Study E reports on the effect of various novel bioactive calcium titanium and calcium titanium zirconium orthophosphates on the osteoblastic phenotype of human bone-derived cells (HBDC) and compares these observations to those for cells on implant materials already clinically used, i.e. HA-coated titanium and porous titanium surfaces.

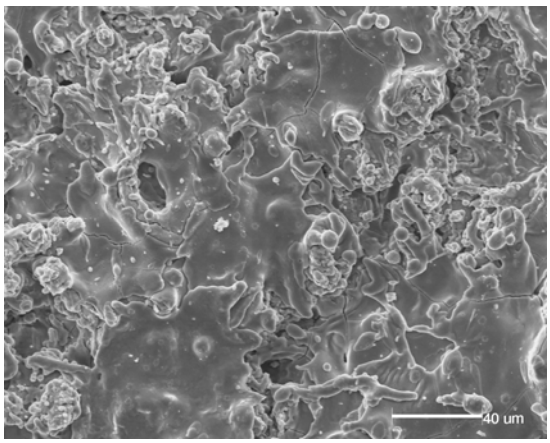
II/2 Materials and Methods

II/2.1 Test Materials

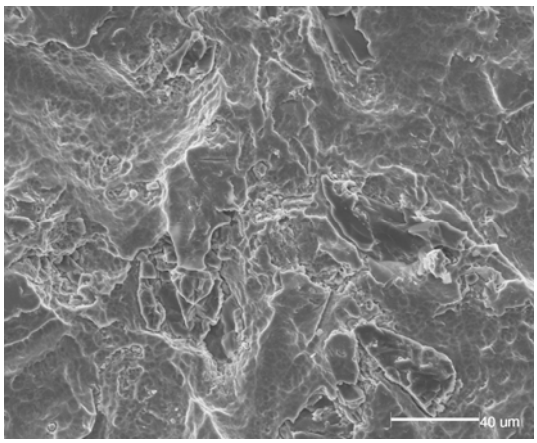
II/2.1.1 Test Materials Study D

All titanium substrates were made from commercially pure titanium (cp Ti) grade 2 (ASTM-F67). The materials were titanium (Ti) discs of 10 mm in diameter and 2 mm thick. Four implant surfaces were examined in the present study. The first test material was a roughened titanium surface with deep profile structure (material denominated: Ti-DPS). This surface configuration was produced applying an acid-etch technique (combination of anorganic acids) to the grit-blasted surfaces. Blasting was performed using Al_2O_3 particles (grain size 300-600 µm). Two surfaces were created from Ti discs receiving a porous titanium plasma-sprayed coating (samples denominated: Ti-TPS), or a plasma-sprayed porous hydroxyapatite coating (surfaces denominated HA). Uncoated titanium samples with a machined surface served as control and were denominated Ti-ma. Surface roughness of the different specimens was measured using profilometry (UBM microfocus surface-measuring system, UBM Inc., Ettlingen, FRG). Parameters used to quantify surface roughness were: R_a (the

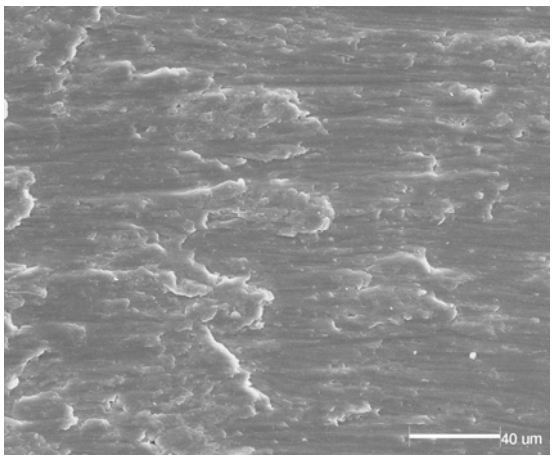
arithmetic mean of departures of the roughness profile from the mean line), R_t (maximum peak to valley height of the profile in the assessment length) and R_z(DIN) (the average of five consecutive values of roughness height which is defined as the distance between the top of the highest peak and the bottom of the deepest valley). Table 7 lists the results of the surface roughness assessment. Ti-TPS showed the highest surface roughness values, closely followed by Ti-DPS. Values for the hydroxyapatite-coated specimens (HA) were significantly lower. And for the smooth surfaces (Ti-ma), surface roughness values were the lowest. The surface morphology of the specimens is illustrated by the scanning electron micrographs presented in Figure 14. Quantitative X-ray diffraction (XRD) analysis (Philips X-ray-diffractometer with Cu tube and Ni filter) and ^{31}P magic angle spinning (MAS)-nuclear magnetic resonance (NMR) measurements were used to characterize the plasma-sprayed hydroxyapatite as described by Vogel et al. (1996). Furthermore, the coating was characterized by the manufacturer using energy dispersive X-ray (EDX), quantitative XRD analysis and Ion-Coupled Plasma (ICP) analysis (Philips Ion-Coupled-Plasma-Spectrometer PU 7450). The results of the ICP elemental analysis of the plasma-sprayed hydroxyapatite are presented in Table 8. XRD analysis confirmed the hydroxyapatite coating to consist at 99% of hydroxyapatite (1% tricalcium phosphate) and the coating to be 60% crystalline. Specimens were packed, sealed and sterilized by gamma-irradiation at their site of production (Friadent Dentsply Inc., Mannheim, FRG).



