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

surfaces (Fig. 16). By day 14, Ti-DPS had more cells than Ti-ma and Ti-TPS (Fig. 16).

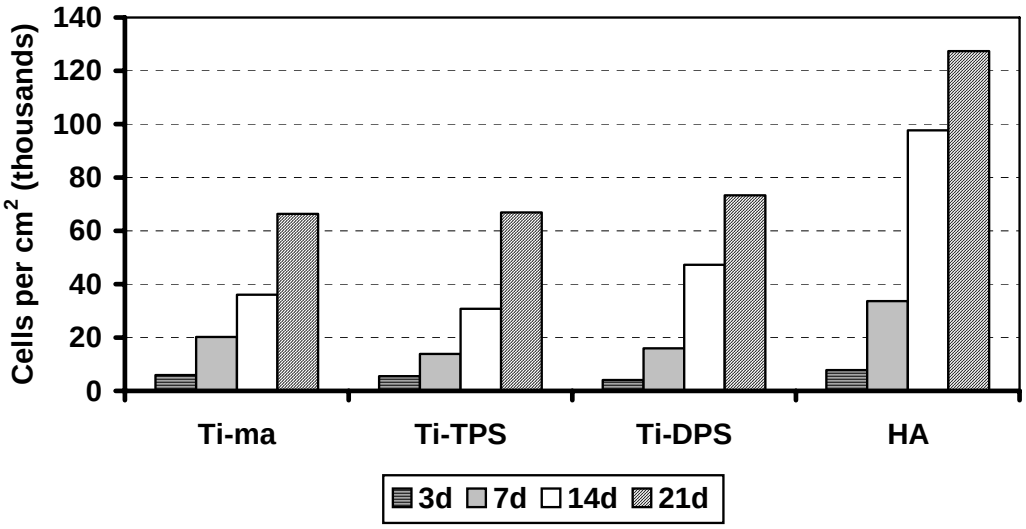


Figure 16. Number of HBDC cultured over 21 days on different dental implant surfaces in study D.

Cellular Differentiation

At day 3, HBDC cultured on Ti-DPS expressed significantly higher mRNA levels for ON compared to cells grown on all other surfaces ($p<0.002$) (Fig. 17(a)). mRNA expression for Col Iα2 and OC was similar for all substrata examined. Furthermore, mRNA expression for ALP was higher in cells grown on Ti-DPS ($p<0.02$) and Ti-TPS ($p<0.05$) compared to cells cultured on Ti-ma. For OP higher mRNA levels were expressed by HBDC cultured on HA compared to cells grown on all other substrata ($p<0.05$) (Fig. 17(a)). Protein production by HBDC for Col I and ALP was significantly higher, when these cells were cultured on Ti-TPS and Ti-DPS compared to HA ($p<0.02$) (Fig. 18(a)). The same was true when comparing OP protein levels on Ti-TPS and Ti-DPS to these on Ti-ma and HA ($p<0.04$). Furthermore, HBDC on Ti-DPS and Ti-TPS expressed more Col I and ALP protein

than cells on Ti-ma, however, this was not statistically significant. For OC and BSP, protein levels were highest, when cells were grown on Ti-DPS ($p<0.05$). ON protein expression was similar for all substrata tested (Fig. 18(a)).

At day 7, HBDC cultured on HA expressed significantly higher mRNA levels for all bone-related genes than cells on the titanium surfaces ($p<0.05$). mRNA expression for Col Iα2, ALP, OP and OC were similar for all titanium substrata, while cells on Ti-TPS and Ti-DPS expressed more ON mRNA than HBDC on Ti-ma (Fig. 17(b)). HBDC cultured on HA expressed significantly higher levels for all bone-related proteins tested compared to identical cells grown on all other implant surfaces. ($p<0.008$) (Fig. 18(b)). Col I protein levels were significantly higher for cells grown on Ti-DPS compared to HBDC cultured on Ti-ma and Ti-TPS ($p<0.05$). The same was true when comparing BSP protein levels on Ti-DPS to those on Ti-TPS ($p<0.04$) (Fig. 18(b)).

At day 14, mRNA expression for Col Iα2 and ALP was similar for all substrata examined. Cells grown on HA ($p<0.04$), Ti-ma ($p<0.01$) and Ti-TPS ($p<0.02$) expressed significantly higher mRNA levels for ON compared to identical cells grown on Ti-DPS (Fig. 17(c)). OC mRNA levels were higher on Ti-TPS, Ti-DPS and HA compared to Ti-ma ($p<0.02$) (Fig. 17(c)). At day 14, significantly more cells were found on HA compared to the titanium substrata (Fig. 16). Bone-related protein levels tested were significantly higher on HA compared to all titanium surfaces ($p<0.05$) (Fig. 18(c)). The same was true when comparing ALP ($p<0.02$) and OP ($p<0.04$) protein levels on Ti-DPS to these on Ti-ma surfaces. OC protein levels on Ti-DPS were significantly higher compared to those for cells grown on Ti-TPS and Ti-ma ($p<0.01$). ALP protein expression was higher in cells grown on Ti-TPS compared to cells on Ti-ma ($p<0.01$) (Fig. 18(c)).

