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Research Article

Interaction of Cells with Organic-Inorganic Nanocomposite Coatings for Titanium Implants

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Abstract In order to evaluate the biocompatibility and bioactivity of recently described nanocomposite coatings, consisting of polyelectrolyte multilayers interspersed with calcium apatite crystals, extensive cell culture tests have been applied to coated and uncoated chemically etched titanium plates. The coatings had top calcium phosphate layers $(PLL/PGA)_{10}CaP[(PLL/PGA)_5CaP]_4$ (**A**) or top polyelectrolyte multilayers, $(PLL/PGA)_{10}CaP[(PLL/PGA)_5CaP]_4(PLL/PGA)_5$, (**B**), where PLL and PGA are poly-l-lysine and poly-l-glutamic acid respectively and CaP is calcium deficient apatite. Before testing coated samples were crosslinked and subsequently stored for extended periods of time (18 or 32 weeks) under dry, sterile conditions. Nonspecific activities, e.g. cell adhesion, proliferation, vitality, activity of mitochondrial dehydrogenases and cell morphology in contact with the material surfaces were tested using the cell-line MC3T3-E1, while for testing osteoblast specific activities, (collagen type I synthesis and alkaline phosphatase activity) SAOS-2 cells were used. The results show excellent biological properties of chemically etched titanium which were even surpassed by surfaces covered with coating **B**, while coating **A** (top crystal layer) adversely affected cell proliferation and performance. The effect is explained by morphological observations, showing inhibited spreading of the cells on the rough surfaces of coating **A**. The results also show that coatings, when shelved under dry and sterile conditions are stable for up to 8 months.

Keywords nanocomposite coatings; titanium; polyelectrolyte multilayers; calcium deficient apatite; cell culture tests

1 Introduction

Novel organic-inorganic nanocomposite coatings for artificial implants, to be used for the replacement and/or repair of bone and teeth have recently been designed and characterized by some of us [2,3,4,5]. The design is based on principles used in some forms of biomineralization, e.g. to first lay down an organic matrix and then grow calcium phosphate crystals "*in situ*" upon/within this matrix. The organic matrix in our design consisted of polyelectrolyte multilayers (layers of poly-L-lysine, PLL and poly-L-glutamic acid, PGA). The inorganic phase was co-adsorbed in the form of layers of amorphous calcium phosphate, ACP, which were transformed "*in situ*" into octacalcium phosphate or calcium deficient apatite by immersing the material into a metastable calcifying solution. Preliminary "*in vitro*" biological tests showed that when the coatings were topped with an additional polyelectrolyte multilayer, the resulting smooth surfaces effectively induced cell adhesion and proliferation. In this paper we describe the results of more extensive cell culture experiments, which confirm previous findings and demonstrate the remarkable stability of our coatings.

2 Materials and methods

Disks (d $=$ 15 mm; thickness 1.5 mm) from commercially pure ASTM grade 2 and/or grade 4 chemically etched titanium (received by courtesy of Dentaurum, J. P. Winkelstroeter AG, Germany and Sano, Italy) were used as substrates. Coatings were prepared [5] by alternately depositing $(PLL/PGA)_n$ multilayers and layers of amorphous calcium phosphate (ACP) and immersing the thus coated titanium plates into a metastable calcifying solution. This procedure yielded coatings **A** of the composition: $(PLL/PGA)_{10}CaP[(PLL/PGA)_5CaP]_4$. Subsequently an additional $(PLL/PGA)_5$ multilayer was deposited onto some of the coatings, yielding coating **B** of the composition $(PLL/PGA)_{10}CaP[(PLL/PGA)_5CaP]_4(PLL/PGA)_5$. Here PLL is poly-l-lysine, PGA is poly-L-glutamate and CaP is octacalcium phosphate or calcium deficient apatite. Before cell culture experiments all coatings were subjected to a crosslinking procedure [1]. In order to obtain information about the shelf-life of the coatings, samples were kept

