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Hemocompatible, pulsed laser deposited coatings on polymers

Anwendung der Pulslaserbeschichtung zur Abscheidung von hämokompatiblen Beschichtungen auf Polymeroberflächen

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Abstract

State-of-the-art non-thrombogenic blood contacting surfaces are based on heparin and struggle with the problem of bleeding. However, appropriate blood flow characteristics are essential for clinical application. Thus, there is increasing demand to develop new coating materials for improved human body acceptance. Materials deposited by vacuum coating techniques would be an excellent alternative if the coating temperatures can be kept low because of the applied substrate materials of low temperature resistance (polymers). Most of the recently used plasma-based deposition techniques cannot fulfill this demand. However, adequate film structure and high adhesion can be reached by the pulsed laser deposition at room temperature, which was developed to an industrial-scaled process at Laser Center Leoben. Here, this process is described in detail and the resulting structural film properties are shown for titanium, titanium nitride, titanium carbonitride, and diamond-like carbon on polyurethane, titanium and silicon substrates. Additionally, we present the biological response of blood cells and the kinetic mechanism of eukaryote cell attachment. In conclusion, high biological acceptance and distinct differences for the critical delamination shear stress were found for the coatings, indicating higher adhesion at higher carbon contents.

Keywords: cell adhesion; diamond-like carbon; pulsed laser deposition; titanium; titanium carbonitride; titanium nitride.

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Zusammenfassung

Derzeit verwendete Oberflächen für den Blutkontakt basieren im Allgemeinen auf Heparin, wobei es in der klinischen Verwendung leicht zum Problem von Blutungen kommen kann. Nichtsdestotrotz ist aber ein optimaler Blutfluss entscheidend, wodurch sich zunehmend die Forderung nach der Entwicklung von neuen Materialien mit verbesserter Körperakzeptanz stellt. Mittels Plasmaverfahren auf Oberflächen abgeschiedene Werkstoffe könnten zukünftig eine Alternative zu Heparin bieten, wenn die Temperaturen während des Herstellprozesses auf nahezu Raumtemperatur abgesenkt werden könnten, um vor allem die vielfach verwendeten Kunststoffe mit geringer Temperaturbeständigkeit beschichten zu können. Als eine der wenigen Techniken bietet sich dafür die Pulslaserabscheidung (PLD) an, welche zu einem industriell einsetzbaren Prozess am Laserzentrum Leoben entwickelt wurde. Diese Arbeit beschreibt die Hauptmerkmale dieses Prozesses und die sich ergebenden strukturellen Filmeigenschaften von Ti, TiN, Ti(C,N) und diamantähnlichem Kohlenstoff (DLC) auf Polyurethan-, Titan- und Siliziumoberflächen. Zudem werden die biologische Antwort von Blutzellen und die kinetischen Haftungsmechanismen von Eukaryotzellen dargestellt. Zusammengefasst zeigen diese Schichtwerkstoffe hohe biologische Akzeptanz und deutliche Unterschiede in den kritischen Scherspannungen zur Delamination, wobei eine höhere Zelladhäsion bei höheren Kohlenstoffgehalten erreicht wird.

Schlüsselwörter: diamantähnlicher Kohlenstoff; Pulslaserbeschichtung; Titan; Titancarbonitrid; Titannitrid; Zellanhaftung.

Introduction

Materials used in blood-contact devices (e.g., heart valves, artificial hearts, stents, capillary tubes) have often been chosen based on their physical characteristics, such as flexibility or rigidity, mechanical strength, transparency, degradability, etc. [5]. Moreover, cost-effectiveness, ease of processing, and sterilization have also been important considerations when selecting a particular material. Thus, optimal thrombogenicity – the tendency to encourage blood clotting (hemostasis) – might not always be achieved. Hemostasis involves the adsorption of blood proteins, followed by platelet adhesion and activation. Shear stresses triggered by flowing blood

strongly influence these cell adhesion processes on vessel walls in human body or the biomaterial's surface. The resulting mechanical tension affects the receptor-ligand bonds at the contact areas, e.g., fibroblasts adhere to the extracellular matrix through focal contact areas, for which number and surface increase with the mechanical tension applied to the cell [7, 28]. Detachment of cells occurs at hydrodynamic stresses above a threshold, statistically following apparent first-order kinetics. The threshold stress for each cell depends on cell size, the nature of the substrate, and the presence of adhesion proteins at the plasma membrane surface [9, 15].

