture, 30μm deep, showing more alignment of collagen in grooves (<-) than in gap. C) 0° degree. D) 45° degree rotation of same area as C, a position in which birefringent structures aligned with the grooves are extinguished. Similar results were found on Ti substrata. (x20)

the orientation of collagen became parallel to the grooves, as is evident by comparing figures 13A&B and 13C&D. By a 45° degree rotation of the field shown in figure 13A&C, the collagen that was not evident in figures 13B&D. By the same token collagen fibres evident in figure 13B&D are not evident in figure 13A&C. Furthermore in the smooth gap areas of the 3μm deep surfaces, collagen was found to be oriented perpendicular to the grooves as well as diagonally within the gaps. (Figure 13A&B). The collagen orientation in the gaps of the 30μm surfaces was similar to that observed on 3μm deep surfaces. (Figure 13 Gaps). Similar results were observed on Ti substrata (data not shown).

4.   Nodule Quantification:

A.   Nodule counts:

The HA and Ti substrata without cells were tested to insure that the light that is emitted from the surfaces was not an optical artifact. The results displayed in figure 14 show no fluorescence under U.V. light. The appearance of a mineralized nodule labelled with tetracycline examined under U.V. light is shown in figure 15. Also visualized (figure 15) are small (< 35μm) globules labelled with tetracycline that were too small to be counted as discrete mineralized nodules.

The numbers of nodules on smooth and grooved surface
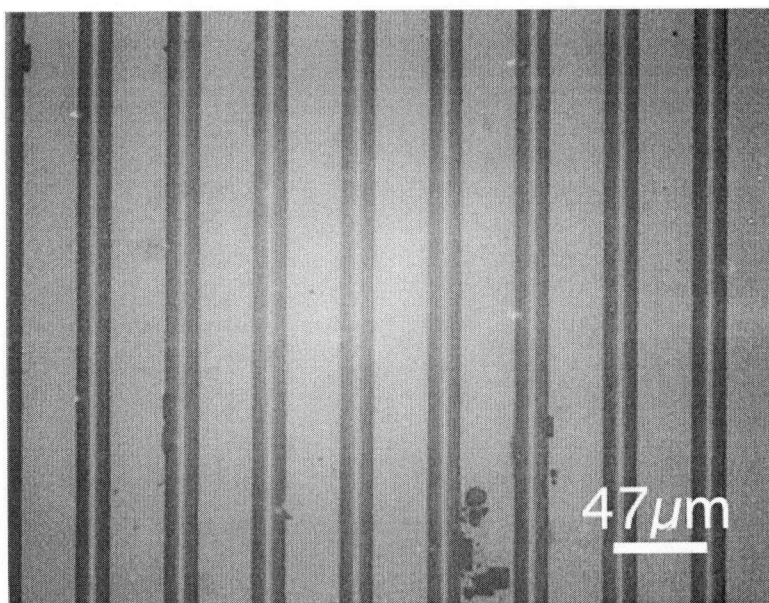
A
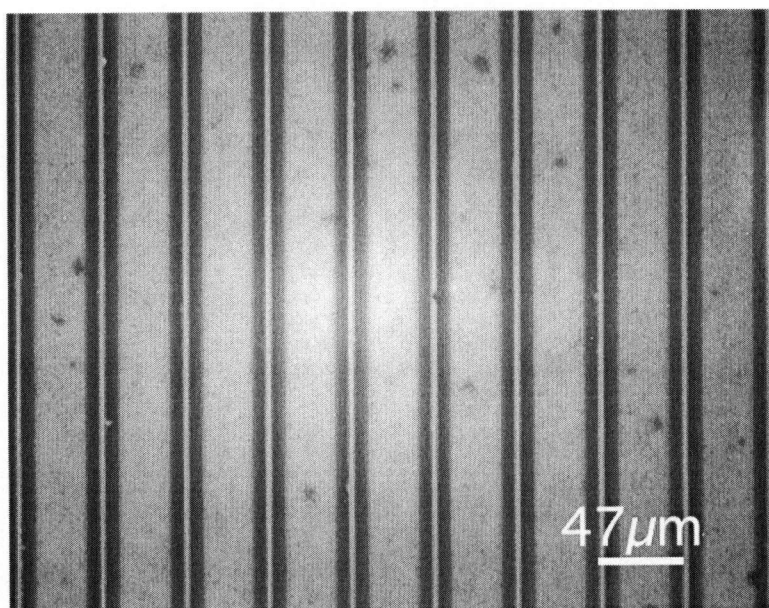
B

Figure 14. **Tetracycline controls**. A&B). Cells were not present but the substrata (10μm deep) were incubated for 1.5 weeks with mineralization supplements and tetracycline in the culture medium. Fluorescence under U.V. light (488nm) was not observed. A) HA surface. B) Ti surface.
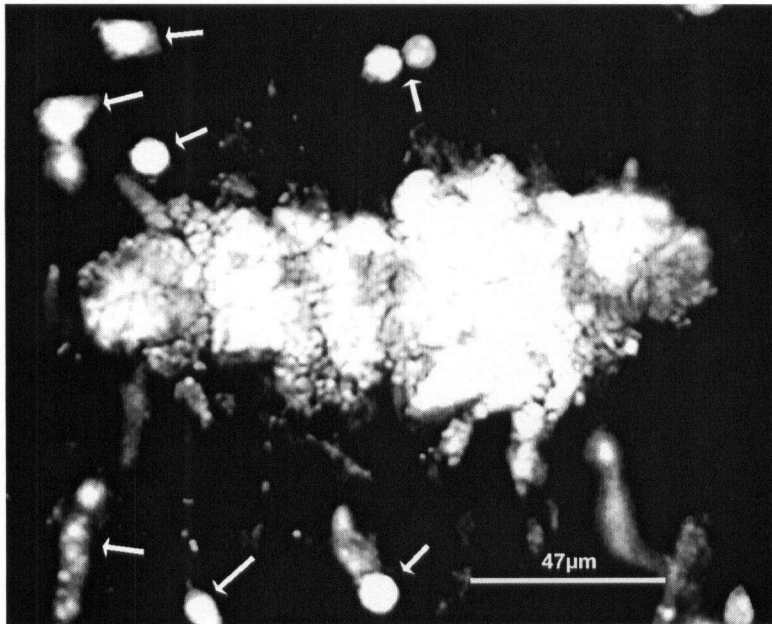
Figure 15. **Tetracycline labelled nodule**. Osteoblasts cultured on micromachined substrata for 4 weeks with mineralization supplements and tetracycline. A nodule observed under U.V. light (488nm) on a HA coated surface. Arrows represent globules labelled with tetracycline that were too small to be counted as discrete mineralized nodules.

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topographies are given in figure 16. HA coated grooves were found to produce significantly more mineralized nodules at all depths than Ti coated grooves. (Table 1). The number of nodules increased with depth of grooves with the 30μm deep surface was found to produce more nodules then shallower depths on both HA and Ti surfaces. When the experiment was

repeated similar results were obtained. It should be noted that this experiment is a two factor factorial design with the factors being type of surface at two levels (HA & Ti) and the second factor being depth of grooves at four levels (smooth, 3, 10, 30μm). The dependent variable is the number of nodules. Such a design enables one to test for interactions between the factors, in this experiment the interaction concerns whether the effect of coating (HA & Ti) varies depending on the topography of the surface. The statistical analysis of the data indicated that the effects of coating and depth were both significant ($p < 0.01$). Moreover the interaction effect was also significant ($p < 0.05$). (Table 1).

The same analysis was applied to the HA coated smooth gaps which produced significantly more nodules then the Ti coated smooth gaps. (Figure 17). Significant differences were also observed between patterns and between surfaces. (Table 2) but no significant interaction effect between pattern and surface was observed. The 50μm gap and 30μm depth

61
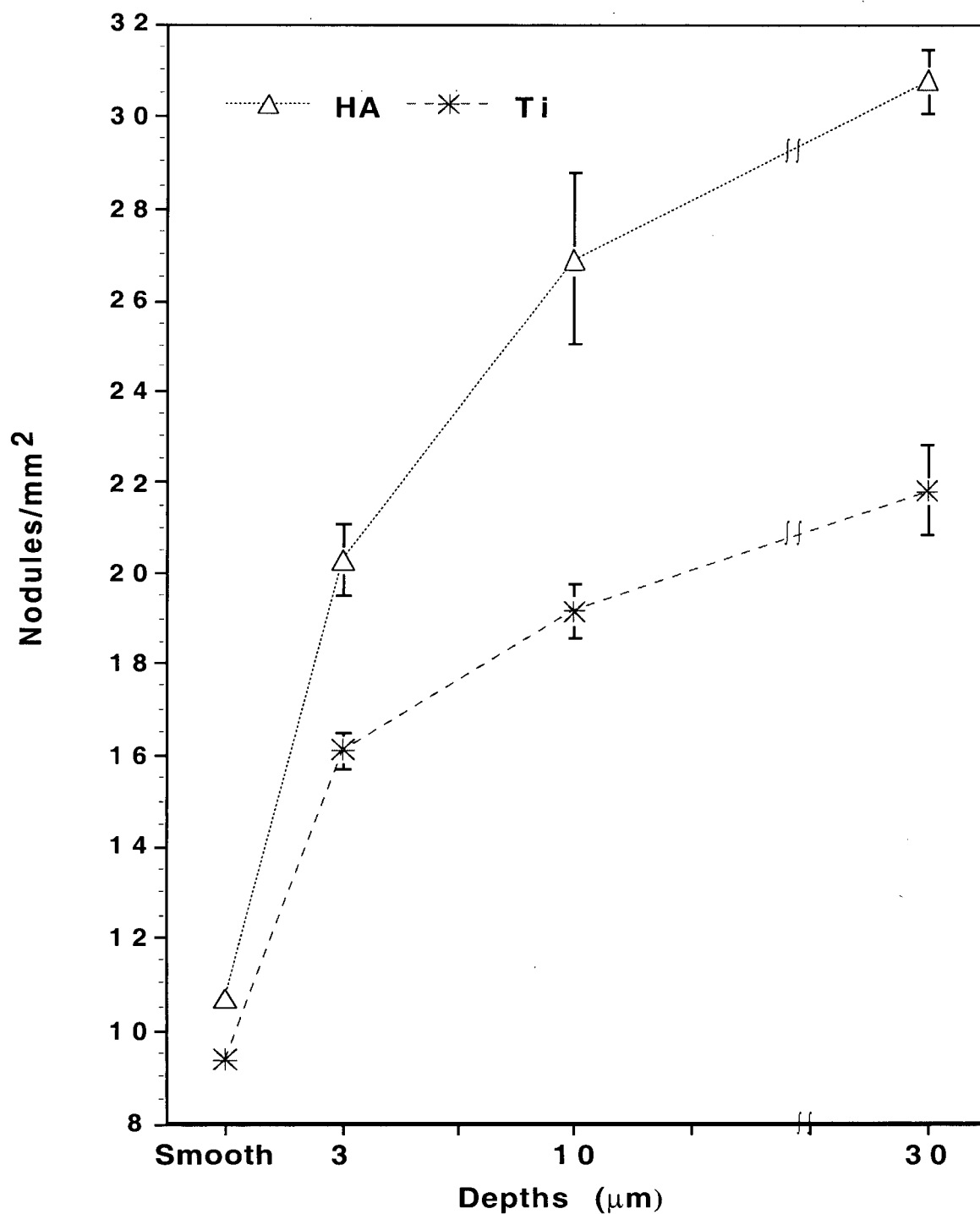
Figure 16. **Nodule counts on grooved substrata.** A significant difference in nodule counts was found between HA and Ti grooves, and significant differences were also observed between depths (p≤0.05). Osteoblasts cultured on micromachined substrata for 4 weeks with mineralization supplements and tetracycline. Number of nodules (± Std. Dev.) in the grooved areas. Repeat experiments showed a similar pattern.