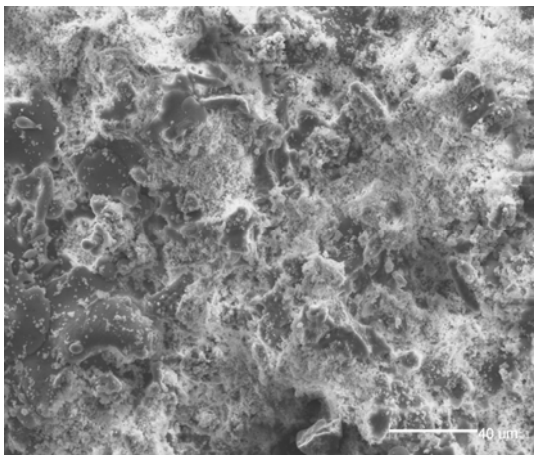
(a) Ti-TPS



(b) Ti-DPS



(c) Ti-ma



(d) HA

Figure 14. Scanning electron micrographs of the dental implant surfaces examined in study D. (a) Ti-TPS (titanium plasma-sprayed coating), (b) Ti-DPS (titanium substrate with deep profile structure), (c) Ti-ma (machined titanium surface), (d) HA (hydroxyapatite plasma-sprayed coating). Bar = 40 μm (original magnification: x500).

Table 7 Surface roughness assessment of dental implant surfaces examined in study D

Biomaterial	**Surface roughness**		
	R_zDIN (µm)	R_a (µm)	R_t (µm)
Ti-ma	1.02±0.33	0.15±0.04	1.56±0.62
Ti-TPS	14.77±2.37	3.43±0.63	20.49±3.10
Ti-DPS	13.06±1.82	2.91±0.53	19.11±3.86
HA	7.60±1.00	2.07±0.36	12.55±1.83

The values are mean ±SD.

Table 8 ICP elemental analysis of the hydroxyapatite coating

Elements	Atomic composition (atom%)
Ca	24.43±0.79
P	14.61±0.45
O	60.96±0.34
Ca/P ratio	1.67±0.11

The values are mean ±SD.

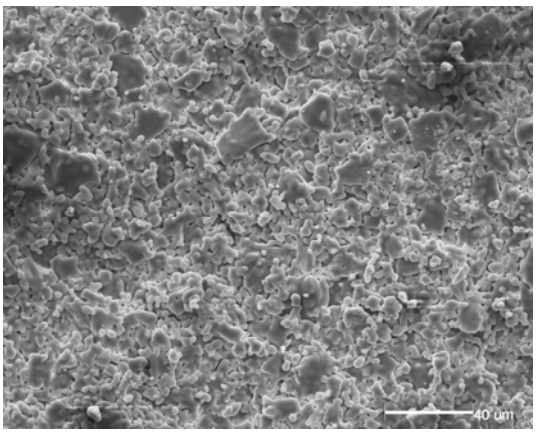
II/2.1.2 Test Materials Study E

Novel calcium titanium and calcium titanium zirconia orthophosphates have been synthesized from $Ca_3(PO_4)_2$ (TCP), $CaTi_4(PO_4)_6$ (CTP) and $CaZr_4(PO_4)_6$ (CZP) (Lugscheider et al. 1995, Berger et al. 1996, Ploska & Berger 1997) in order to