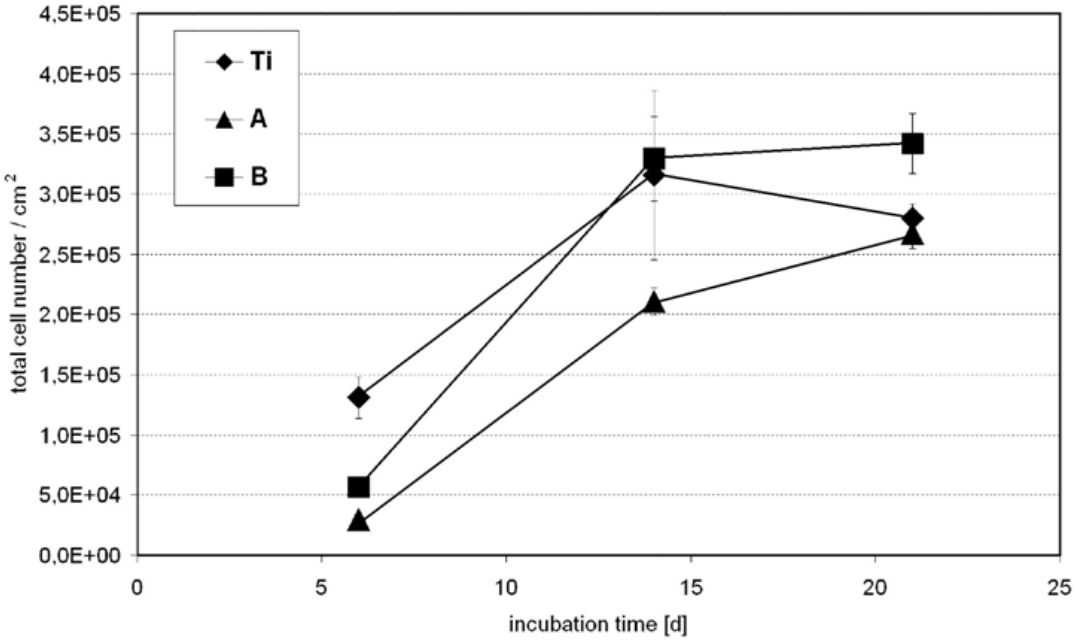

Figure 1: Proliferation of MC3T3-E1 cells on coatings aged for 18 weeks prior to testing. Lines are added for guidance of the eye only. Initial cell density: 5.6×10^3 per cm^2.

before testing for 18 and/or 32 weeks under dry and sterile conditions.

The cell culture tests were carried out using two types of cells. The cell line MC3T3-E1 (mouse embryo, fetus calvaria fibroblasts, DSMZ Braunschweig, DSM ACC 210) was used for determining common cell parameters (initial cell adhesion, proliferation, activity of mitochondrial dehydrogenases and cell morphology) while the cell line SAOS-2 (human osteogenic sarcoma, DSMZ Braunschweig, DSM ACC 243) was used for testing of osteoblast specific cell parameters (synthesis of collagen type I and the activity of alkaline phosphatase [ALP]). Samples were seeded with cells and incubated for up to 21 days in a specific cell medium at 37 °C, 5% CO_2 and 80% humidity. MC3T3-E1 cells were incubated in α-MEM, 10%FBS, 3% Pen/Strep medium while SAOS-2 cells were incubated in McCoy's 5A, 15% FBS, 3% penicillin/streptomycin. At day 14 the medium was supplemented with ascorbic acid (50 μg/mL) and ß-glycerol (10 mM) to stimulate osteoblast-like cell differentiation processes.

In tests of *initial cell adhesion* (4 h incubation), *proliferation* (up to 21 days) and *vitality* (e.g. the percentage of living cells vs. total cells by trypan blue staining) the cell number was determined after cell detachment by manually counting in a cell counter chamber. *Cell activity* was determined by testing the enzymatic conversion of tetrazolinum salts into formazan (Cell proliferation kit II, Roche Diagnostics). For *SEM analysis* (EVO LS10, Carl Zeiss NTS Oberkochen) samples were washed with PBS, dehydrated with isopropanol, dried and sputtered with gold plasma. *Synthesis of collagen type I* was quantified by immunoassays for the collagen specific C-terminal peptide (Metra CICP, Osteomedical) and *Alkaline phosphatase (ALP)* activity was determined from the cell lysate by testing the enzymatic conversion of p-nitrophenylphosphate into p-nitrophenol (test kit from Sigma). One-way ANOVA and t-tests were used to determine statistically significant differences between coatings and controls.

3 Results and discussion

We confirmed both by EDX and XRD spectra that the crosslinked coatings consist of PLL/PGA/calcium deficient apatite nanocomposites and provide good coverage of the titanium surfaces [5]. Initial *cell adhesion* was generally around 80% on both etched titanium and on sample **B** (top PE layer) but significantly lower (about 35%) on sample **A**. According to the *proliferation* tests on 18 weeks old samples (Figure 1), in the early incubation period the highest number of cells was consistently detected on etched titanium. A fast increase of cell numbers between day 7 and 14 was observed on both uncoated titanium and sample **B**, with the rate being greater on sample **B**, indicating cell stimulating processes induced by the coating. On sample **A** cell proliferation was significantly delayed. During the later incubation periods proliferation was slower on all samples, presumably because of aging processes and of the nearly confluent surface coverage achieved by the cell population already until day 14. The above data are in remarkable