<pre>
              * * * A N A L Y S I S   O F   V A R I A N C E * * *

              NODCOUNT
        by   DEPTH
              SURFACE

              UNIQUE sums of squares
              All effects entered simultaneously

                              Sum of   Mean   Sig
      Source of Variation   Squares   DF   Square   F   of F

      Main Effects   580011574   4 145002893.506   80.785   .00
          DEPTH   464544753   3 154848251.014   86.270   .00
          SURFACE   115466821   1 115466820.981   64.330   .000

      2-Way Interactions   18321759   3   6107253.088   3.403   .017
          DEPTH   SURFACE   18321759   3   6107253.088   3.403   .017

      Explained   598333333   7 85476190.470   47.621   .00

      Residual   2283148148   1272   1794927.789

      Total   2881481481   1279   2252917.499

      1280 cases were processed.
      0 cases (.0 pct) were missing.
</pre>

Table 1. **Analysis of Variance of the grooved areas.** Nodcount (Nodule counts). Surface, (Ti and HA coatings). Depth, (smooth , 3, 10, 30μm deep). Significant differences were observed by type of surface and depths. There was a significant difference in interaction between surface and depth.

63

A

Nodules/mm² vs Depths (μm)

Legend: 50μm (△), 100μm (✳), 150μm (○), 200μm (⊠)

X-axis: Smooth, 3, 10, 30
Y-axis: 0, 5, 10, 15, 20, 25, 30, 35, 40

Depths (μm)

A

B

Nodules/mm² vs Depths (μm)

Legend: 50μm (△), 100μm (✳), 150μm (○), 200μm (⊠)

X-axis: Smooth, 3, 10, 30
Y-axis: 0, 5, 10, 15, 20, 25, 30, 35, 40

Depths (μm)

B

Figure 17. **Nodule counts on smooth gaps.** A,B) Osteoblasts cultured on micromachined substrata for 4 weeks with mineralization supplements and tetracycline. Nodules counted in the areas of smooth gaps surrounded by grooves. A significant difference was observed between HA and Ti, between depths, and between gap sizes (50, 100, 150, 200μm) ($p \leq 0.05$). A) HA smooth gaps, the 50μm gap in the 30μm deep grooves produced the most nodules. B) Ti smooth gaps 50μm gap in the 30μm deep grooves produced the most nodules. Repeat experiments showed similar patterns, see table 3. There was considerable variation in the number of nodules counted in the gaps. (Error bars represent standard errors).

```
* * *   A N A L Y S I S   O F   V A R I A N C E   * * *

                COUNTS
        by      PATTERN
                SURFACE

                UNIQUE sums of squares
                All effects entered simultaneously
```
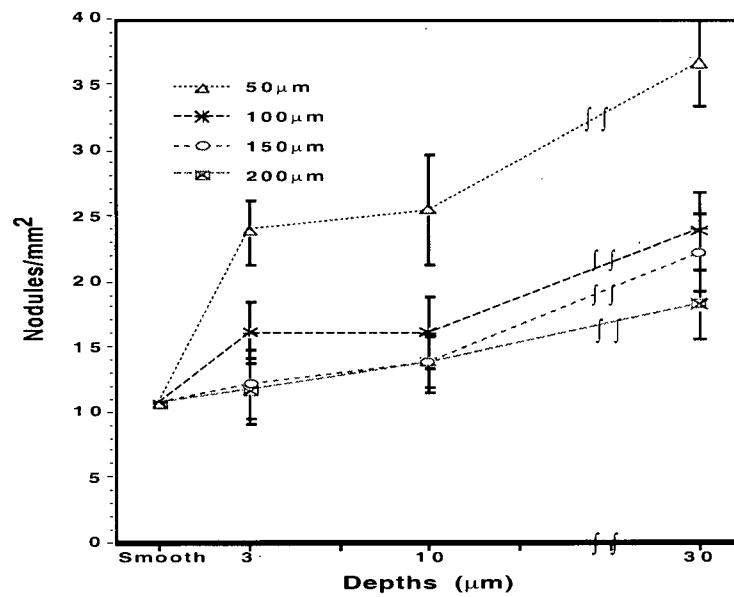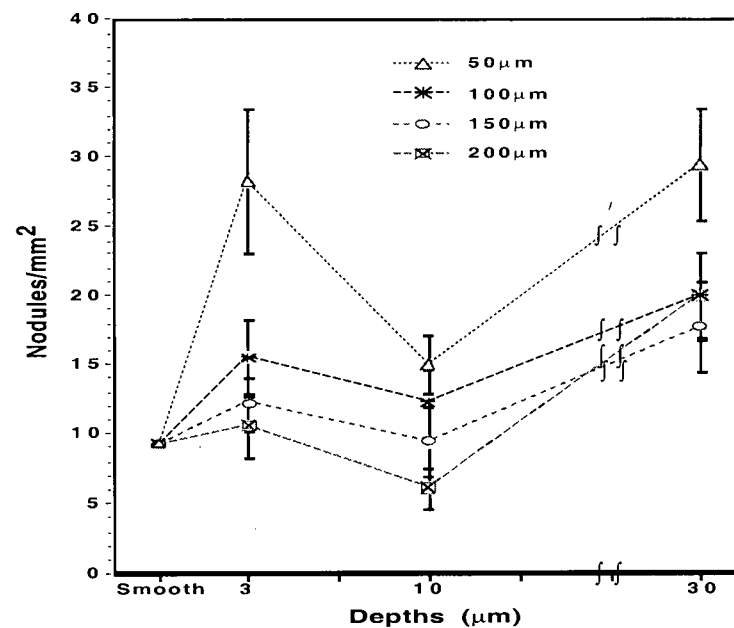
Source of Variation	Sum of Squares	DF	Mean Square	F	Sig of F
Main Effects	336400694	13	25876976.432	19.224	.00
PATTERN	322555556	12	26879629.627	19.969	.00
SURFACE	13845138	1	13845138.091	10.286	.001
2-Way Interactions	19898148	12	1658179.012	1.232	.256
PATTERN SURFACE	19898148	12	1658179.012	1.232	.256
Explained	362509259	25	14500370.369	10.773	.000
Residual	1041836420	774	1346041.886		
Total	1404345679	799	1757629.135		

800 cases were processed.
0 cases (.0 pct) were missing.

Table 2. **Analysis of Variance of nodule counts in the smooth gaps**. Counts, (nodule counts). Surface, (Ti and HA coatings). Pattern, (gap size at each depth). Significant differences observed by type of surface and pattern, but no significant difference in the interaction between surface and pattern.

was found to have more nodules than other depths and gap sizes on both HA and Ti surfaces. (Figure 17). Considerably more variation was noted in the data gathered from the gaps, possibly because only relatively small areas were available for counting. When repeated, the experiment showed similar results. (Table 3).

B. % Area of Nodule on Ridge:

To determine if nodules were being formed preferentially on the ridges or within the grooves, the relative areas of the nodule located on the ridge and groove were measured. Table 4 shows the percentage of a nodule that lies on a ridge. The results showed no significant difference between HA and Ti, nor was there a significant difference found among depths. However the least percentage area of a nodule that was located on the ridge was on deeper grooves. This is what would be expected if the formation of a nodule was favoured by the microenvironment formed as a result of restricted diffusion in the grooves.

5. Alkaline Phosphatase Activity:

A. Optimum pH:

The optimal pH had to be established for the enzymatic reaction for the cells used in this study. In two experiments the optimum pH was observed to be 9.6. (Figure 18).

B. Controls:

Nodule Formation on HA & Ti Smooth Gaps (Nodules/mm^2 ±Std. Dev.)					
Surface Type	Smooth		Smooth Gaps		
HA	14.6±2.9	50μm	100μm	150μm	200μm
Ti	10.7±1.4				
HA 3μm		21.1±9.5	14.4±10.9	14.4±10.3	13.9±8.7
HA 10μm		24.4±12.2	17.8±9.8	13.9±9.5	10.6±8.4
HA 30μm		26.1±11.0	17.8±12.7	13.3±10.6	13.9±8.7
Ti 3μm		16.1±13.7	12.8±8.3	15.0±10.4	12.2±11.3
Ti 10μm		10.6±9.9	13.3±10.6	10.6±8.4	13.9±12.4
Ti 30μm		16.1±11.1	15.0±9.7	17.2±13.2	10.6±8.4

Table 3. **Nodule formation on HA & Ti smooth gaps**. Repeated experiment of osteoblasts cultured on micromachined substrata for 4 weeks with mineralization supplements and tetracycline. Nodules were counted in the smooth gaps. Table showed the 50μm gap 30μm deep HA coated surface produced more nodules/mm^2.