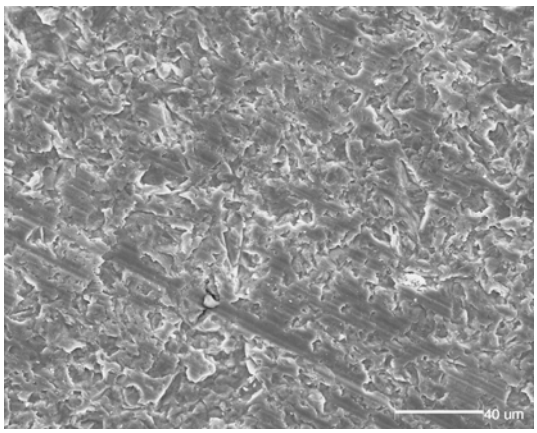
develop compounds based on calcium orthophosphates which are bone-bonding but less soluble than hydroxyapatite and suitable for generating coatings on implants of titanium of titanium alloy. CTP and CZP are known to be biocompatible and to bond directly to bone (Smukler-Moncler et al. 1992). CTP and CZP are members of the sodium zirconium phosphate (NZP) family and crystallize in the NASICON (sodium superionic conductor)-type lattice. NZP compounds have a hexagonal network which is characterized by a high stability and a great flexibility (Alamo 1993). A variety of NZP compounds have been created because they have a number of interesting features: a low thermal expansion coefficient and low thermal anisotropy (Alamo 1993). Furthermore, they facilitate ion-exchange and redox reactions and have the capability of chemical absorption. By exchanging sodium by calcium, calcium zirconia (CZP) and calcium titanium orthophosphates (CTP) can be produced. As a result CTP, CZP and compounds (CTZP) containing both Ti and Zr in various amounts were created (Ploska & Berger 1997). Thus, recently novel compositions in the system $CaTi_xZr_{4-x}(PO_4)_6$ with x = 0-4 were described and characterized with regard to solubility (Lugscheider et al. 1995, Berger et al. 1996, Ploska & Berger 1997). These studies showed that these novel calcium titanium and calcium zirconium phosphate ceramics are less soluble than HA (Ploska & Berger 1997). Moreover, such compounds are suitable for plasma-spraying onto titanium substrata and have a thermal expansion coefficient similar to titanium and titanium alloy. Thus heat-treatment of the sprayed coatings can be applied to increase crystallinity without creating tensions between the coating an the underlying titanium substrata.

In study E, the following five surfaces were created from these novel calcium titanium or calcium titanium zirconia phosphates and compared to titanium and hydroxyapatite surfaces. Ten mm discs were prepared by compressing powder (grain size 40 µm) and sintering at 1350-1400°C. This procedure fabricated sintered

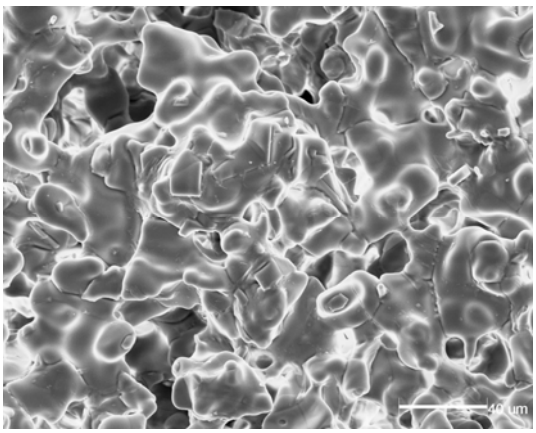
$CaTi_4(PO_4)_6$ (material denominated: CTP-S1), sintered $46CaO{\cdot}23TiO_2{\cdot}31P_2O_5$ (material denominated: CTP-S2), sintered $CaTiZr_3(PO_4)_6$, (material denominated: CTZP-S1), sintered $46CaO{\cdot}23ZrO_2{\cdot}31P_2O_5$ (material denominated: CTZP-S2) and sintered $55CaO{\cdot}20TiO_2{\cdot}31P_2O_5$ (material denominated: CTP-S3). The remaining two surfaces were created from commercially pure titanium (Ti) discs of 10 mm diameter receiving a deep profile structure produced by acid-etching a sand-blasted surface (material denominated: Ti-DPS); or Ti-discs receiving a plasma-sprayed porous hydroxyapatite coating (samples denominated: HA). The surface roughness of the specimens was characterized by profilometry using a Hommel T 8000 Surface Profile Measuring System with a diamond stylus (Hommel Inc., Germany). Parameters used to quantify surface roughness were: R_a (the arithmetic mean of departures of the roughness profile from the mean line), R_z (the average of five consecutive values of roughness height which is defined as the distance between the top of the highest peak and the bottom of the deepest valley). Surface roughness data and thermal expansion coefficients are listed in Table 9. The ceramic specimens were sterilized at 300°C for 3 h. Phase transformations do not occur below 600°C. HA and Ti-DPS specimens were sterilized by γ-irradiation by the manufacturer (Friadent Dentsply Inc.). The surface morphology of the novel calcium titanium phosphate specimens is illustrated by the scanning electron micrographs presented in Figure 15. The HA and Ti-DPS specimens represent commonly used dental implant surfaces as described above (II/2.1.1).



