Direct laser writing-mediated generation of standardized topographies for dental implant surface optimization

Rainer Wittig(a),b)  
Institut für Lasertechnologien in der Medizin und Messtechnik (ILM) an der Universität Ulm, 89081 Ulm, Germany

Erik Waller(b) and Georg von Freymann  
Arbeitsgruppe Optische Technologien und Photonik und Forschungszentrum OPTIMAS, Fachbereich Physik, Technische Universität Kaiserslautern, 67663 Kaiserslautern, Germany

Rudolf Steiner  
Institut für Lasertechnologien in der Medizin und Messtechnik (ILM) an der Universität Ulm, 89081 Ulm, Germany

(Received 14 October 2011; accepted for publication 18 May 2012; published 16 July 2012)

The functionalization of dental implants, aiming at the improvement of long-term acceptance, is of pivotal interest in dental research. Bone, connective tissue, and oral epithelium are in direct contact to the implant surface and exhibit distinct requirements for proper growth and differentiation. The authors applied direct laser writing and atomic layer deposition for the generation of TiO2-coated micro and nanostructures which were subsequently tested for colonization and growth behavior of SaOs-2 cells, an osteosarcoma cell line revealing osteoblastic properties. Structures composed of rigid posts and flexible rods provide a matrix, which—when spaced adequately—favor the three-dimensional growth and proliferation of SaOs-2 cells. The results provide a proof of concept for the optimization of dental implant surfaces using generic techniques which deliver highly standardized structure motifs supporting the biological functions of the tissues affected. © 2012 Laser Institute of America.

Key words: direct laser writing, dental implant, titanium dioxide, 3D scaffold, cell growth

I. INTRODUCTION

Dental implant complications represent a serious problem for the individuals concerned. Implant failure, leading to loss of the construct and the need for further dental prosthesis, occurs in approximately 5–8% of cases. Major problems during implant adaptation to the body are (i) reduced formation of bone tissue, which impairs stable integration of the construct, (ii) parallel orientation of collagen fibers to the implant surface, which interferes with proper adhesion of connective tissue, and (iii) a lack of differentiation of epithelial cells, which inhibits a regular stratification of the oral epithelium and a tight junction between this tissue and the implant. These complications frequently lead to destabilization of the constructs and to periimplantitis and may successively result in dental implant loss.

In the past, attempts for an optimization of the dental implant surface mainly focused on the interaction between titanium (as the material of choice for most constructs) and bone tissue. Modification of the titanium surface, e.g., by altering its hydrophilicity, by providing integrin binding ligands or growth factor motifs, or by structural modifications, or combinations of the latter, have been analyzed for an enhancement of cell growth, adhesion, and/or differentiation of osteoblasts, osteoblastlike cells, or mesenchymal stem cells. While such functionalizations frequently induced alterations in one of the parameters mentioned above, many of them have not been evaluated for an optimization of the balance between proliferation and differentiation, which is required for optimized osseointegration. A series of recent publications described elevated proliferation in line with osteogenic differentiation during growth of osteoblastlike cells on biphasic structural motifs composed of micro and nanostructures, which was in part functionally validated by in vivo experiments. The results suggest that a systematic analysis of cellular phenotypes during growth on titanium surfaces containing different biphasic structural motifs may facilitate the identification of further improvements on the way to an optimized osseointegration of dental implants.

The influence of substrate functionalization on soft tissues involved in dental implant adaptation has been studied with less intensity. Topography-dependent alterations in adhesion and signalling of gingival fibroblasts have been described. In vivo, different implant surface structures affected the orientation of collagen fibers; however, the optimum perpendicular orientation was not observed in this study. Growth factor coating of implants was found to alter the interaction between soft tissue and implant surface, but the orientation of fibroblasts and collagen fibers was not affected. Considering the precision required for the fabrication of tailored structures for proper differentiation of the affected tissues, rather arbitrary surface designs resulting from processes like acid-etching, machining, or sandblasting may be of minor quality and not sufficient for an optimal interaction...
between cells and structures. To this end, we suggest direct laser writing (DLW) as a generic approach to produce highly standardized structures on dental implant surfaces, which meet the requirements of cell types under investigation. As a proof of concept, we generated structures which enabled three-dimensional growth and enhanced proliferation of osteoblastlike cells on titanium surfaces.