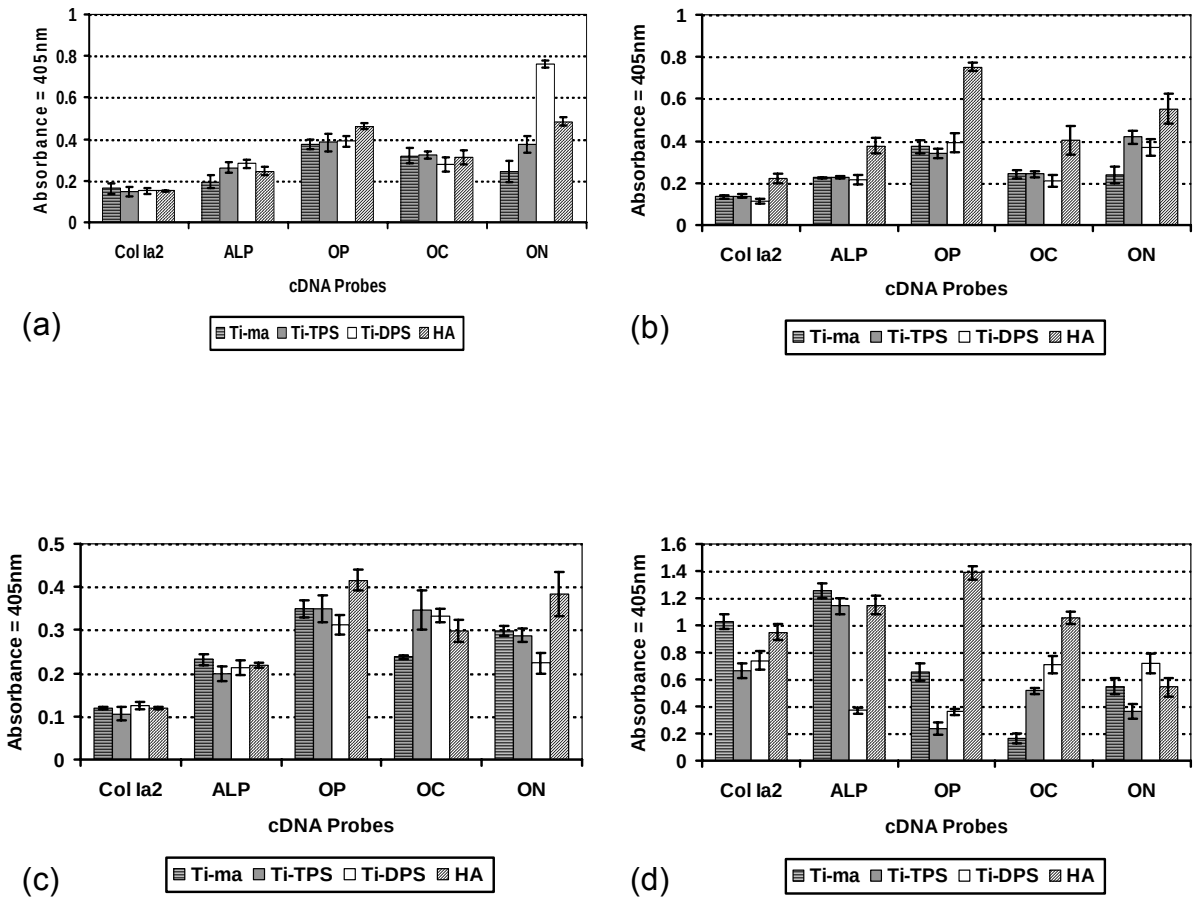


Figure 17. The temporal expression of osteogenic mRNA by HBDC cultured on different endosseous implant materials for 3 weeks in study D. (a) Day 3, (b) day 7, (c) day 14, (d) day 21. Cellular mRNA expression by HBDC is at (a) 3, (b) 7, (c) 14, and (d) 21 days of culture on Ti-ma, Ti-TPS, Ti-DPS, HA. Results are normalized to the internal control β-actin mRNA for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean ± standard deviation. Col Ia2, procollagen Iα2; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; and ON, osteonectin.