Chemistry, elasticity, and topography are dependent on the material itself and its manufacturing process. Differences between bulk and surface properties can be achieved by surface modification techniques, which either change the existing surface or apply an additional layer on the surface. Plasma techniques offer a great variety for such changes on metal, ceramics, and polymer surfaces. Plasma sterilizes and cleans surfaces (from manufacturing residues) but also promotes adhesion for subsequent deposits (further coatings, proteins, cells) by chemical, mechanical, and topographical changes. Ablation for removing the outermost layer, implantation of oxygen or nitrogen atoms, radical formation on the surface, and deposition of polymers, metals or ceramic materials of generally some nm to $\sim$50 μm thickness are the basic processes. Many of the plasma-based deposition processes (e.g., magnetron sputtering, plasma-activated chemical vapor deposition, ion-assisted deposition or arc evaporation) require elevated temperatures ($>$150–200°C) to reach the desired properties of the surfaces. However, numerous medically applied polymers can withstand only temperatures of up to 70–100°C. Thus, increasing focus is given to the development of low temperature techniques such as pulsed laser deposition (PLD).

The present study shows the application of PLD for depositing titanium (Ti), titanium nitride (TiN), titanium carbonitride [Ti(C,N)], and diamond-like carbon (DLC) thin films on titanium, silicon, and polyurethane substrates of hemocompatible use.

Materials and methods

Film deposition by RT-PLD

In PLD, a high-power laser beam is periodically focused onto a target material provoking instantaneous evaporation and ionization of the surface atoms and, hence, forming dense plasma inside the plume of the evaporated material. These ablated atoms, electrons, and ions are driven away from the target at high speeds into the vacuum of controlled conditions and strike the surface of a substrate leading to the nucleation and growth of thin films with the same chemical composition as the evaporated material (Figure 1). After evacuation of the chamber to pressures $<2\times10^{3}$ Pa, we used targets of grade 2 titanium (Eurotitan GmbH, Solingen, Germany) and electrocarbon graphite (99.7% carbon, Hoffmann & Co., Steeg, Austria) for the ablation with the pulsed

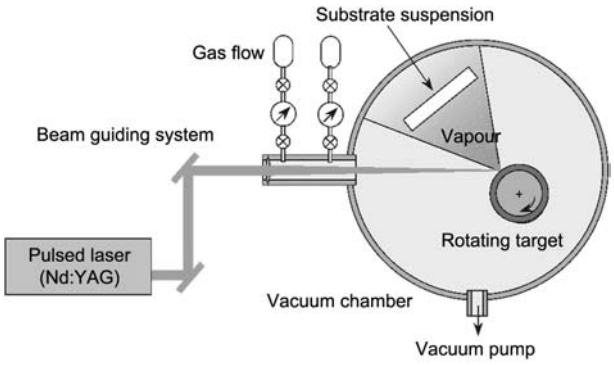

Figure 1 Principles and technical set-up of PLD coating.

Nd:YAG laser systems (Powerlite, Continuum Inc., Santa Clara, CA, USA) of 1064 nm wavelength, 10 ns pulse duration, and 50 Hz repetition rate. The deposition resulted in 50 nm thick films on polyurethane (PU; Chronothane P, Cardiotech Inc., Wilmington, MA, USA), 300 nm thick films on silicon wafers (B-doped, Wacker Chemie AG, München, Germany) and grade 2 titanium (Eurotitan GmbH) substrates. Silicon wafers had 2″ diameter and 500 μm thickness, titanium discs 2 mm thickness. The PU foils (thermal stability up to $\sim$65°) of 100 μm thickness were cut to 50×50 mm^2 squares. To prevent any damage of the polymer substrates, the substrate temperature during deposition was always kept at room temperature.

In general, gas molecules can be introduced inside the reaction chamber to react with the atoms or molecules from the plume to reactively deposit films. Even if inert gases are used, an increase of the deposition pressure confines the plume expansion by increasing the number of molecules that collide with the highly polar, forward peaked and cone-shaped plume, allowing a homogeneous, thickness- and composition-uniform deposition over several cm^2 of substrate surface [8]. For obtaining the different coatings, we used pure argon atmosphere for DLC growth from electrocarbon targets and Ti growth from titanium targets. The latter targets were also used for deposition of TiN films in nitrogen (4.6 purity; Linde Gas GmbH, Stadl-Paura, Austria) and Ti(C,N) films in nitrogen-acetylene (C_2H_2, 99.5% purity; Linde Gas GmbH) atmospheres with low [0.5 sccm (standard cubic centimeters per minute)] and high (2.5 sccm) gas flow resulting in lower [Ti(C,N)^{l}] and higher [Ti(C,N)^{h}] carbon content.