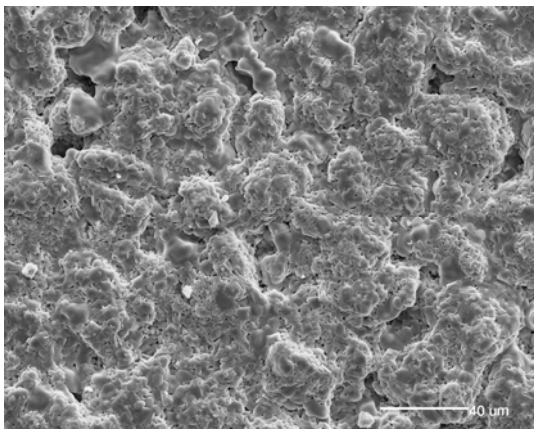
(a) CTP-S1



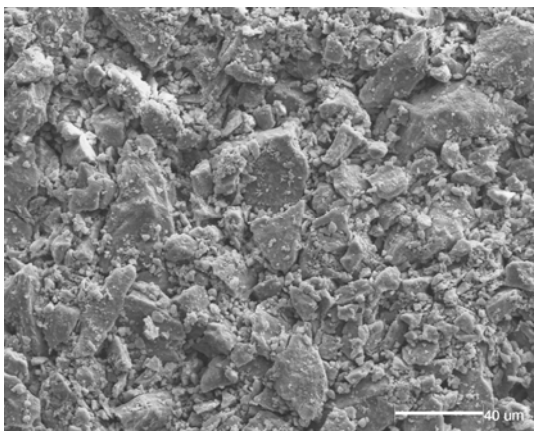
(b) CTP-S2



(c) CTZP-S1



(d) CTZP-S2



(e) CTP-S3

Figure 15. Scanning electron micrographs of the novel calcium titanium phosphates examined in study E. (a) CTP-S1 (sintered $CaTi_4(PO_4)_6$), (b) CTP-S2 (sintered $46CaO{\cdot}23TiO_2{\cdot}31P_2O_5$), (c) CTZP-S1 (sintered $CaTiZr_3(PO_4)_6$), (d) CTZP-S2 sintered $46CaO{\cdot}23ZrO_2{\cdot}31P_2O_5$), (e) CTP-S3 ($55CaO{\cdot}20TiO_2{\cdot}31P_2O_5$). Bar = 40 µm (original magnification: x500).

Table 9 Surface roughness and thermal expansion coefficient of the different biomaterials examined in study E (compared to titanium alloy)

Biomaterial	**Surface roughness**		**Thermal expansion coefficient**
	R_z (mean) [µm]	R_a (mean) [µm]	[$x10^{-6}K^{-1}$]
Ti-DPS	13.06	2.91	9.1
HA	7.60	2.07	11
CTP-S1	17.72	3.03	6.3
CTP-S2	16.36	0.26	8
CTZP-S1	27.35	5.53	5.3
CTZP-S2	17.23	2.38	4.3
CTP-S3	15.48	2.27	6
Ti-6Al-4V	-	-	8

II/2.2 Cell isolation and cultures

Human bone-derived cells (HBDC) were grown from small pieces of vertebral bone harvested from healthy female patients under 15 years of age using a modification of the method developed by Bellows et al. (1986) as described in part I (I/2.2). In brief, bone was morselized into about 1 mm^3 pieces, washed several times in 0.02% (w/v) trypsin (Sigma, USA) in phosphate buffered saline (PBS), and digested for 90 min at 37°C with 0.02% (w/v) trypsin (Sigma, USA) in PBS. Digested cells were cultured in α-Minimal Essential Medium (α-MEM, Gibco Laboratories, USA), 10% (v/v) fetal calf serum (FCS, Gibco Laboratories, USA), 2mM L-glutamine

(Gibco Laboratories, USA), 25mM Hepes Buffer (Gibco Laboratories, USA), 30 µg/ml penicillin, 100 µg/ml streptomycin (Gibco Laboratories, USA) and 0.1M L-ascorbic acid phosphate magnesium salt (Wako Pure Chemicals, Osaka, Japan). Permission to use discarded human tissue was granted by the Human Ethics Committee of the University of New South Wales (Ethic clearance CEPIHS 97043).