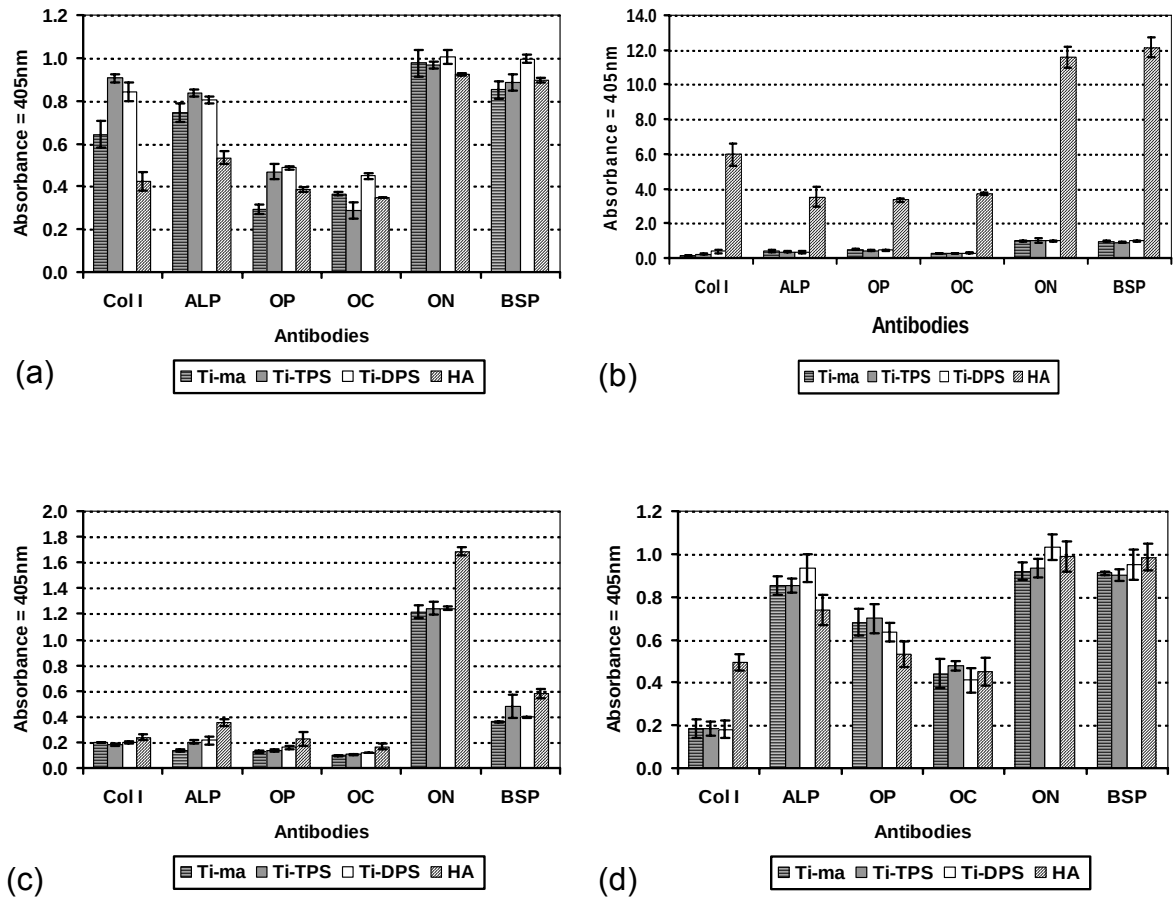


Figure 18. The temporal expression of bone-related proteins by HBDC cultured on different implant surfaces for 3 weeks in study D. (a) Day 3, (b) day 7, (c) day 14, (d) day 21. Intracellular protein expression by HBDC is at (a) 3, (b) 7, (c) 14, and (d) 21 days of culture on Ti-ma, Ti-TPS, Ti-DPS, HA. Results are normalized to the internal control β-actin protein for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean $\pm$ standard deviation. Col I, type I collagen; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; ON, osteonectin; and BSP, bone sialoprotein.

At day 21, HBDC cultured on HA expressed significantly higher mRNA levels for OP ($p<0.02$) and OC ($p<0.05$) compared to identical cells grown on the titanium surfaces (Fig. 17(d)). Also the expression of ALP mRNA was more abundant with cells on HA than on Ti-DPS ($p<0.002$). Col Iα2 mRNA levels, however, were higher on Ti-ma compared to Ti-TPS ($p<0.02$). Furthermore, HBDC cultured on Ti-ma and Ti-TPS expressed more ALP mRNA than identical cells grown on Ti-DPS ($p<0.03$), while cells grown on Ti-DPS expressed more ON mRNA than cells cultured on Ti-TPS ($p<0.04$). At the protein level, HBDC grown on HA expressed more Col I protein than cells on Ti-ma, Ti-TPS and Ti-DPS ($p<0.0001$). ALP protein levels, however, were higher on Ti-DPS ($p<0.03$) than on HA ($p<0.05$) (Fig. 18(d)). The same was true comparing OP protein levels on Ti-TPS, Ti-DPS and Ti-ma to HA ($p<0.03$) (Fig. 18(d)). OP and OC protein levels were significantly higher in cells grown on Ti-TPS ($p<0.018$) compared to those on Ti-DPS ($p<0.03$). Protein expression for Col I, ON and BSP was similar for all titanium surfaces tested (Fig. 18(d)). HA had the highest cell numbers at the end of the incubation period followed by Ti-DPS (Fig. 16).

II/3.2 Results Study E

Cellular Proliferation

All substrates supported continuous cellular growth for 21 days (Fig. 19). At day 14, HA, CTP-S2, CTZP-S1 and CTZP-S2 surfaces displayed higher cell numbers than the titanium surfaces (Fig. 19). By day 21, HA, CTP-S1, CTZP-S2 and CTP-S3 had more cells than Ti-DPS; surfaces of HA and CTP-S1 had the highest number of HBDC (Fig. 19). At day 14 and 21, a multilayer of cells and extracellular matrix had formed on all substrata (Fig. 20).