% of Nodule on Ridge (%± Std. Dev.)		
Depth	**HA**	**T i**
3 μm	23±9	20±7
10 μm	22±9	19±8
30 μm	20±11	18±5

Table 4. **Percent of Nodule on ridge.** Osteoblasts cultured on micromachined substrata for 4 weeks with mineralization supplements and tetracycline. Areas of nodules located on the ridge were calculated using NIH Image software. Table shows a trend that nodules prefer the grooves over the ridge, but no statistical significance. ($p>0.05$).
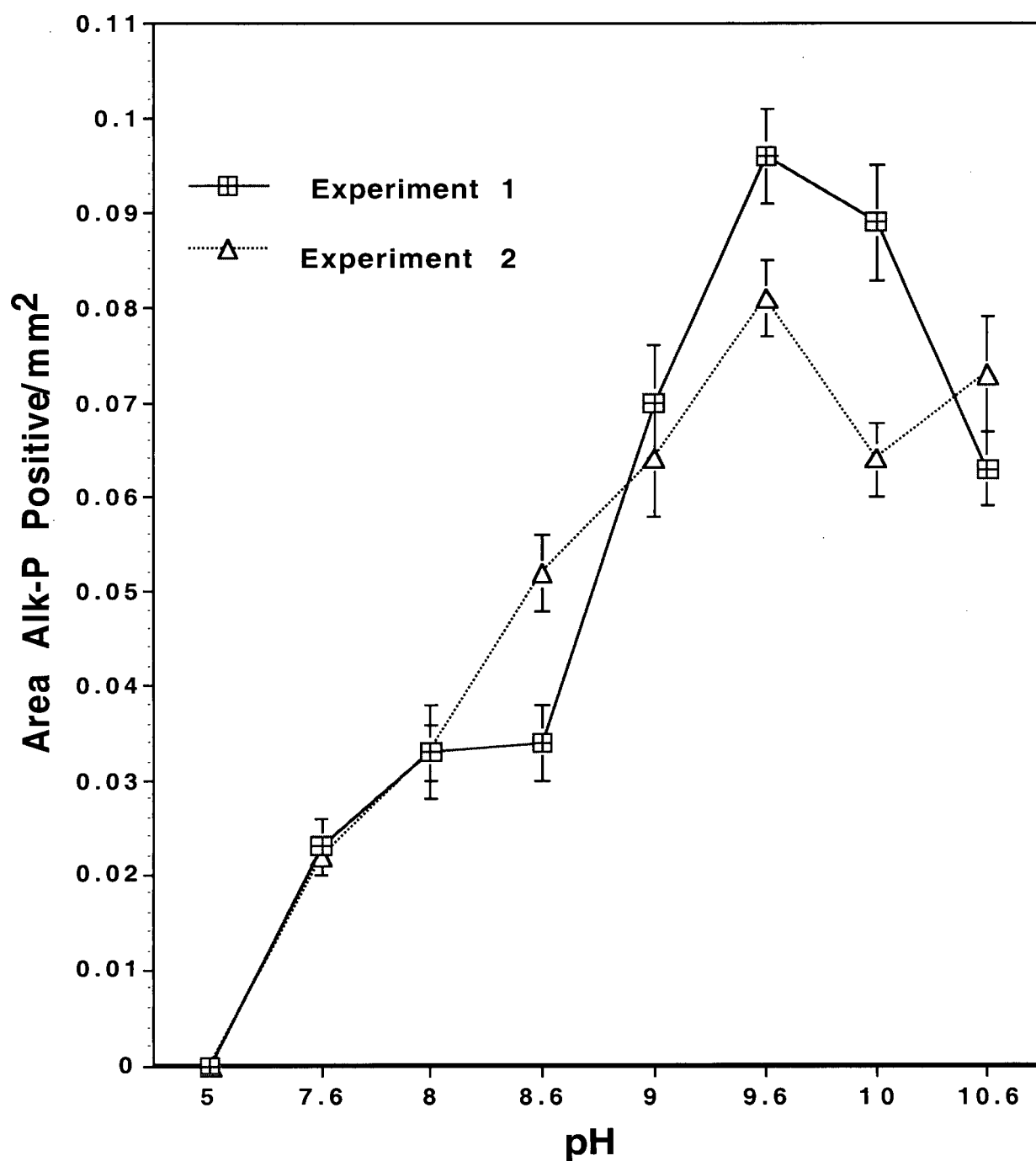
Figure 18. **The effects of pH on Alk-P activity.** Osteoblasts were cultured on smooth Ti substrata for 2 weeks with mineralization supplements. Alk-P activity was determine using naphthol AS-MX phosphate as the substrate and Fast Blue RR as the diazonium dye. % Areas of Alk-P activity/mm^2 were calculated from images using NIH Image software. The two experiments show results of pH 9.6 as optimum. (Error bars represent standard error).

To insure that the reaction is specific to Alk-P a number of controls were done including the following: a) surfaces incubated in media alone (ie. no cells), b) epithelial cells on surfaces (epithelial cells lack this enzymatic ability), c) Ti smooth controls plated with osteoblasts but no substrate was added, d) the cells were heat treated, and e) the pH reduced to 5.0 to ensure that acid phosphatase was not being detected. No stain was observed in any of these five control conditions. (Figure 19).

Using the reaction solution at a pH of 9.6 on osteoblasts cultured on Ti smooth surfaces, a positive reaction was visible and clear differences between the controls and pH 9.6 were evident. (Figure 19).

C.   Alk-P activity:

Alk-P activity on HA and Ti coated grooved substrata was found to have significantly more % area of positive Alk-P activity/mm^2 on HA than Ti grooved substrata for both 2 week cultures and 3 week cultures. (Figure 20). Furthermore there was a significant effect of depth of the grooves but no significance in the interaction between depths and surface. Similar to the data for nodule formation, there was an increase in Alk-P activity as depth increased. (Figure 20). It should be noted that the % area of positive Alk-P activity/mm^2 decreases from the 2 week to 3 week cultures.

When Alk-P activity was examined in the smooth gaps a

70
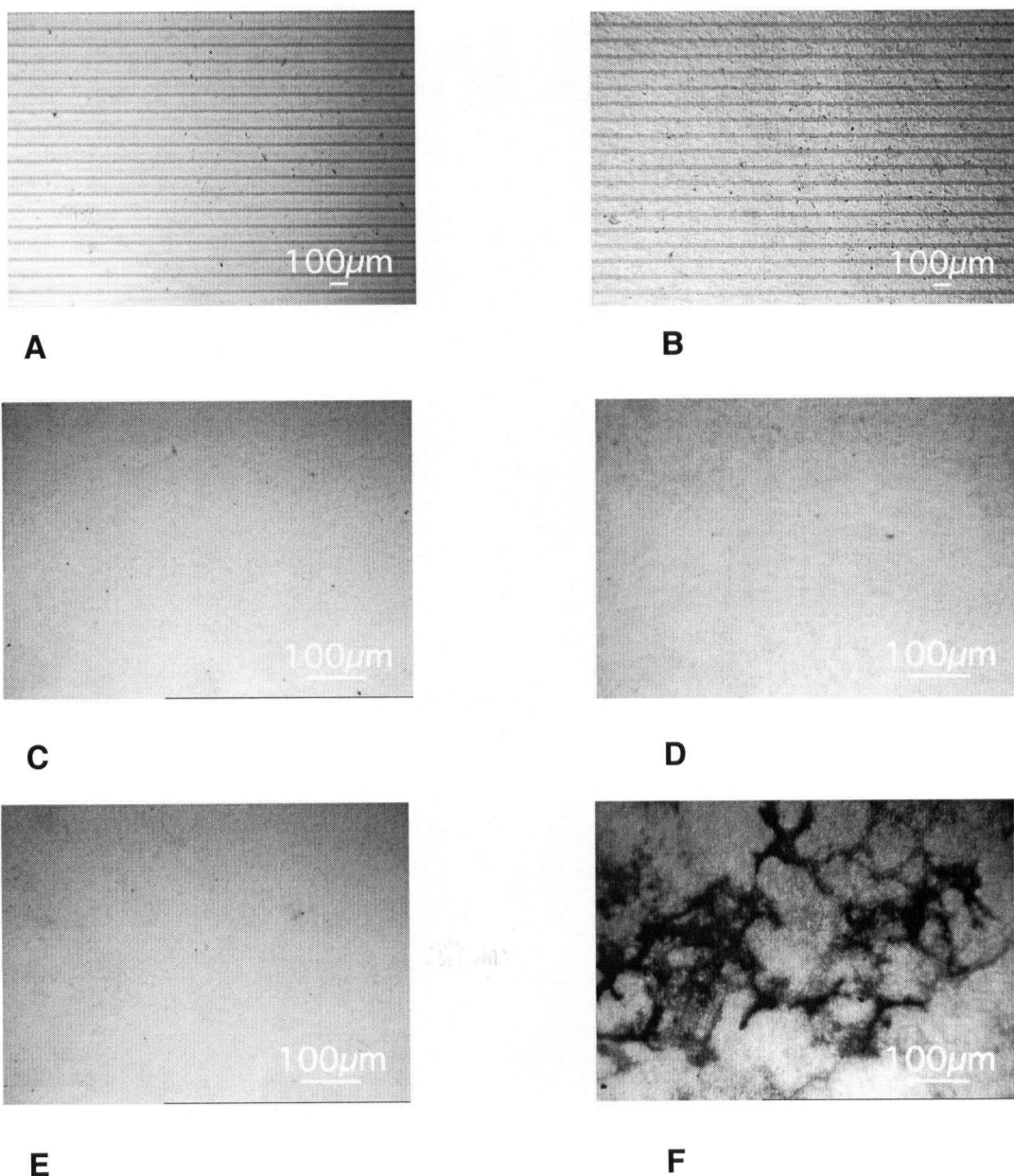
Figure 19. **Alkaline phosphatase activity**. A,B,C,D,E,F). A&B) 5μm deep 100μm pitch HA coated surfaces. A) Control with no cells present; stain was not deposited on HA. B) Control with epithelial cells that do not exhibit Alk-P were cultured on the HA surface for 2.5 weeks, no staining was observed. C,D,E,F) Osteoblasts cultured on Ti smooth substrata. C) Control with no substrate added. D) Control with osteoblasts heat-treated using a steam bath for 10 minutes prior to staining for Alk-P activity. No positive reaction observed. E) Control for pH. Staining for Alk-P activity at a pH of 5.0. No positive reaction observed. F) Staining Alk-P activity at a pH of 9.6. Noticeable positive staining reaction.
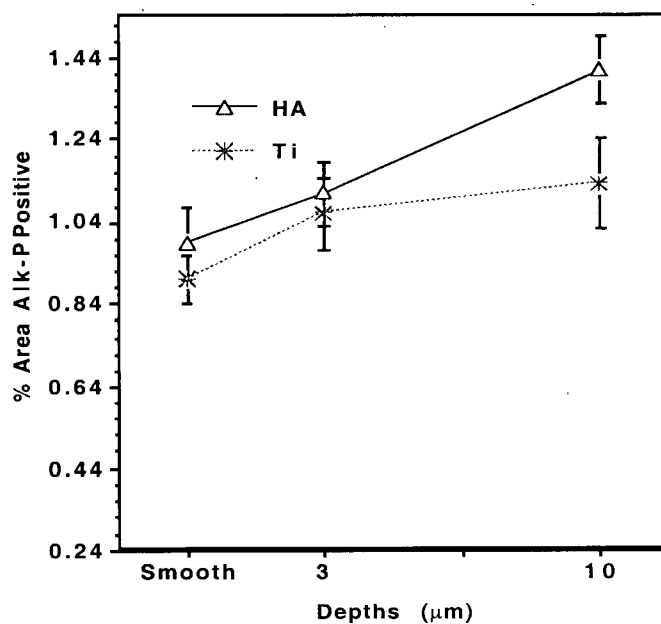
A
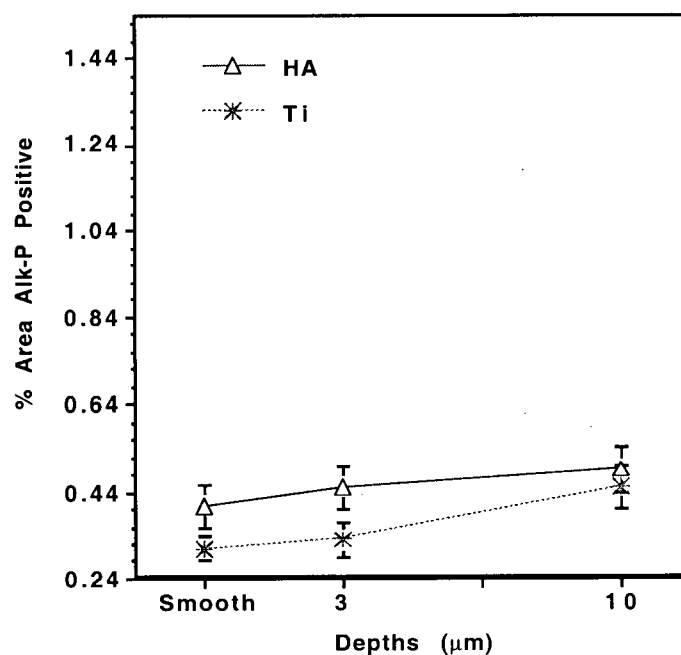
B