II. EXPERIMENTAL METHOD

A. Direct laser writing

Three-dimensional biphasic structures were created via two-photon absorption with a modified nanoscribe photonic professional direct laser writing system (Nanoscribe GmbH, Eggenstein-Leopoldshafen, Germany). The system is based on a 100 MHz pulsed erbium-doped and frequency doubled femtosecond fiber laser in the near infrared wavelength range (pulse duration 150 fs at 780 nm) as a light source, an acousto-optical device modulating the intensity, an inverted microscope for high numerical aperture focusing (numerical aperture NA = 1.4, oil-immersion), a three-axis piezo-electrical device for accurate motion of the sample (PhysikInstrumente), and a motorized stage for large-area coarse structuring [Fig. 1(a)]. Used photoresists include negative resists IP-L and IP-G (both commercially available from Nanoscribe). IP-L is dropcast onto a glass slide and developed by immersion in isopropanol for 20 min. IP-G is dropcast or spin-coated onto a glass slide and prebaked for 1 h at 100°C. Development is performed by immersion in 1-methoxypropan-2-yl acetate for 20 min. No postbake is required. Typical laser power for writing the structures is in the range of 10–20 mW, measured at the entrance pupil of the objective. The writing speed for the structures was set to nominal 100 μm/s, but varies with the complexity of the structures.

B. Atomic layer deposition (ALD)

After the lithography step and the development of the samples, ALD is used to coat the three-dimensional structures with 50 nm TiO₂ (anatase phase) to ensure biofunctionality and to provide photo-toxicity under illumination with UV-light. The ALD process is the only process that allows truly homogeneous film growth on three-dimensional structures since it is not a directed process. The coating is performed with a Cambridge Savannah system with 1667 cycles at 250°C. Precursors were tetrakis(dimethylamino)titanium and H₂O.

C. Scanning electron microscopy (SEM)

SEM images were taken with a Hitachi SU-8000. The structures were not additionally coated.

D. Cell culture and proliferation analysis

SaOs-2 cells were kindly provided by Prof. Ignatius (Institut für Unfallchirurgische Forschung und Biomechanik, Ulm University). The cells were cultured in DMEM-medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For fluorescence imaging, cells were seeded at a density of 100 cells/mm² and grown for 48 h. Cell proliferation of structured and neighboring plain fields (1 mm² each) was determined by counting of 4',6-diamidino-2-phenylindole (DAPI) stained nuclei 48 h post cell seeding. Two—three fields per structure were counted; the data represent mean values ± standard deviation.

E. Cell staining and fluorescence microscopy

Prior to staining, cells were fixed in phosphate buffered saline containing 4% formaldehyde. Vinculin staining for visualization of focal adhesion contacts (FACs) was carried out using a mouse monoclonal anti vinculin antibody (ab18058/clone SPM227, Abcam, Cambridge, UK) and an Alexa 488-conjugated goat antimouse secondary antibody (A11029, Invitrogen, Darmstadt, Germany). Actin microfilaments were visualized using phalloidin-tetramethylrhodamine isothiocyanate (Sigma-Aldrich, Taufkirchen, Germany). Nuclear staining was performed using DAPI (Sigma-Aldrich). Fluorescence microscopy was carried out using Zeiss microscopes (Axiovert 40CFL and LSM510) and software (AxioVision Rel. 4.8 and LSM5). Cell areas were determined by an implemented algorithm in the AxioVision software.

FIG. 1. Direct laser writing mediated generation of micro and nanostructures. (a) Optical setup for direct laser writing. (b) Schematic illustration of structures composed of rigid posts and flexible rods (upper left image) as well as exemplary SEM images of square and honeycomblike structures (upper right image: square, lateral view; lower left image: square, top view; lower right image: honeycomb, top view; post distance: 25 μm), which differ by post arrangement.
III. RESULTS

A. Generation of structures with varying feature sizes

Test structures were composed of rigid posts (5 μm diameter, 13 μm height) connected by thinner and thus more flexible rods [elliptical shape, long axis 780 nm, short axis 300 nm, distance from substrate 10 μm, see Fig. 1(b)]. The posts were arranged in either a square pattern or a honeycomblike pattern. The distance between posts has been varied between 10 and 50 μm in 10 μm steps for square patterns. In addition, honeycomb patterns and square patterns with 25 μm post distance were created alongside for comparison. Each sample contained three square fields of 1 mm² size for each structure type. The rigid posts have been written with piezopositioning, the connecting mesh of thinner rods with the motorized stage of the lithography system.