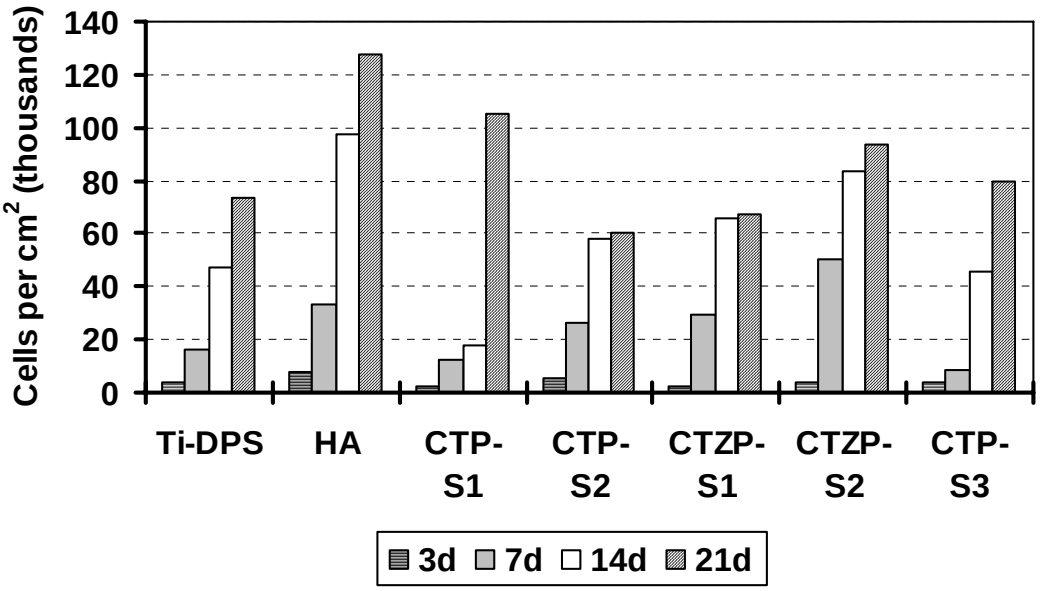
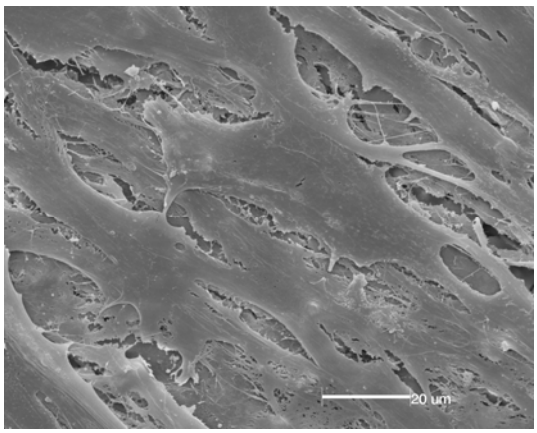


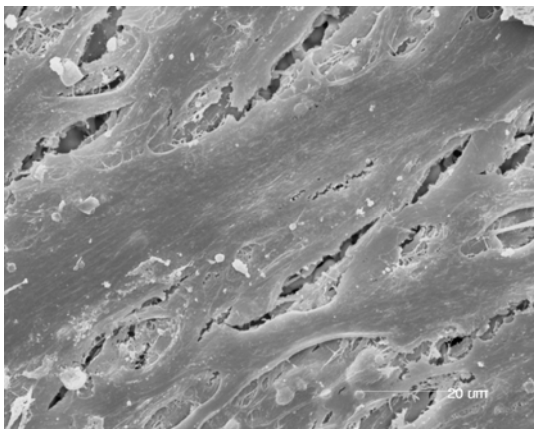
Figure 19. Number of HBDC cultured over 21 days on different implant materials in study E.

Cellular Differentiation

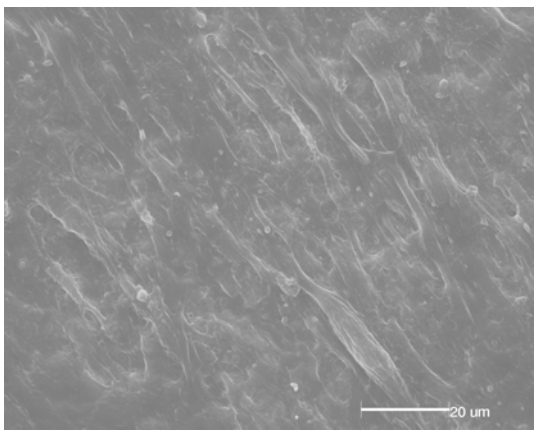
At day 3, HBDC cultured on CTZP-S2 expressed significantly higher mRNA levels for Col Iα2 compared to cells grown on HA ($p<0.04$) (Fig. 21(a)). mRNA expression for ALP and OC was similar for all substrata examined. For OP higher mRNA levels were expressed by HBDC cultured on CTP-S3 and CTP-S1 compared to cells grown on Ti-DPS and HA, however, this was not statistically significant. Cells grown on Ti-DPS expressed significantly more ON mRNA than HBDC cultured on all other surfaces (Fig. 21(a)). Protein production by HBDC for OP was significantly higher, when these cells were cultured on CTP-S3 compared to all other surfaces tested ($p<0.05$) (Fig. 22(a)). For Col I, however, protein levels were highest, when cells were grown on Ti-DPS ($p<0.02$). The same was true when comparing ALP protein levels on Ti-DPS to these on HA, CTP-S1, CTZP-S1 and CTP-S3 ($p<0.03$).