Figure 20. **Alkaline phosphatase activity on grooved substrata.** A,B) Alkaline phosphatase activity expressed as a percentage of area stained positive by osteoblasts cultured on micromachined substrata. Observations made on grooved areas. A significant difference was found between HA and Ti, and significant differences were also observed between depths ($p \leq 0.05$). A) 2 week cultures. B) 3 week cultures, notice a decrease in AlK-P activity. Repeat experiments showed similar patterns. (Error bars represent standard error).

significant difference was observed between HA and Ti substrata for both 2 weeks and 3 week cultures. (Figure 21). Furthermore significant differences were observed among the different depths. As with the nodule data, there was considerably more variation in the data from the gaps but most often the 50 μm gap size had more Alk-P activity then all the other gap sizes. This was observed in both 2 and 3 weeks and on both HA and Ti surfaces. Furthermore, generally there was an increase in Alk-P activity as the depth of the micromachined grooves increased. (Figure 21). Also evident from figure 21 was a decrease in Alk-P activity between weeks 2 and 3.

6. Correlation of Tetracycline Incorporation and Alk-P activity:

The correlation between Alk-P activity and the nodule counts was also examined. (Figures 22 & 23). Examining the data from the grooved areas revealed an excellent correlation (r=0.958) between the two methods where the Alk-P activity levels at 2 weeks were high but a lower correlation was observed in the 3 weeks data when the Alk-P activity had decreased. (Figure 22). The correlation was not as high for the gaps and the pattern was different in that the 3 week Alk-P cultures demonstrated a higher correlation then the 2 week Alk-P cultures. (Figure 23).

To demonstrate the relationship of mineralization and Alk-P activity the Alk-P stain was used on cultures labelled with tetracycline.
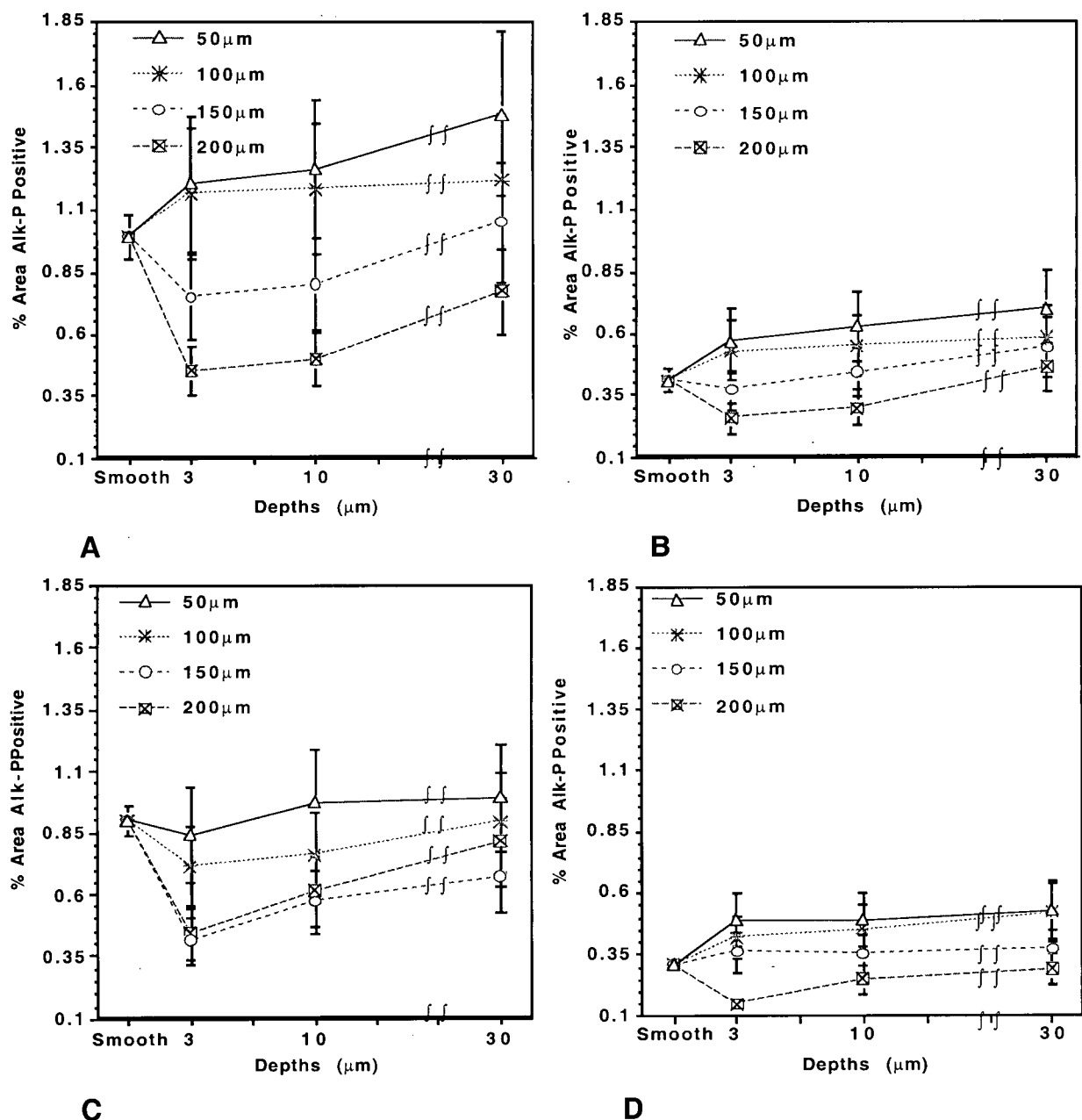
Figure 21. **Alkaline phosphatase activity on smooth gaps**. A,B,C,D) Alkaline phosphatase activity expressed as a percentage of area stained positive by osteoblasts cultured on micromachined substrata. Data observed from the smooth gaps located within the grooves. A significant difference was found between HA and Ti, and significant differences were also observed between depths, and between gap sizes (50, 100, 150, 200μm) (p≤0.05). All showed that 50μm pattern and 30 μm depth had more Alk-P activity. A&B) HA substrata. A) Cultured for 2 weeks. B) Cultured for 3 weeks, showing less Alk-P activity. C&D) Ti substrata. C) Cultured for 2 weeks. D) Cultured for 3 weeks, showing less Alk-P activity. (Error bars represent standard error).
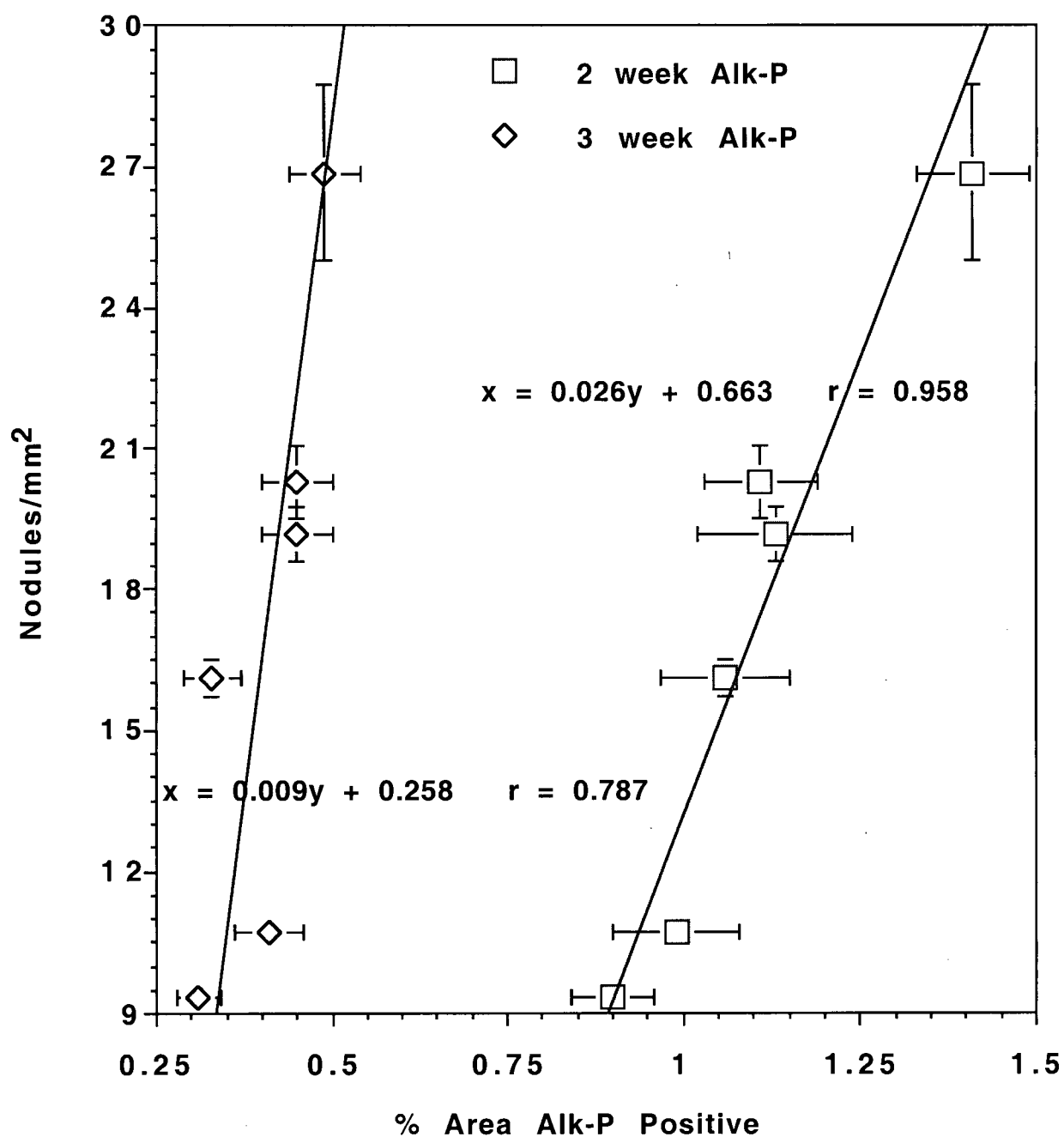
Figure 22. **Correlation between Alk-P & Tetracycline (grooves).** A correlation graph of nodules counted with % area Alk-P activity in the grooved areas. A lower correlation was observed at 3 week cultures. Repeated experiments observed similar results. (Error bars represent standard error).

