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Increased acellular and cellular surface mineralization induced by nanogrooves in combination with a calcium-phosphate coating

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ABSTRACT

The current work evaluated the influence of nanoscale surface-topographies in combination with a calcium phosphate (CaP) coating on acellular and cellular surface mineralization. Four groups of substrates were produced, including smooth, grooved (940 nm pitch, 430 nm groove width, 185 nm depth), smooth coated, and grooved coated. The substrates were characterized by scanning/transmission electron microscopy and atomic force microscopy. Osteoblast-like MC3T3 cells were cultured on the substrates for a period up to 35 days under osteogenic conditions. Differentiation was observed by alkaline phosphatase assay and PCR of collagen I (COLI), osteopontin (OPN), osteocalcin (OC), bone-morphogenic protein 2 (BMP2), and bone sialoprotein (BSP). Mineralization was quantified by a calcium assay and Alizarin Red staining. In addition, acellular mineralization was determined after incubation of substrates in just cell culture medium without cells. Results showed that a reproducible nano-metric (~50 nm) CaP-layer could be applied on the substrates, without losing the integrity of the topographical features. While no relevant differences were found for cell viability, cells on smooth surfaces proliferated for a longer period than cells on grooved substrates. In addition, differentiation was affected by topographies, as indicated by an increased expression of OC, OPN and ALP activity. Deposition of a CaP coating significantly increased the acellular mineralization of smooth as well grooved substrate-surfaces. However, this mineralizing effect was strongly reduced in the presence of cells. In the cell seeded situation, mineralization was significantly increased by the substrate topography, while only a minor additive effect of the coating was observed. In conclusion, the model presented herein can be exploited for experimental evaluation of cell–surface interaction processes and optimization of bone-anchoring capability of implants. The model showed that substrates modified with CaP-coated coated nanogrooves display enhanced *in vitro* mineralization as compared to unmodified controls or substrates modified with either nanogrooves or CaP coatings. However, our results also indicated that acellular mineralization assays are not necessarily predictive for biological performance.

Statement of significance

The manuscript describes the possibility to combine the mechanical properties of nanosized topographies with the biochemical properties of a calcium phosphate based coating for improvement of surface mineralization.
Interestingly, our results demonstrate that further incubation of our surfaces in SBF type media allowed all surfaces to mineralize rapidly to a high extent. Moreover we prove that nanotexture be used to can stimulate and organize mineralization and that the combination surface of a CaP coating and a nanotexture has the potential to be effective as a bone-implant surface. Such experiments will be of considerable interest to those in the research community and industry, who are focusing on bio-mineralization processes and optimization of modern bone-implants.

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1. Introduction

To improve bone-anchoring of dental and orthopedic implants, manipulation of the material surface forms an essential target [1–4]. The use of dedicated topographies has been studied extensively and was found to influence the bone-to-implant response in *in vitro, in vivo* and clinical studies [5–8]. In addition to topography, changing the (bio-)chemical surface properties of the implant material significantly affects its interaction with the host-tissues [9,10]. In this regard, calcium phosphate coatings are well-described, as they can result in the formation of natural bone-like apatite [11]. Multiple studies confirmed that such coatings significantly improve dental and orthopedic implant performance, by means of enhanced biocompatibility, osteoconduction, or even osteoinduction [12–14].

The effect of implant surface-configuration on tissue-response starts at cellular and molecular levels [15], and can be mediated by several mechanisms. For example, conventional surface roughness by grit-blasting might improve the implant function by stimulation of osteogenic differentiation and cellular mineralization, as suggested by an *in vitro* study of Wall et al. [16]. The underlying topographical influence on cell-skeleton organization, focal adhesion formation, and associated signaling pathways has been found for features with sizes down to the nano-scale [17]. However, roughness is a rather abstract parameter, which is independent of the structure and form of the measured topography. As an alternative, defined organized topographies were utilized for *in vitro* setups and fine-tuning of cell behavior by variation of the feature shape and size [18,19]. Especially topographies with features on the submicron and nano-sized scale were studied extensively during the past decade. Due to their dimensions, nanotopographies are thought to interact with cells in a more "natural" manner, resembling interaction with the fibrillar extracellular matrix (ECM) [20].

In addition to the effects of texture, the contribution of calcium-phosphate coatings on implant performance may be dual. On the one hand, CaP can facilitate cell-independent acellular surface mineralization [21]. On the other hand, chemical stimulation of cells can occur through calcium-sensing receptors (CasR), through which differentiation, ECM deposition, and mineralization can be induced [22]. Combining two cues influencing cell-behavior, i.e. texture and surface chemistry, can result in synergetic responses, a phenomenon that was already proven *in vitro* for roughened surfaces [23,24].