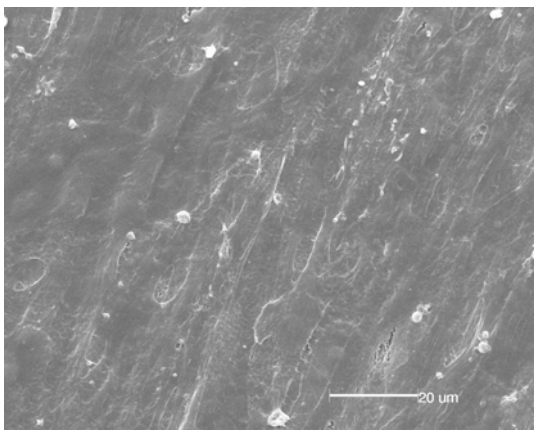
(a) HBDC on CTP-S1 at 14d



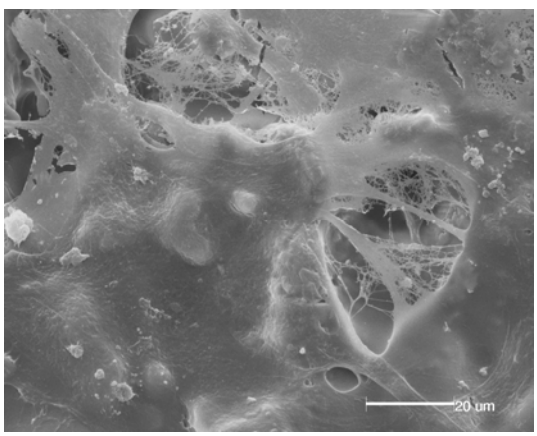
(b) HBDC on CTP-S1 at 21d



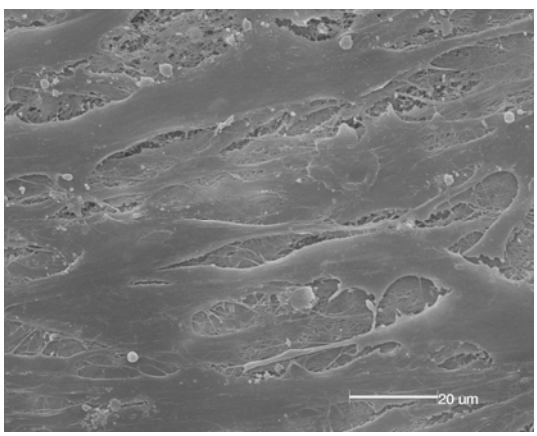
(c) HBDC on CTP-S2 at 14d



(d) HBDC on CTP-S2 at 21d

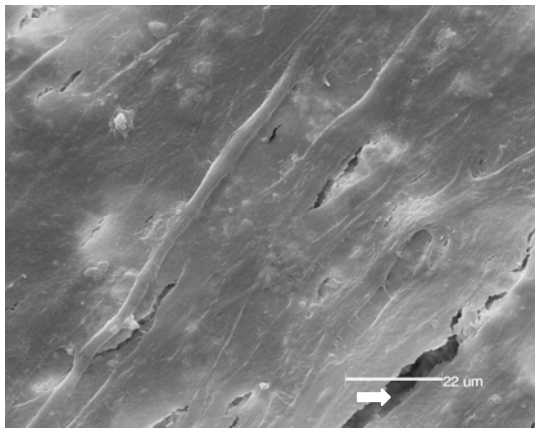


(e) HBDC on CTZP-S1 at 14d

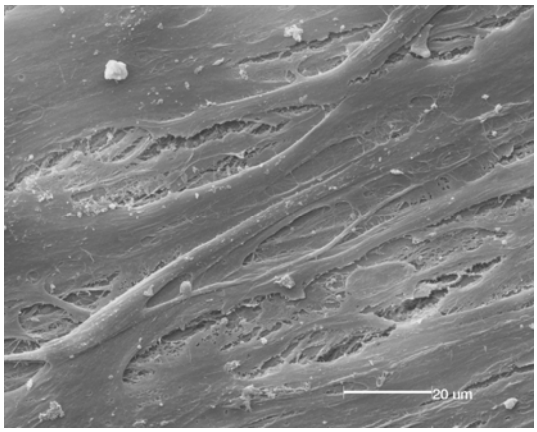


(f) HBDC on CTZP-S1 at 21d

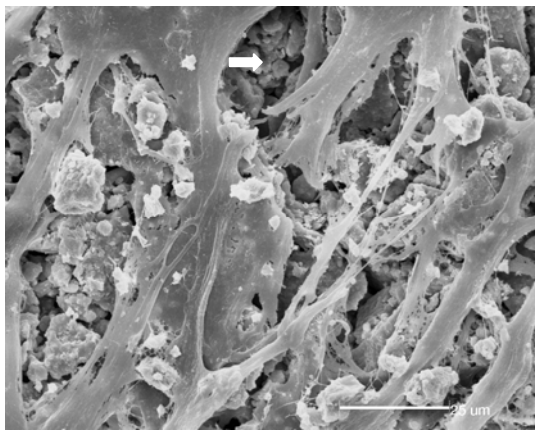
Figure 20. (a-f) Scanning electron micrographs of the novel calcium titanium phosphates showing a multilayer of human bone-derived cells (HBDC) and extracellular matrix covering the substrate surface after 14d and 21d of incubation. (a) CTP-S1 at 14 days, (b) CTP-S1 at 21 days, (c) CTP-S2 at 14 days, (d) CTP-S2 at 21 days, (e) CTZP-S1 at 14 days, (f) CTZP-S1 at 21 days. The drying procedure caused some rupturing of the covering layer. Bar = 20 µm (original magnification: x1000).



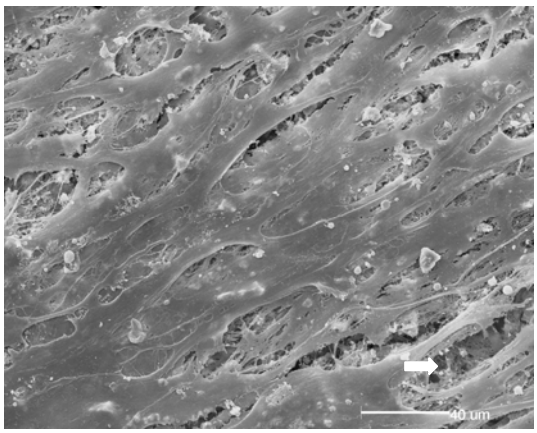
(g) HBDC on CTZP-S2 at 14d. A multilayer of HBDC and extracellular matrix has formed. The drying procedure caused some rupturing of the covering layer. The ceramic surface is visible (arrow). Bar = 22 µm.



(h) HBDC on CTZP-S2 at 21d. A multilayer of HBDC and extracellular matrix has formed. The drying procedure caused some rupturing of the covering layer. Bar = 20 µm



(i) SEM image of HBDC and extracellular matrix on CTP-S3 at 14d. The drying procedure caused some rupturing. The ceramic surface is visible (arrow). Bar = 25 µm.



(j) HBDC on CTP-S3 at 21d. A multilayer of HBDC and extracellular matrix has formed. The drying procedure caused some rupturing of the covering layer. Fibrillar components are visible (arrow). Bar = 40 µm.

Figure 20. (g-j) Scanning electron micrographs of the novel calcium titanium phosphates showing a multilayer of human bone-derived cells and extracellular matrix covering the substrate surface after 14d and 21d of incubation. (g) CTZP-S2 at 14 days, (h) CTZP-S2 at 21 days, (i) CTP-S3 at 14 days, (j) CTP-S3 at 21 days.