75
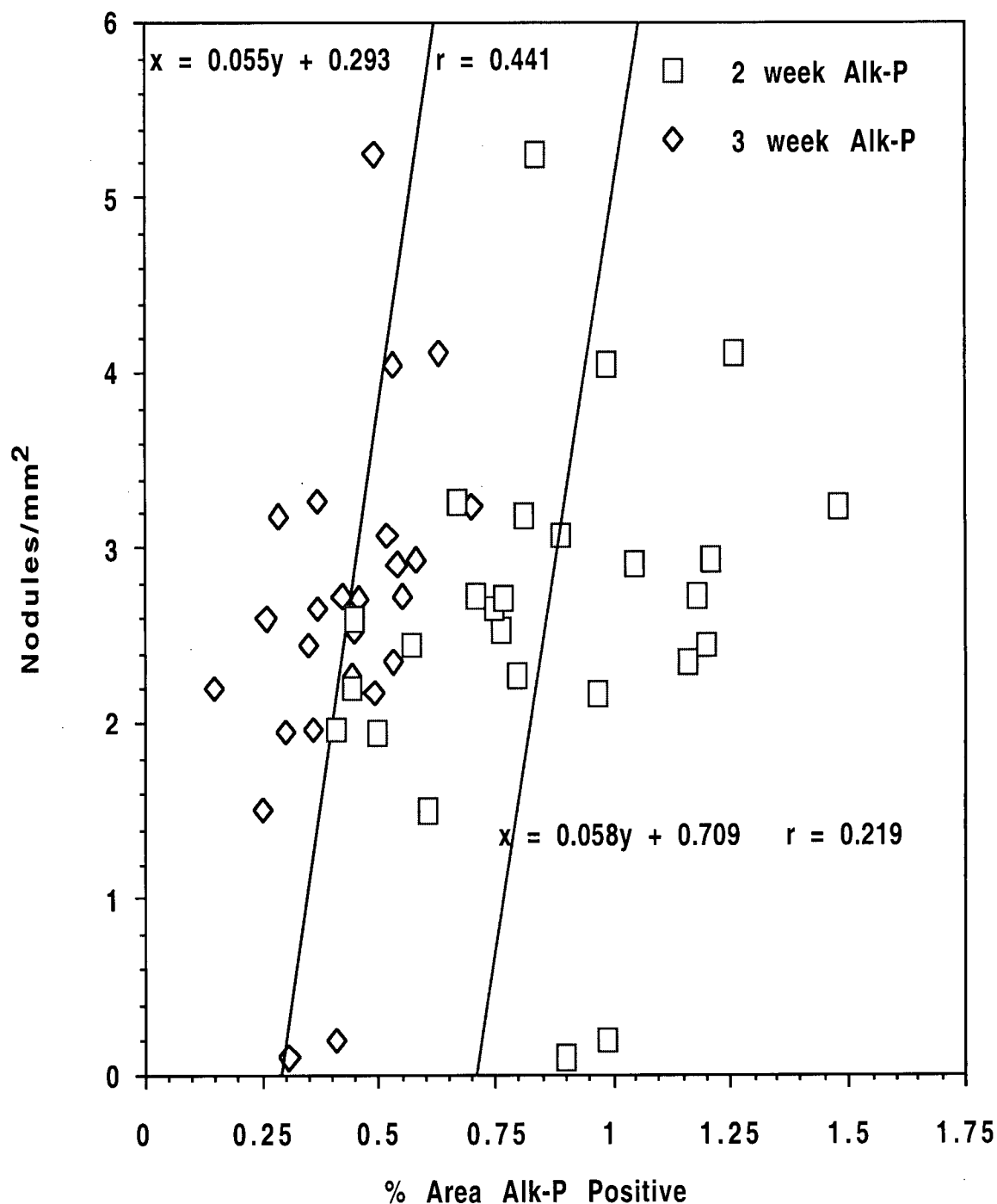
Figure 23. **Correlation between Alk-P & Tetracycline (smooth gaps).** A correlation graph of nodules counted with % area Alk-P activity in the smooth gaps within the grooves. Lower correlation than grooves figure 22 and lower at 2 weeks was observed. Repeated experiments observed similar results. (Error bars not shown because of the high variation).

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Since the stain was able to be seen under the same U.V. light (488nm) evidence of the relationship was illustrated in figure 24 which showed that the tetracycline labelling could be found in the vicinity of Alk-P activity.