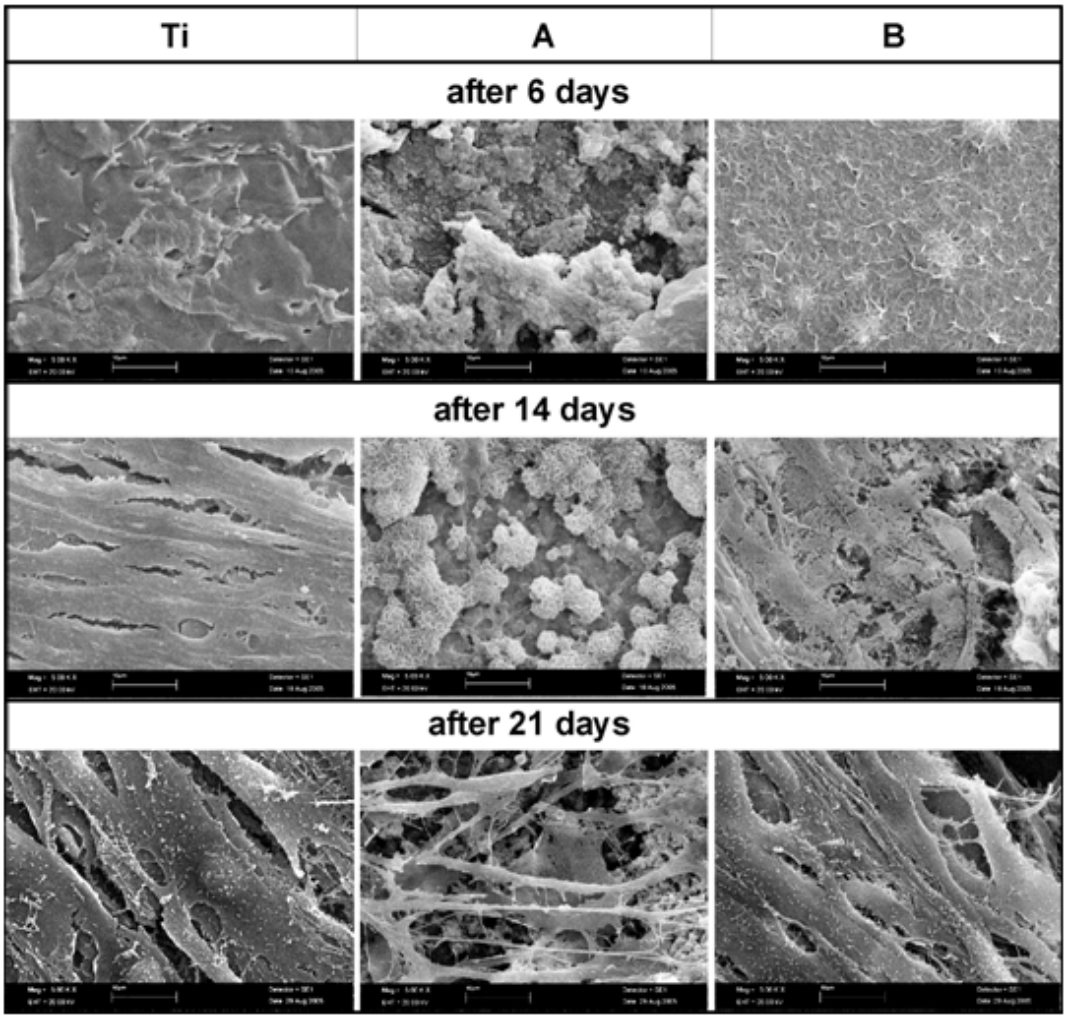

Figure 2: SEM pictures showing time dependent changes in the morphology of MC3T3-E1 cells seeded onto 18 weeks old material surfaces of chemically etched titanium (Ti), and titanium plates covered with coatings **A** and **B**.

accordance with previous results [2,3,5], obtained in a different biological laboratory (Parogene, Strasbourg cedex, France) on fresh material surfaces.

The *cell vitality* was quite high (88%–97%) on all material surfaces, indicating that cell survival is influenced to a much lesser extent than cell adhesion and proliferation. The results for the *cell metabolic activity* (mitochondrial dehydrogenases) positively correlate with the corresponding data from the proliferation test. *Collagen type I synthesis*, as tested by SAOS-2 cells seeded onto material surfaces aged for 32 weeks, also proceeded with the highest rate and reached the maximum amount of collagen type I on surfaces coated with nanocomposite **B**. The *ALP activity* of SAOS-2 cells seeded on coatings **B** was nearly constant over the whole incubation period of 21 days, while the ALP activity on coating **A** and/or on uncoated titanium strongly decreased during longer incubation periods. These data (not shown) indicate a high capability of sample **B** for osteogenic stimulation and cell differentiation effects, while sample **A** showed the statistically lowest level of osteogenicity.

The poor performance of coatings with top crystal layer can be explained on hand of the SEM pictures shown in Figure 2. On the surfaces of etched titanium and sample **B** the cells show a well spread phenotype indicating desired cell-surface interactions. In contrast, cells seeded onto sample **A** appear spindle-shaped, rejecting the surface rather than interacting with it (second column in Figure 2). Apparently the exposed rough crystal surfaces inhibit pronounced cell spreading and thus impede cell performance.

All above findings indicate a clear tendency of a tissue specific cell stimulation of nanocomposite coatings with final polyelectrolyte multilayers, as opposed to coatings with top calcium phosphate layers. The remarkable stability of our coatings for up to 8 months is also apparent from the results.

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