Fig 21

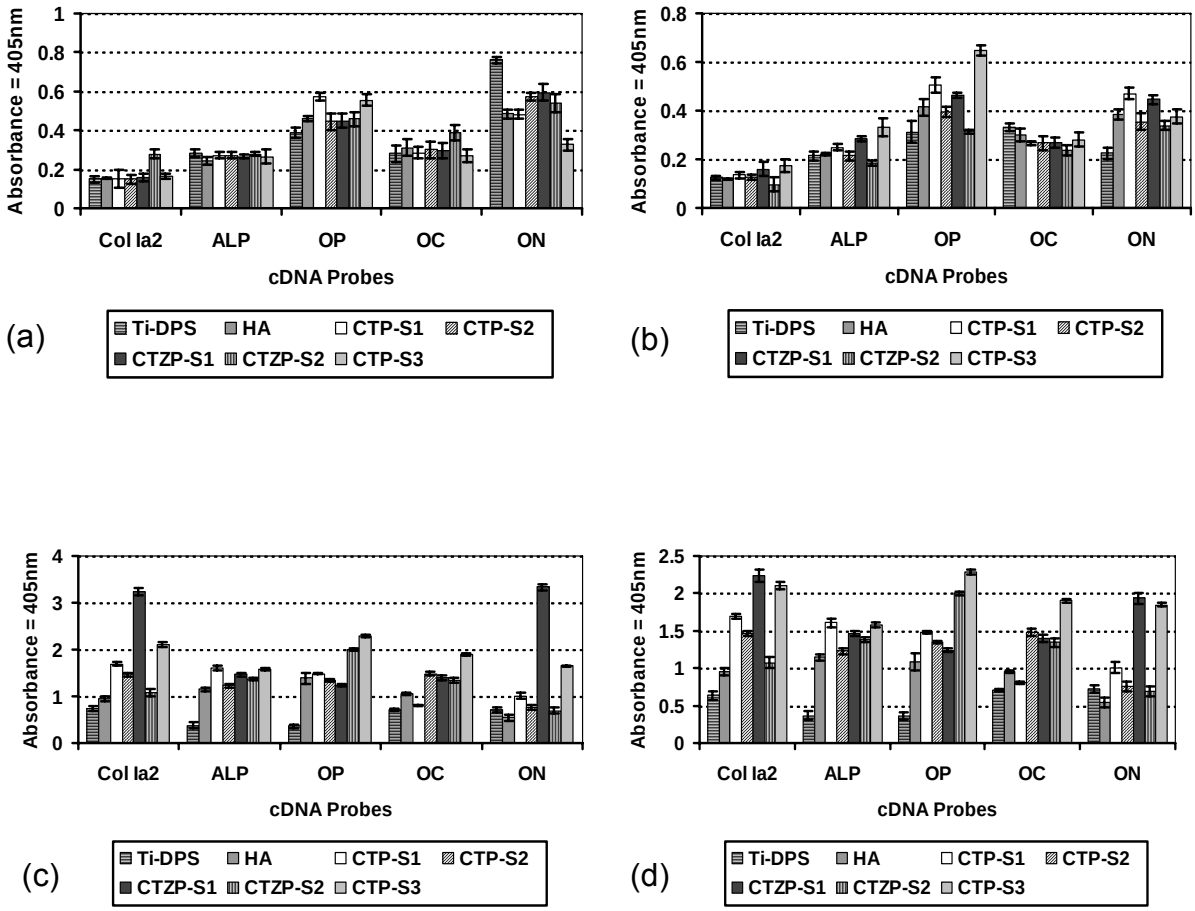


Figure 21. The temporal expression of osteogenic mRNA by HBDC cultured on different endosseous implant materials for 3 weeks in study E. (a) Day 3, (b) day 7, (c) day 14, (d) day 21. Cellular mRNA expression by HBDC is at (a) 3, (b) 7, (c) 14, and (d) 21 days of culture on Ti-DPS, HA, CTP-S1, CTP-S2, CTZP-S1, CTZP-S2, CTP-S3. Results are normalized to the internal control β-actin mRNA for each time point and each substratum. Three runs of experiments were performed in which there were three replicates. All values are mean $\pm$ standard deviation. Col Ia2, procollagen Iα2; ALP, alkaline phosphatase; OP, osteopontin; OC, osteocalcin; and ON, osteonectin.

Cells grown on HA and CTZP-S1 expressed more Col I protein than HBDC on CTP-S1 ($p<0.04$) (Fig. 22(a)). HBDC cultured on CTP-S3 expressed more OP protein than cells on all other surfaces ($p<0.05$) and more BSP protein than cells on HA, CTP-S1, CTZP-S1 and CTZP-S2 ($p<0.03$). The same was true when comparing protein expression for ON on CTP-S3 to that on HA, CTP-S1, CTP-S2 and CTZP-S2 ($p<0.02$). Furthermore, cells grown on CTZP-S2 expressed more ALP, ON and BSP protein than cells on HA (Fig. 22(a)). This was not statistically significant, however.

At day 7, cells grown on CTP-S3 expressed significantly higher mRNA levels for ALP compared to identical cells grown on Ti-DPS, HA, CTP-S1, CTP-S2 and CTZP-S2 ($p<0.05$) (Fig. 21(b)). The same was true when comparing mRNA levels for OP on CTP-S3 to these on Ti-DPS, HA, CTP-S2, CTZP-S1 and CTZP-S2 ($p<0.04$). Furthermore, mRNA levels for ALP, OP on CTP-S1 and CTZP-S1 were significantly higher compared to cells on Ti-DPS, HA, CTP-S2 and CTZP-S2 ($p<0.05$) (Fig. 21(b)). The same was true when comparing mRNA expression for ON on CTP-S1 and CTZP-S1 to that on Ti-DPS and CTZP-S2 ($p<0.05$). Furthermore, HBDC on CTZP-S1 had more Col Iα2 mRNA than cells on Ti-DPS, HA, CTP-S1, CTP-S2 and CTZP-S2 ($p<0.05$) (Fig. 21(b)). At the protein level, HBDC cultured on HA, CTP-S3 and CTP-S1 expressed significantly higher protein levels for all osteogenic markers compared to Ti-DPS, HA, CTP-S2, CTZP-S1 and CTZP-S2 ($p<0.04$) (Fig. 22(b)). Furthermore, protein expression for Col I, ALP and OC was significantly higher for cells grown on CTP-S3 compared to HBDC cultured on CTP-S1 ($p<0.04$).