B. Three-dimensional growth of SaOs-2 cells in square and honeycomb structures

Fluorescence staining of microfilaments and FAC (via FAC bound vinculin) revealed a three-dimensional growth of cells in both square and honeycomb structures, which was evident through the identification of cytoplasmic protrusions touching posts and rods of the structures, thus allowed cell growth in three dimensions (scale bar: 20 μm). When defined through presence of fluorescence signals from the cytoskeleton and FAC during epifluorescence microscopy, the growth area of cells in both square and honeycomb structures was significantly smaller when compared to plain surfaces.

IV. DISCUSSION

In this study, direct laser writing combined with atomic layer deposition was successfully applied for the generation of TiO₂-coated structures at the micro and nanometer scale, which were subsequently analyzed for biocompatibility.
growth, adhesion, and differentiation is incomplete and requires further investigation. Biphasic structures, as generated by acid-etching of surfaces and subsequent sputter-mediated deposition of TiO$_2$ nanonodules, were found to improve dental implant osseointegration, but also to impair biological functions of fibroblasts. These results underline (i) the need for an identification of optimized structures for all tissues affected during dental implant acceptance and (ii) the importance of spatially resolved surface structuring, which assures the presence of an optimized micro-environment for each tissue affected. In this study, we provide evidence for the regulation of proliferative activity of osteoblast-like cells by the presence and size of highly ordered 3D structural motifs at the micrometer scale. In addition, the formation of FAC at both lateral and apical structural elements and a reduction of the growth area occupied suggest that our matrix provides a scaffold which is well accepted for 3D growth by osteoblast (-like) cells. The differentiation-promoting effects of three-dimensional growth of osteoblast (-like) cells induced by the presence of artificial 3D scaffolds have been demonstrated by others. Since proper osteogenesis requires a delicate balance between proliferation and differentiation, further studies are needed to analyze the influence of our scaffolds on hallmarks of osteoblast differentiation, such as alkaline phosphatase activity, osteocalcin secretion, or hydroxyapatite deposition.

The requirement for precision of features at the nanometer scale for biological applications has been demonstrated. Regular spacing of features and the degree of flexibility of the micro-environment (determining mechanically induced signal transduction in target cells) have profound impact on physiology of various cell types, thus justifying research on designer extracellular matrices for medical applications. Direct laser writing has already been successfully employed for the controlled generation and functionalization of 3D structures for biological applications at the submicrometer scale. Photoresists such as ORMOCER and SU8 have been proven for their biocompatibility, and 3D structures generated have been demonstrated to support the development of characteristic phenotypes for a variety of cell types, among them granulosa cells, fibroblasts, chondrocytes as well as endothelial, epithelial, and cancer cells. Recent research of our lab addressed the impact of structure flexibility and scaffold functionalization on cellular properties. However, the method lacked the potential for structuring areas of sufficient size and sub-100 nm resolution.

To this end, we combined stage- and piezo-electrical scanning to fabricate structures covering areas up to 1 cm$^2$. Within this, the creation of sub-100 nm features and minimum feature separations down to 300 nm have been targeted and achieved by the development of new photoresists, which have been thoroughly characterized for two-photon polymerization by femtosecond laser pulses and were also shown to be suited for two-photon polymerization using continuous-wave (cw) diode lasers at wavelengths down to 532 nm.

While the present work focuses on the influence of large-area structures with submicron feature sizes on osteoblast-like cells, the new photoresists should enable the generation of well-defined biphasic structures containing precise details at the sub-100 nm scale for future
applications. Further progress in structuring of crocked surfaces should then facilitate the generation of tailored topographical features for an optimized interaction of dental implants with both hard and soft tissues.

ACKNOWLEDGMENTS

This study was supported by a grant from the German Research Foundation (ST31490/23-1 to R.S. and FR1671/6-1 to G.v.F.). The authors thank Eva Winkler and Petra Kruse for expert technical assistance, Andreas Frölich for running part of the atomic-layer coatings, and Herbert Schneckenburger and his group for valuable support in fluorescence microscopy. G.v.F. is cofounder of Nanoscribe GmbH, commercializing direct laser writing and corresponding photoresist materials.