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Nanoscaled periodic surface structures of medical stainless steel and their effect on osteoblast cells

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Abstract

Nanoscaled lamellar surface structures have been prepared on medical stainless steel AISI 316LVM surfaces by chemical etching of the decomposed phases. The effect of this structure on osteoblastic cells has been investigated. Long filopodia were developed by the cells perpendicular to the lamellar structure while almost no or only short filopodia were formed parallel to the lamellae. These results are explained in terms of a topographical influence of the nanostructure. During the growth process of the filopodia a nearly flat surface was recognized parallel to the lamellae while a topographical change was sensed perpendicular to the structure, which was preferred by the cells.

Keywords: Nanostructures; Stainless steel; Osteoblasts

1. Introduction

Over the years, material research has led to the development of numerous metals and alloys for use in biomedical applications [1]. Besides titanium-based materials, stainless steel plays an important role, due to its corrosion resistance and excellent mechanical properties. Its scope of application ranges from clinical devices such as stents and artificial joints to surgical tools. One of the major tasks of biomaterial research is the functionalization of the material surface to improve the biocompatibility according to a specific application. However, the correlation between material properties and the reaction of a biological system is usually very complex. One of the first events that takes place when a foreign material comes into contact with a living body is protein adsorption. This depends, among other things, on the physico-chemical properties of the surface and its topography [2,3]. The subsequent cellular adhesion is determined by various surface properties, like its topography [4–6], and also a dependence on the previously formed protein layer, as discussed in Ref. [7]. Many studies have focused on the functionalization of the material surface by applying defined micro- and nanostructures to it and observing the reaction of the biosystem. For example, enhanced human osteoblast cell adhesion and proliferation has been observed on stainless steel 316LS after CO_2 laser treatment [8].

In this study, nanoscaled periodic surface structures were generated on medical stainless steel AISI 316LVM and their influence on osteoblastic cells was observed. AISI 316LVM is an austenitic stainless steel used for permanent surgical implants. Its main components are less than 0.03 wt.% C, 17–19 wt.% Cr, 13–15 wt.% Ni and 2.25–3 wt.% Mo according to the American Iron and Steel Institute (AISI). Cr is introduced to improve the corrosion resistance, Ni to stabilize the austenite and Mo primarily to increase the resistance against pitting corrosion, but is also efficient in promoting solid solution hardening [9]. In mixed systems with two or more components and a miscibility gap or a eutectic, decomposition processes can be used to obtain self-organized periodic surface structures.

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If an etchant is available, which affects the different phases in the sample at different rates, a surface structure can be obtained by chemical etching [10,11]. The resulting structure usually depends on the decomposition kinetics, the etching time and the orientation of the individual grains. Austenitic stainless steels with a low carbon percentage are well known for their eutectoid decomposition with a lamellar phase structure, which was utilized in this study to generate fine and periodic surface structures and investigate their influence on osteoblastic cells.

2. Material and methods

2.1. Preparation of the substrates

Cold-rolled AISI 316LVM stainless steel samples (Ergste Westig GmbH, Schwerte, Germany) with a diameter of 15 mm and a height of 1.5 mm were ground, polished and ultrasonic-cleaned in a distilled water/ethanol mixture. The samples were then etched in an ultrasonic bath (Sonorex, Bandelin Electronic, Germany) using a commercially available "V2A-Beize" (solution of hydrochloric and nitric acid in water, Wirtz-Buehler GmbH, Düsseldorf, Germany), which is commonly used for a visualization of the microstructure as well as etching austenite, δ-ferrite or the carbide structure. An etching time of 15 min at room temperature was chosen since after this time the surface structure was optimally visible. The employment of ultrasound during the etching process leads to an accelerated erosion rate with additional cavitation pitting [12,13], provides a uniform corrosion of the sample and is essential for the generation of a distinct surface structure. The correlation between the eutectic decomposition process in stainless steel and the lamellar surface structure was verified by annealing a sample at 900 °C for 2 h and quenching it to room temperature, so that the time between entering the two-phase region of the Fe–C system and a freezing of diffusion at lower temperatures was too short for a phase separation. After quenching, the sample was polished and etched as described above, and the resulting surface structure was compared to the unquenched samples.

2.2. Surface characterization

Characteristic surface parameters and height profiles of the etched samples were obtained from atomic force microscopy (AFM) measurements and compared to the results of polished and machined surfaces. Ten scans at random positions with a scan area of 15 × 15 μm were carried out for each sample using a CP Research atomic force microscope (ThermoMicroscopes/Veeco, USA) in contact mode, followed by analysis of the topography. Moreover, the macroscopic roughness was examined with a Mitutoyo SJ-201 profilometer. Five random positions with a length of 0.8 mm each were investigated for each of the three samples.

2.3. Cell cultivation

Before starting the cell experiments, etched, polished and machined samples as well as conventional glass coverslips (12 mm diameter; Marienfeld GmbH & CoKG, Lauda, Germany) were rigorously cleaned to remove any chemical residues thoroughly. Therefore the samples were sonicated at 40 °C in a series of several cleaning solutions for 10 min each: (i) 2% sodium dodecyl sulfate (Serva, Hamburg, Germany), (ii) distilled water, (iii) 5% Extran (Merck, Darmstadt, Germany), (iv) double-distilled water and (v) isopropyl alcohol. Osteoblastic cells MG63 (ATCC No. CRL-1427, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen) in a humidified 5% CO_2 atmosphere at 37 °C. For the analysis of cell growth on modified stainless steel samples, the cells were detached from the culture dish by incubating with Accutase (PAA, Cölbe, Germany) and diluted to a concentration of 50,000 cells ml^{-1}. The etched and reference samples as well as a glass coverslip were placed into the cavities of a 24-well plate (Costar, Vitaris AG, Baar, Germany) and covered with 1 ml of the cell suspension. The cells were grown on the surfaces for 48 h.

2.4. Scanning electron microscopy

After cultivation on the samples, the cells were rinsed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 7 mM $Na_2HPO_4{\cdot}2H_2O$, 1.5 mM KH_2PO_4) and fixed for 15 min in ice-cold glutaraldehyde (6% in PBS, pH 7.4, Merck, Darmstadt). After four rinses in PBS, cells were dehydrated in ascending acetone concentrations (30%, 50%, 75% and 90%, and four times at 100%). After critical point drying the samples were coated with gold and analyzed in a Zeiss DSM 940 (Carl Zeiss, Oberkochen, Germany) scanning electron microscope.

2.5. Cell viability tests

Cell growth on the etched samples was investigated by using cell counting and the WST cell activity test (Roche Diagnostics, Mannheim, Germany). The results were compared to polished and machined samples. Four samples of each type were placed into the cavities of a 24-well plate for each time step (three surfaces, four samples, three time steps, 36 samples total) and covered with the cell medium (50,000 cells ml^{-1}). Cell activity and cell number were determined after 3, 5 and 7 days. Cell activity was analyzed using the reagent WST 1 (Roche Diagnostics, Mannheim, Germany). After incubating the cells for 30 min with the WST reagent 1:10 in DMEM at 37 °C, the absorption of the supernatant was quantified in a photometer (Fluor Plus, Tecan GmbH, Crailsheim, Germany). Cell proliferation was determined by electronic cell counting using a cell analyzer (CASY 1 TTC, Schärfe System, Reutlingen, Ger-

ID	Title	Pages
1302	Nanoscaled periodic surface structures of medical stainless steel and their effect on osteoblast cells	6