At day 14, cells grown on all novel ceramic substrata expressed more Col Iα2 ($p<0.04$), ALP ($p<0.04$) and OP mRNA ($p<0.003$) than cells cultured on the titanium surfaces (Ti-DPS) (Fig. 21(c)). OC mRNA levels were significantly higher on CTP-S2, CTZP-S1, CTZP-S2 and CTP-S3 compared to Ti-DPS ($p<0.006$). HBDC grown on CTZP-S2 and CTP-S3 expressed significantly higher levels of Col Iα2 ($p<0.05$)

and ON mRNAs ($p<0.02$) compared to all other substrata (Fig. 21(c)). The same was true when comparing OC mRNA levels on CTP-S3 to those on all other surfaces ($p<0.033$) and OP mRNA levels on CTP-S3 to these on Ti-DPS, CTP-S1, CTP-S2, CTZP-S1 and CTZP-S2 ($p<0.012$) (Fig. 21(c)). Moreover, cells cultured on CTP-S1 had more ON mRNA than identical cells grown on Ti-DPS, HA, CTP-S2 and CTZP-S2 ($p<0.04$) (Fig. 21(c)). The same was true when comparing mRNA levels for Col Iα2 and ALP on CTP-S1 to HA ($p<0.05$). Col I protein levels were significantly higher for CTP-S1 and CTP-S3 compared to all other surfaces ($p<0.008$) (Fig. 22(c)). The same was true when comparing ALP protein levels on HA ($p<0.02$) and CTP-S3 ($p<0.03$) to these on Ti-DPS, CTP-S1, CTP-S2, CTZP-S1 and CTZP-S2 surfaces. Moreover, significantly higher protein levels for OC were expressed by cells grown on CTP-S2 compared to all other surfaces ($p<0.03$) (Fig. 22(c)). Furthermore, HBDC on CTP-S2 also expressed more BSP protein than cells grown on Ti-DPS, CTP-S1 and CTZP-S1 ($p<0.01$) and more ON protein than cells on Ti-DPS, CTP-S1 and CTP-S3 ($p<0.05$). The same was true when comparing ON and BSP protein levels on HA to Ti-DPS, CTP-S1 and CTP-S3 ($p<0.03$) (Fig. 22(c)).

At day 21, HBDC cultured on the novel ceramic substrata expressed significantly higher mRNA levels for Col Iα2 ($p<0.05$), ALP ($p<0.04$), OP ($p<0.003$) and OC ($p<0.043$) compared to identical cells grown on the titanium surfaces (Fig. 21(d)). Furthermore, by day 21 significantly more cells were found on HA, CTP-S1, CTZP-S2, and CTP-S2 compared to the titanium substrate (Fig. 19). Also the expression of ALP, OP and OC mRNAs was more abundant with cells on HA, than Ti-DPS ($p<0.035$). More importantly an enhanced expression of Col Iα2 ($p<0.03$), ALP ($p<0.05$) and ON mRNAs ($p<0.0034$) was found on CTP-S1 and CTP-S3, compared to HA (Fig. 21(d)). Cells on the latter 2 surfaces had a similar pattern to those on HA but mRNAs were expressed more abundantly (Fig. 21(d)). HBDC

cultured on CTP-S3 expressed significantly more mRNA for OP and OC than cells on Ti-DPS, HA, CTP-S1, CTP-S2 and CTZP-S1 ($p<0.04$). mRNA expression for Col Iα2 ($p<0.05$) and ON ($p<0.023$) was highest on CTZP-S1, followed by CTP-S3. On CTP-S1 surfaces, more ON mRNA was noted compared to Ti-DPS, HA, CTP-S2, CTZP-S2 ($p<0.05$). At the protein level a different pattern was observed. Cells grown on Ti-DPS, CTP-S1 and CTP-S3 expressed more OP protein than cells on HA, CTP-S2, CTZP-S1 and CTZP-S2 ($p<0.03$) (Fig. 22(d)). Protein expression by HBDC cultured on HA and CTP-S1 was significantly higher for OC than in cells on CTZP-S1, CTZP-S2 and CTP-S3 ($p<0.05$) (Fig. 22(d)). The same was true comparing BSP protein levels on HA and CTP-S1 to these on CTZP-S1 ($p<0.02$). Furthermore, protein production for Col I was significantly higher in cells grown on HA ($p<0.018$) and CTP-S3 ($p<0.002$) compared to all other surfaces. Protein expression for ON was similar for all substrata tested (Fig. 22(d)). HA had the highest cell numbers at the end of the incubation period followed by CTP-S1 (Fig. 19).

II/4 Discussion and Conclusions Part II

II/4.1 Discussion Study D

In implant dentistry there has been an ongoing effort to enhance and accelerate osseointegration of dental implants by optimizing their implant surface design (Keller 1998). This is related to the fact that treatment outcomes in dental implantology are critically dependent on implant surface designs that optimize the biological response during the different mechanisms by which bone becomes juxtaposed to an endosseous implant surface (Davies 1998, Keller 1998). The mechanisms by which endosseous implants become integrated in bone can be subdivided into three distinct phases (Davies 1998). The first, osteoconduction relies