7. Time-Lapse Cinemicrography:

This study was done to investigate osteoblast behaviour on HA surfaces using differential interference optics and video microscopy. The videos showed osteoblasts assuming an elongated shape on grooved surfaces, and after one week osteoblasts located in the gaps were aligned with the grooves forming the border of the gaps. (Figure 25). At 2 weeks, however, some cells were observed that were not aligned with the grooves as the culture became more dense and multi-layers formed. (Figure 25F). In addition, observations were made on cells cultured on a 5μm deep 100μm pitch HA coated surface to follow the formation of a nodule. The resulting video showed that osteoblasts in the periphery of the nodule apparently experienced mitosis more frequently than osteoblasts adjacent to the nodule, but there were insufficient observations for statistical analysis. To confirm that the aggregation of cells was an actual nodule, tetracycline labelling was preformed for an additional week. It can be seen that tetracycline was incorporated into this nodule figure 26B.
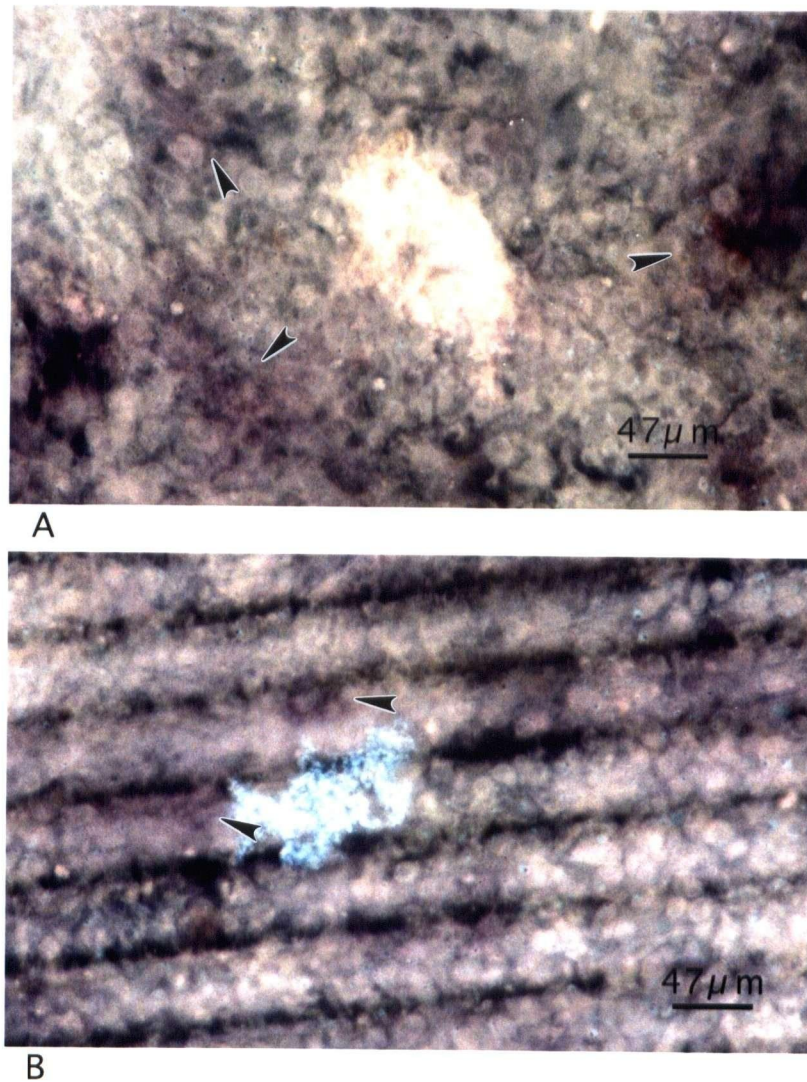
Figure 24. **Alk-P & Tetracycline labelled nodules**. Osteoblasts cultured on micromachined substrata for 3 weeks with mineralization supplements and tetracycline. Stained for Alk-P activity. Observed under U.V. light (488nm). Notice the nodules fluorescing and the purple-blue stain of Alk-P activity in the vicinity of the nodules (<-). A,B) HA coated substrata. A) Smooth. B) 10μm deep grooved.
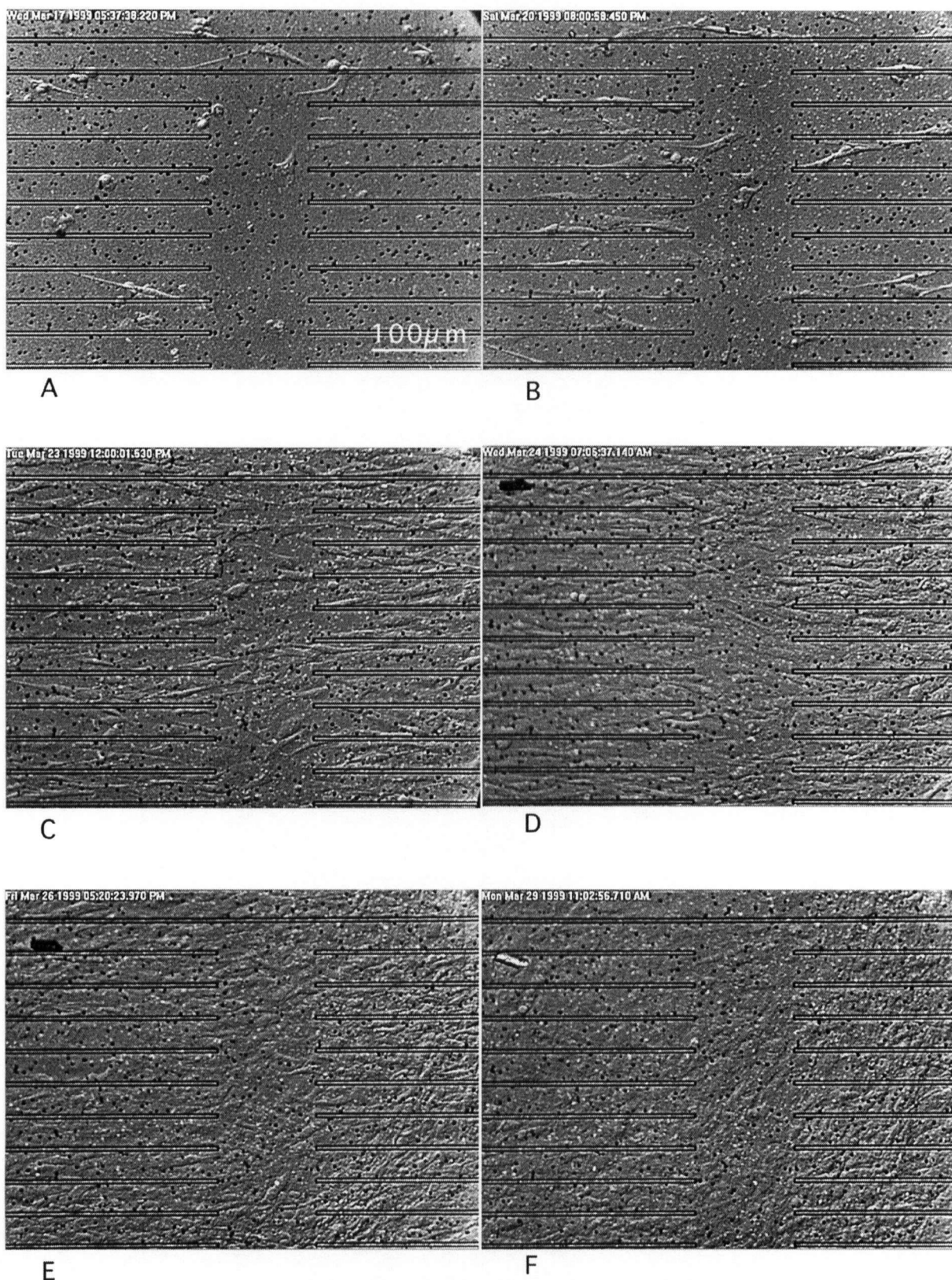
Figure 25. **Time-lapse series**. Time-lapse differential interference contrast micrographs of osteoblasts cultured on 3μm deep micromachined substratum. A) 24hrs. B) 4 days. C) 1 week. D) 8 days. E) 10 days. F) 2 weeks.

79
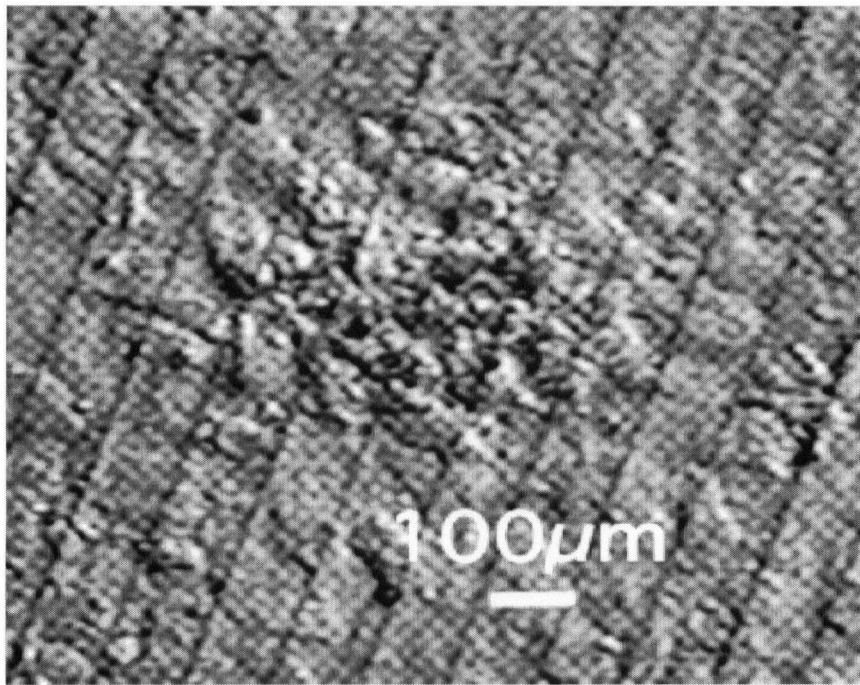
A
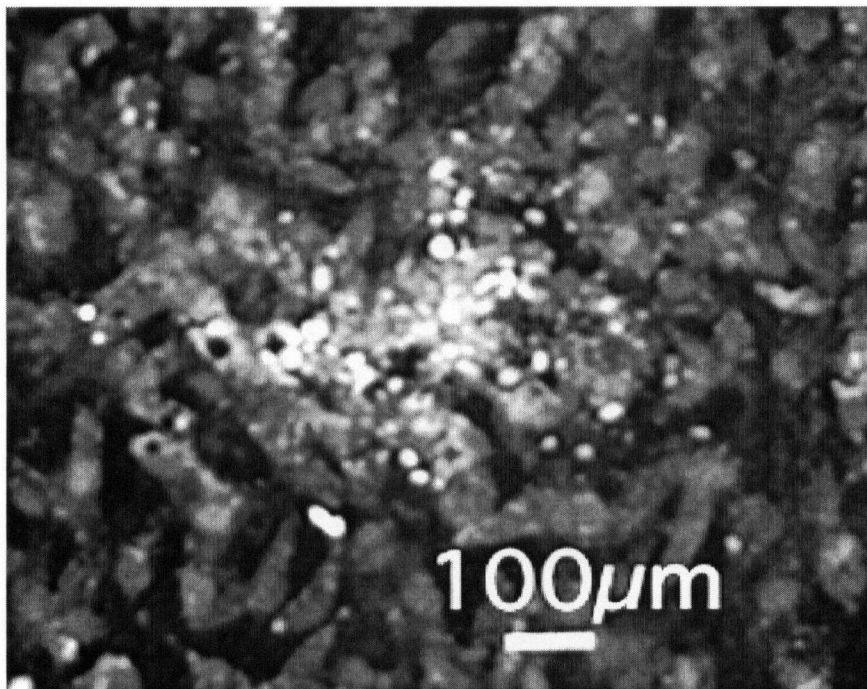
B

Figure 26. **Development of tetracycline labelling of a nodule during one week**. Osteoblasts cultured on HA coated micromachined surface, 5μm deep, 100μm pitch. A) Differential interference contrast time-lapse movie made for 4 weeks with mineralization supplements. B) The same area was photographed under U.V. light after an additional week with tetracycline in the medium. Tetracycline has been incorporated into the areas of the cell aggregate indicating it is a developing nodule.