II/2.3 Cellular quantitative *in situ* hybridization assay (QISH)

The calcium phosphate surfaces were preincubated in 500 µl of culture medium for 24 hours without cells. HBDC were seeded at a density of 2.83×10^4 cells/cm^2 on the different implant surfaces for 3, 7, 14 and 21 days. QISH was performed as described above (I/2.3). In brief, cells were harvested from the test surfaces by trypsinization using 0.1% trypsin/0.02% EDTA in phosphate-buffered saline solution (PBS) and counted with a hemocytometer. Equal amounts of cells (1×10^4) from each material tested at each time point were placed into wells of 96-well plates and centrifuged at 1,000 rpm for 10 min. The supernatant from each well was vacuum aspirated using a fine needle and cells were dried to the plates for 30 min in a fan forced incubator at 37°C. Cells dried to the plates were fixed by immersing the entire plates in 10% (v/v) formalin buffered in PBS and probed applying photobiotin labeled cDNA plasmids. The cells were probed for the following mRNA molecules: alkaline phosphatase (ALP), pro-collagen Iα2 (Col Iα2), osteonectin (ON), osteopontin (OP), and osteocalcin (OC). β-Actin was used as a positive control and respective vectors were used as negative controls. The biotin label on the cDNA plasmids was detected using multiple applications of streptavidin and a biotinylated ALP and subsequently visualized by the *p*-nitrophenyl phosphate (*p*-NPP) assay as described above in part I (I/2.3). Quantitation was carried out by measuring the

optical density of the yellow colour (*p*-NP), and it was read by a TIM-200 plate reader (InterMed, Denmark) at 405 nm. The results were normalized to the internal control β-actin mRNA (Appendix A6).

II/2.4 Cellular quantitative immunocytochemistry assay

HBDC were cultured on the different test surfaces for 3, 7, 14 and 21 days. At these predetermined time points, cells were harvested and equal amounts of cells centrifuged onto the 96-well microtiter plates as described above in part I (I/2.4). The expressed intracellular proteins were detected using monoclonal antibodies for ALP (Sigma), Col I (Amersham), as well as polyclonal antibodies for OP (LF-123), ON (BON-I), OC (LF-32) and bone sialoprotein (BSP, LF-83) as described above (I/2.4).

The presence of a biotin label on the $F(ab)_2$ fragment of the secondary antibody was quantitated using a one-step application of streptavidin, followed by biotinylated ALP and subsequently visualization by the p-NPP assay (Appendix A7). An amount of 5 mM of levamizole was added to inhibit endogenous ALP activity. Quantitation was performed as described above (I/2.4), and the results were normalized to the internal control β-actin protein.

II/2.5 Statistical analysis

Three separate studies were performed. Assays were run in triplicate for each material and at each time point and the data were analyzed using student's t-test as described in part I (I/2.5) (Zreiqat et al. 1996, Zreiqat & Howlett 1999, Zreiqat et al.

1999a, 1999b, 2003, Knabe et al. 2004), and significance considered achieved at $p<0.05$.

II/2.6 Scanning electron microscopy

Additional specimens were prepared for scanning electron microscopy (SEM). Specimens of each test material incubated for 21 days without cells served as controls. The cell cultures were prepared for SEM analysis by rinsing the cells grown on the different substrata three times in 0.1 M cacodylate-buffered solution, pH 7.2 (Appendix B8) and fixed in 4% glutaraldehyde (Sigma, USA) in 0.1 M sodium cacodylate-buffered solution (Appendix B8) at 4°C for 15 minutes. Subsequently the specimens were washed with cacodylate buffer 0.1 M, pH 7.2 three times and dehydrated in ascending concentrations of ethanol, viz. 30%, 50%, 70%, 80%, 90% and 96%, finally immersed in absolute ethanol for ten minutes each, after which the specimens were immersed for 10 min each in three baths of hexamethyldisilazane (HMDS, Sigma, # H 4875). Each specimen was then air-dried for 24 hours. The dried specimens were glued onto aluminium stubs, sputter-coated with gold and examined in a CamScan MaXim at an accelerating voltage of up to 20 kV.

II/3 Results

II/3.1 Results Study D

Cellular Proliferation

All substrates supported continuous cellular growth for 21 days (Fig. 16). At 3, 7, 14 and 21 days, HA surfaces displayed higher cell numbers than the titanium