Combinations of an organized topography together with a CaP material for instance were shown in an *in vitro* study by Zhao et al. [25]. These authors produced ordered micropatterns of different sizes into bulk hydroxyapatite (HA), by simple molding using nylon sieves. The experiment showed that bone marrow stromal cells increased their osteogenic response with decreasing topography sizes. However, the logical follow-up to further decrease the size of the topography proved problematic, as nanotopographies cannot be made with the described methodology. As an alternative, various methods exist to apply a CaP coating onto a material surface, but also the combination of a coating with an organized uniform nanotopography is challenging. Due to the heterogeneity in composition and thickness, most coating techniques cannot preserve the underlying complex nano-structures [26]. Therefore, the current study utilized a controlled "biomimetic" wet calcium-phosphate deposition technique to coat nanogrooved surfaces [27], in order to evaluate the influence of combined nanotopographical and biochemical cues on cellular mineralization behavior. We postulate (1) that with these thin coatings it will be possible to preserve the topographical structure on nanogrooved surfaces, allowing cellular recognition and adaptation to the topography, and (2) that a combination of topographical and biochemical cues will synergistically increase the mineralization of the surfaces by facilitation of the cellular and the acellular surface mineralization.

2. Materials and methods

2.1. Substrate production

2.1.1. Polystyrene solvent casting

A grooved pattern was introduced into a silicon master wafer by means of laser interference lithography as described before [28]. This wafer was then used as a mold for reproduction of the topography into tissue culture plastic by solvent casting. For this purpose, polystyrene (PS, Acros, Geel, Belgium) was dissolved in chloroform in a proportion of 1:6 (w/v) and casted on the silicon master. As controls, smooth surfaces were produced. After evaporation, the substrates were treated by radiofrequent glow-discharge (Harrick, Ossining, USA) in Argon gas for 5 min at an atmospheric pressure of about 10^{-2} mbar. Glow discharge treatment increased the wettability and sterilized the polystyrene surfaces. The substrates were used directly for cell culture, or were additionally coated with calcium-phosphate. For the experiments round-shaped substrates (smooth, grooved) with a diameter of 1.1 cm ($A \approx 1\ cm^2$) were punched out.

2.1.2. Calcium phosphate coating

A wet-chemical coating method was applied, as described previously [27]. In brief, the polystyrene replicates were immersed in a solution at 37 °C, which contained 2 mM Na_2HPO_4 (Merck, Darmstadt, Germany), 147 mM NaCl (Merck), 2.5 mM $CaCl_2$ (Merck), and 100 mM urea (Invitrogen, Karlsruhe, Germany). Urease (type III from Jackbeans, U1500-20kU, Sigma–Aldrich, Taukirchen, Germany) was resuspended in a 200 mM Na_2HPO_4 (Merck) buffer solution to a concentration of 1 Unit/μL. Under continuous stirring, urease was added to the solution in a proportion of 1 Unit/mL (600 μl in 600 mL coating solution). After 60 min the substrates were washed in 100% Ethanol and dried to air.

2.2. Substrate characterization

2.2.1. Atomic force microscopy (AFM)

An atomic force microscope (Catalyst, Bruker, Santa Barbara, CA, USA) was used to analyse substrates in tapping mode with a 118 μm long silicon cantilever (NW-AR5T-NCHR, NanoWorldAG, Wetzlar, Germany). This type of AFM probe has a nominal resonant frequency of 317 kHz and nominal spring constant of 30 N/m, and is equipped with a high aspect ratio (7:1) portion of the tip with a nominal length of >2 μm and a half-cone angle of <5°. The nominal radius of curvature of the AFM probe tip was less than 10 nm. Height images of each field/sample were captured in ambient air at 50% humidity. The analyzed field was scanned at a rate of 1.0 Hz and 512 scanning lines.

2.2.2. Scanning electron microscopy (SEM)

The substrates were lyophilized and sputter-coated with gold (10 nm thick layer). Surface topography was examined by scanning electron microscopy (JEOL 6310, Jeol, Japan).

2.2.3. Transmission electron microscopy (TEM)

To define the thickness and homogeneity of the coating, the substrates were embedded in Araldite epoxy resin (Huntsman Corporation, Salt Lake City, UT, USA). After polymerization, ultra-thin sections were made and observed using the transmission electron microscope (JEOL JEM-1010). The thickness of the coating was