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IV.   Discussion

This thesis used micromachined surfaces coated with HA and Ti to investigate osteogenesis *in vitro*. Although the precise mechanisms of mineralization are still under investigation, this thesis provides evidence to support a role for material surface properties in the production of bone-like tissue in cell culture.

Many properties of biomaterials such as their chemistry, crystallinity, and topography affect cellular responses *in vitro* and *in vivo* (Boyan *et al*, 1993; Massas *et al*, 1993; Dubois *et al*, 1998; Ong *et al*, 1998; Brunette and Chehroudi, 1999). The topography of the surface affects the production of bone-like nodules on Ti surfaces *in vivo* (Chehroudi *et al*, 1992), and *in vitro* (Brunette, 1988; Brunette *et al*, 1991; Boyan *et al*, 1993; Brunette and Chehroudi, 1999), on cell culture plates (Davies and Matsuda, 1988b) and on HA surfaces (Chang *et al*, 1999). Moreover it has been found that more nodule formation is associated with HA surfaces than with Ti surfaces (Massas *et al*, 1993). This thesis investigated the effects of well defined topographies, the use of HA, and their interactions on nodule production *in vitro*. A summary of the results of this thesis follows:

1) A thin, dense coating of hydroxyapatite resulted in significantly more bone-like nodules than were formed on a titanium coating.

2) A grooved, micromachined topography produced an increased number of

bone-like nodules.

3) An interaction was found between topography and chemistry. The increase in nodule formation produced using a HA coating was greatest when associated with the deepest surface feature of topography.

4) Alkaline phosphatase, an enzyme thought to be involved in the initiation of mineralization, may be a good leading indicator of the amount of nodule production *in vitro.*

5) The finding that the deepest surface feature produced the most nodules is consistent with the concept that a development of a suitable microenvironment enhances mineralized tissue production.

6) The results regarding the role of collagen orientation in formation of nodules were equivocal. More nodules were found on grooved surfaces than on smooth surfaces, and the collagen was aligned with these grooves. However, similar numbers of nodules were found in smooth gaps on the substrata where there was no obvious alignment of collagen.

The following components of the study will now be discussed: hydroxyapatite coating, the effects of topography on cell orientation, the combined effects of topography and chemistry on mineralized nodule production, alkaline phosphatase as a mineralization marker, and collagen involvement in mineralization.

HA coatings:

Scanning electron microscopy and surface roughness measurements indicated that although there was a small (1μm) increase in ridge width, the depth of surface features did not differ between micromachined Ti and HA surfaces. The topographies of the HA and Ti surfaces were considered to be identical for practical purposes since the change in width was small and the depth of feature has consistently been a more important factor than lateral spacings in determining cell behaviour (Chehroudi and Brunette, 1995). Furthermore the HA coating was found to be uniform and dense, a result confirmed by other studies using sputter coating methods (Jansen *et al*, 1993; Wolke, 1997; Wolke *et al*, 1998). The degree of crystallinity of HA could not be definitely determined because standards established for thin HA films of known crystallinity are not available (Lacefield; personal communication), however, x-ray diffraction clearly demonstrated that HA crystals were present (figure 5).

Effects of Topography on cell orientation:

It has been shown that surface topography affects cell orientation (Brunette, 1986; 1988; Brunette *et al*, 1991; Chehroudi and Brunette, 1995; Ratkay, 1995; Qu *et al*, 1996). In this thesis the contact-guided cell locomotion seen on HA-coated grooved (figure 25) surfaces was similar to the observations of Qu *et al* (1996), in which the osteoblast-like cells orientated their long axis with the grooves, and elongated in the direction

of the grooves on Ti substrata. Orientation of osteoblasts has also been related to the orientation of a naturally occurring substrate (Baslé *et al*, 1998). Baslé *et al* (1998) observed human osteoblast-like cells, aligned with collagen fibers on xenogenic bone biomaterial. Thus, my results are in agreement with numerous studies showing that cell orientation is affected by topography (Brunette and Chehroudi, 1999).

Effects of topography and chemistry on mineralized nodule production:

It has been previously observed that microfabricated grooved Ti substrata enhance the production of mineralized nodules (Brunette, 1988; Brunette *et al*, 1991; Chehroudi *et al*, 1992; Chehroudi and Brunette, 1995) a finding that was confirmed in this study. On average, approximately twenty three percent more bone-like nodules were found on HA than on Ti coated substrata. The increase in nodules ranged from 12% to 29%, thus HA produced approximately 2.5 times more nodules on the deepest groove relative to a smooth surface. These results are in general agreement with the work by Morgan *et al* (1996), who found that HA surfaces were associated with 8 times more mineral formation than sandblasted Ti surfaces. The difference in results could be attributable to their use of a different cell line (UMR 106-01 BSP) at 2.5 times the plating density than that employed in my study. In addition they used a plasma-sprayed HA

coating and measured elevated mineral formation rather than nodules. Similarly, Vercaigne *et al* (1998), who also used a plasma sprayed HA coated Ti implant, found in an *in vivo* study in goats that the percentage of bone contact was 2.5 times greater on HA implants compared to Ti. These two studies as well as my results, conform to the general rule that HA surfaces increase mineralization compared to Ti surfaces (Kay, 1993; Soballe, 1993).

Results from this study showed that regardless of coating, surfaces with the deepest grooves (30μm) and narrowest gaps (50μm) produced more mineralized nodules than shallower grooves and wider gaps. Studies by Ratkay (1995) and Chehroudi *et al* (1992; 1997) found similar results. Ratkay, using the same cell populations as used in this study, showed that 30μm deep grooves on Ti coated micromachined surfaces produced more bone-like nodules than smooth surfaces, although she used a plating density twice that used in this thesis. The topographies she used were also different in that the two groove depths were 19μm and 30μm with a pitch of 39μm. Furthermore, she used L-ascorbic acid in the supplements and replaced the media three times a week and used Von Kossa staining to assess bone-like nodules.

An *in vivo* study in rats, by Chehroudi *et al* (1992), using Ti coated micromachined implants, found that mineralization tissue (assessed by

transmission electron microscopy (TEM)) was adjacent to deep grooved surfaces (30μm) but not with smooth surfaces. Another rat study by Chehroudi *et al* (1997), also using Ti coated micromachined surfaces and assessing the bone index by radiographs and computer image processing, found that in the range 30μm to 120μm deep, the bone-like foci formation decreased as the depth of grooves increased. A study by Khakbaznejad (2000, in preparation), used the same topography as this thesis with the same cell populations and found that smooth gaps produced more bone-like nodules than the grooves, and the production of nodules decreased as depth of feature increased. The contradictory results between Khakbaznejad and this thesis could be attributed to the numerous variations in methods such as cell density, supplements, feeding frequency, and nodule assessment. Khakbaznejad used twice the plating density than that used in this thesis. Furthermore he used L-ascorbic acid in supplements which were fed three times a week, and bone-like nodules were assessed by Von Kossa staining rather than tetracycline labelling. It, thus, appears that bone-like nodule formation in response to topography is very sensitive to the specific culture conditions. Nevertheless, the conditions used in this thesis produced results that mimicked the performance of implants *in vivo* in that HA increased mineralized tissue production as did microfabricated grooved topographies.

A novel finding in this study was that an interaction was found between topography and surface chemistry ($p < 0.05$). That is, the increase in nodule production on HA surfaces was greater than the amounts expected by adding the effects of HA and groove depth. As far as is known, an interaction between chemistry and microfabricated topographies in the production of bone-like tissue *in vitro* has not previously been reported.

Deep grooves ($30\mu m$) and narrow gaps ($50\mu m$) have confined areas in which diffusion would be expected to be restricted, therefore it is possible that the concentration of regulatory factors in these areas differs from that found in the bulk medium. Such a situation could be called a microenvironment and indeed this possibility was proposed by Chehroudi *et al* (1992). It is possible that the combination of deep micromachined surfaces and HA would allow regulatory factors to achieve concentrations that promote mineralized tissue formation (Chehroudi *et al*, 1992; Chehroudi and Brunette, 1995). Another possibility is that the microenvironment found in HA substrata contains higher levels of Ca^{2+} and PO_4^{-3} ions which are released from the HA (Morgan *et al*, 1996; Keller, 1998).

In this thesis the long acting ascorbate analogue, L-ascorbic acid-2-phosphate (Asc-P), was used. L-ascorbic acid-2-phosphate has been

used in previous studies (Hitomi *et al*, 1992; Jaiswal *et al*, 1997). Jaiswal *et al* (1997), using mesenchymal stem cells plated at one hundredth of the density of that used in this thesis on tissue culture plates, found that more mineralized area (as assessed by Von Kossa staining) was seen with Asc-P than L-ascorbic acid. These results are in agreement with my preliminary tests that indicated that L-ascorbic-2-phosphate produced more mineralized tissue than L-ascorbic acid (data not shown). Moreover, Jaiswal *et al* (1997), suggested that the increase in mineralized area that they observed in cultures using Asc-P, was the result of changing the media only twice a week rather than three times. They believed that the less frequent media changes allowed the cultures to concentrate their soluble products in the media.

Alkaline Phosphatase:

Alkaline phosphatase plays a crucial role in the initiation of mineralization but is not needed for the enlargement of bone nodules (Bellows *et al*, 1991; 1992). Moreover, there is generally a greater increase in Alk-P activity on HA surfaces compared to Ti surfaces (Massas *et al,* 1993; Ozawa and Kasugai, 1996), as was observed in this thesis. It has been suggested that cultures on HA surfaces have greater Alk-P activity than those on Ti, because there is increased growth and differentiation of osteoblasts on HA substrata (Massas *et al*, 1993; Ozawa

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and Kasugai, 1996). In this thesis I found that the percent area of Alk-P increased with groove depth on Ti and HA surfaces. The importance of substrata topography in mineralization *in vitro* was also observed by Schwartz *et al* (1996). Furthermore they have shown that both growth and differentiation are affected by topography. Their study used chondrocyte cell populations at a density ten times less than that used in this thesis. They employed solid Ti disks that differed in surface roughness and found that course a grit blasted surface had a higher Alk-P activity than fine grit blasted. Moreover, the importance of topography in mineralization *in vitro* was also observed on ceramic substrata by Dubois *et al* (1998). They measured the specific Alk-P activity in behaviour of bone cells on glass, plastic and ceramic and found that ceramic had higher Alk-P activity. These studies, together with the results in this thesis, show that Alk-P activity is affected by the topography and chemistry of the surface (Kieswetter *et al*, 1996).

It has been suggested by Aubin and Turksen (1996) that Alk-P is found in certain stages of differentiation of the osteogenic lineage seen in cultures. Although absent in the earliest stages (mesenchymal stem cells and early osteoprogenitor cells) and the final stage (osteocytes), Alk-P is found in the intermediate stages (late osteoprogenitor cells, pre-osteoblasts and osteoblasts). A study by Sammons *et al* (1994), who

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plated similar cell populations as used in this thesis on a dense, irregular coating of HA, found that Alk-P activity increased within two weeks and decreased thereafter. My results agreed, both on the smooth as well as the grooved Ti and HA surfaces. Similarly, a study by Massas *et al* (1993) who used pure HA substrata also found an increase in Alk-P activity within 2 weeks and a decrease thereafter. In addition, Jaiswal *et al* (1997) who used mesenchymal stem cells plated on tissue culture plates also found an increase within 2 weeks followed by a decrease, within 3 weeks, in Alk-P activity. In this thesis a subsequent decrease of Alk-P was seen at three weeks which may result from the onset of mineralization at which time the progression of mineralization proceeds independently of Alk-P activity (Bellows *et al*, 1991; 1992). Furthermore, the onset of mineralization correlates with the maturation of osteoblasts (Aubin and Turksen, 1996) which leads to a decrease in proliferation and differentiation (Schwartz *et al*, 1993; 1996; Sammons *et al*, 1994; Jaiswal *et al*, 1997). Thus my results conform to what is generally observed with Alk-P activity *in vitro*.

Alk-P is thought to be involved in the initiation of mineralization by releasing phosphate from organic phosphate (Bellows *et al*, 1991; 1992). The activity of Alk-P was measured in this thesis in order to determine whether it correlated with nodule production *in vitro*. The correlation

between Alk-P activity at two weeks and mineralized nodule production at 6 weeks was highest (r=.958) on grooved areas. Studies by Ballanti *et al* (1995) also found a good correlation between Alk-P activity and bone formation (r=.784), using human transiliac bone biopsies and the same two methods as this thesis (tetracycline for bone labelling and an azo-diazonium reaction for Alk-P activity). Moreover Alk-P activity in the vicinity of mineralized area (figure 24), was also noted by Ballanti *et al* (1993). In these studies, Alk-P seems to be a good leading indicator of subsequent mineralization on various substrata. Thus, in the future it would be feasible to screen surfaces for their nodule production at 6 weeks by using Alk-P at 2 weeks thus saving time and expense.

Collagen involvement in mineralization:

Results presented in this thesis demonstrated that collagen was present on the underside of a mineralized nodule, and was interdigitated with mineralized globules, similar to the findings of Davies and Matsuda (1988b) who used cell populations similar to this thesis on bioactive glass. In addition, there were collagen fibers aligned with the grooves on Ti and HA surfaces. The orientation of the collagen fibers may be important since specific orientations of collagen *in vivo* are associated with certain stages of bone production (Schenk and Buser, 1998). Mineralization is initiated by an extracelluar matrix of which collagen is

the major component (Veis, 1993). Surface topography can affect the orientation of collagen fibers deposited by cells (Brunette, 1986b; Davies and Matsuda, 1988b; Chehroudi and Brunette, 1995; Baslé *et al*, 1998). As the structural matrix macromolecules (such as collagen) define the shape and structure of the mineralizing compartment (Veis, 1993). Surface topography could influence mineralization through its effects on collagen orientation.

It should be noted, however, that in this study collagen orientation was similar on HA and Ti coated grooved substrata, therefore, other factors must be responsible for the differences in bone nodule formation between the substrata. However, for both HA and Ti substrata, nodule production did roughly correlate with collagen orientation. That is, most nodules were found where the collagen was aligned best (i.e. grooves), an intermediate number of nodules were found in the smooth gaps where there was some preferred orientation of collagen fibers, and lower numbers of nodules were found on smooth surfaces where the collagen fibers had no preferred orientation. Thus, my results suggest that collagen orientation may have some role in promoting nodule formation; but strong support for this possibility would require the quantification of collagen alignment so that a quantitative correlation could be established.

V. Future Work

My data shows that bone-like nodule formation increases with increasing depth of surface feature. One possible explanation is that the deep grooves restrict diffusion of regulatory and other molecules, thus promoting the development of a microenvironment within the grooves that differs from the bulk medium in the culture. Other studies have used the concepts of restricted diffusion (Miura and Shiota, 2000) and microenvironment (Schmeichel *et al,* 1998) as explanations for cellular behaviour in chondrogenic pattern formation (Miura and Shiota, 2000) or in breast epithelial cell phenotypes (Schmeichel *et al,* 1998). Components of such a microenvironment could be soluble, i.e in the media, or cell-associated or be related to the extracelluar matrix.

A limitation of the ability of this thesis to support the concept of microenvironment is that no direct measurements of the contents of the media within the grooves were done. One approach to extend the findings of this thesis, therefore, would be to sample the media within the grooves (where the microenvironment is thought to develop) and compare it to media sampled well above the grooves and to media sampled from close to the smooth surfaces. Of particular interest would be the concentrations of molecules associated with bone production, such as the growth factors transforming growth factor-ß (TGF-ß) and bone morphogenetic protein-7 (BMP-7), which have both been studied extensively in the field of bone

biology (Bostrom *et al*, 1999). For example, if concentrations of TGF-ß, estimated by using an ELISA technique, were found to be elevated in the grooves, an increase in bone formation would be expected (Bostrom *et al*, 1999). Furthermore, by observing the amount of mineralized tissue produced and comparing the levels of growth factors, it might be possible to determine the regulatory factors that play an important role in mineralization. If a difference in the concentration of growth factors (i.e TGF-ß and BMP-7) was found, a related approach would be to use such data to alter the media on smooth surfaces so that the levels of growth factors were the same as those found within the grooves, and then determine whether there was a similar increased production of nodules. It would be expected that if concentrations of growth factors (i.e TGF-ß and BMP-7) were in the range of 1µg to 10µg for TGF-ß and 3.13µg of BMP-7 (Bostrom *et al*, 1999), then production of nodules on smooth surfaces would increase. The difference between the study proposed here and standard assays of growth factor effects is that the concentrations of growth factors employed in the proposed study would be specified by measurements of levels of regulatory factors in the microenvironment where bone-like tissue formation in cultures was enhanced.

Another area where more information is required is the effect of substrata topography and chemistry in the differentiation of osteogenic

cells. As the differentiation sequence of osteogenic cells advances from mesenchymal stem cells to osteocytes, the cells acquire expressions of several known markers (i.e. alkaline phosphatase and osteocalcin) (Aubin and Turksen, 1996). In the Aubin and Turksen (1996) model, markers for mesenchymal stem cells and early osteoprogenitor cells are not known. However, late osteoprogenitor cells express Alk-P, at the pre-osteoblast stage osteopontin and Alk-P are expressed, and at the osteoblast stage Alk-P, osteopontin, osteocalcin, and bone sialoprotein are expressed and finally at the osteocyte stage osteopontin, osteocalcin, and bone sialoprotein are expressed. This model of osteogenic lineage that Aubin and Turksen (1996) suggested may be examined using grooved and smooth surfaces, as well as HA coated surfaces, to determine whether specific stages of differentiation are affected by substratum topography. The immunolabelling methods used on fetal rat calvaria by Aubin and Turksen (1996) on cultures on grooved and smooth surfaces could be used sequentially over a defined time period to compare the appearance of markers associated with bone-like tissue production such as osteocalcin, osteopontin and bone sialoprotein. One might expect that differentiation would be accelerated on grooved surfaces, therefore the number of cells exhibiting osteocalcin, for example, would be greater at earlier times of culture on grooves compared to smooth surfaces, possibly leading to an

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early onset of mineralization, resulting in formation of more nodules.

Another approach to manipulating the microenvironment would be to alter feeding frequencies (i.e. media changes). By changing media frequencies, the concentrations of soluble products would be expected to be altered. For example, an increase in feeding frequency might be expected to disrupt the development of a microenvironment within the grooves, and thus restrict nodule production to similar levels to those found on smooth surfaces.

It was also found that there was an interaction between topography and chemistry in the formation of mineralized nodules. However, it is not known if this interaction is specific to the particular topography employed in this thesis. To investigate this interaction further, one approach would be to change the V-shaped grooves to grooves with vertical walls at various depths using both Ti and HA coatings. In changing the shape of the grooves, a greater surface area is created, allowing more attachment area for cells (Brunette, 1988). The increased surface area produced by changing groove shape might be expected to have effects similar to increasing surface area by increasing groove depth as in V-shaped grooves. Surface area could also be increased by increasing the number of grooves. Thus, exploring the effects of changing the shape and number of the grooves would allow for the relationship of surface area

and surface geometry to be investigated.

Another aspect of this thesis that warrants further exploration is the process of the development of a nodule. With the use of tetracycline and the appropriate blue light (Frost, 1968), differential interference optics and video microscopy, it is possible to observe the growth of a nodule from the early stages up to full development. Questions that could be answered directly by this approach would be the rate of mineralized tissue accretion through tetracycline incorporation as well as rates of cell recruitment into nodules, and locations where cell division occurs. All these parameters would give useful sight into the cellular dynamics of nodule formation.

Finally, but most importantly, the surfaces and conditions most effective in producing mineralized tissue formation *in vitro* should be tested *in vivo*. In particular the HA-coated 30μm deep grooved surfaces would appear to be most promising. Such *in vivo* investigations would both test the validity of the *in vitro* model and point the way to the utilization of HA-coated micromachined surfaces for clinical use.

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