The detrimental effect of PLD is the small homogenous coating area, limiting the application of PLD in industrial style [1, 26]. Special equipment of target or substrate movement is required to provide a relative motion between the substrate and the beam to scan the selected deposition area across the entire surface of the substrate [18]. High-rate coating is only guaranteed if several laser systems are applied simultaneously (multi-spot PLD) and the ablated plumes are optimal superposed (imitation of a line evaporator) [19]. This attempt has successfully been applied for the worldwide first time at Laser Center Leoben in a PLD coating machine. Applying this concept with four Nd:YAG lasers, we were

able to keep the thickness variation of the coatings lower than 5%. Additionally, the substrates were moved with a relative speed of 54 mm s^{-1} through the plasma plumes during deposition.

Film characterization

Characterization of film structure and growth behavior X-ray photoelectron spectroscopy (XPS) was employed to investigate the chemical bonding in the films using an ''Omicron Multiprobe LT'' system (Omicron NanoTechnology GmbH, Taunusstein, Germany) with a monochromized AlKα (1486.6 eV) X-ray beam and an ''EA 125'' energy analyzer. The resolution of this set-up is better than 0.3 eV, and the analysis took place at a pressure of 4×10^{-9} Pa. The spectrometer was operated in the fixed analyzer transmission mode. All binding energies reported in this study were referenced to the binding energy of the carbon C1s peak at 285.0 eV. The detection sensitivity was approximately 1 mass%. For sputtering an Omicron ''ISE 10'' sputter gun using Ar^+ ions was used. High-resolution transmission electron microscopy (HR-TEM; ''Tecnai G2 F20-TWIN'', FEI Inc., Hillsboro, OR, USA) was used to characterize the growth and atom arrangement in the films. The thin foils were prepared using focus ion beam cutting (''FEI Dual Beam'', FEI Inc.).

Characterization of static cell expression of human fibroblasts The cell expression was tested for fibroblasts in 48-h tests. The human fibroblasts were obtained from healthy donors under compliance with the rules of the Polish medical ethics commission. The cells in a concentration of 1.5×10^5 ml^{-1} in Dulbecco culture medium (Sigma-Aldrich Co., St. Louis, MO, USA) were moved on the surface of materials [standard glass as control material, PU coated with Ti, TiN, and Ti(C,N)[h]] after substrate sterilization in plasma (hydrogen peroxide, 7 hPa, 46±2°C, 1 h) and washed in a physiological salt-buffered solution. Adhering cells were fixed by a solution of 4% paraformaldehyde and 70% methanol (Sigma-Aldrich Co.) and counted using light microscopy. Then, the substrates with the fixed cells were washed in 0.5% bovine serum albumin (ATTC Co., Rockville, MD, USA), marked with antibodies [CD49E (Serotec Co., Martinsried-Planegg, Germany), Alexa Fluor 488 (Invitrogen Co., Carlsbad, CA, USA)], and colored by 7AAD (Merck AG, Darmstadt, Germany). These cells were analyzed by laser confocal microscopy (''Olympus FV-500'' device, Olympus Microscopes, Tokyo, Japan) and by laser scanning cytometry (CompuCyte Inc., Westwood, MA, USA). Cell nuclei visualization was performed by incubation with 7-amonioactinomycin D (Merck AG). The amount of interleukin (IL)-1β was marked by the immunoensimatic method in the incubation fluids of the cell cultivation. The colored reaction was achieved by OPD hydrochloride (Sigma-Aldrich Co.) and analyzed by a single-channel reader assay system (''ELX 800'', Biotek Instruments Inc., Wonoosky, UT, USA) at a wavelength of 492 nm. Afterwards, the cells were detached from the sample surfaces by non-enzymatic cell dissociation solution (Sigma-Aldrich Co.) for light microscopy investigations and analyzed with Trypan blue staining (Sigma-Aldrich Co.).

Dynamic cell expression characterization by shear flow testing with Ax-2 *Dictyostelium discoideum* cells In general, several techniques are used as set-up for studying shear flow-induced cell motility (SFICM) [27]. Among them, the applied radial flow detachment assay, based on the set-up used by Lauffenburger et al., is often used for studying cellular expression on and cell adhesion to solid substrates under controlled flow conditions [10, 11, 13, 14, 25]. *Dictyostelium discoideum* cells are like a model organism for such investigations [13]. For testing, we used *D. discoideum* Ax-2 cells (cell line of Minatec-INP, Grenoble, France), which were grown in axenic medium [2] in agitated suspension (180 rpm). The vegetative cells were harvested during exponential phase (see explanation below) at a density of $2–4\times10^6$ ml^{-1}, pelleted by centrifugation and washed twice in Sörensen phosphate buffer (2 mM Na_2HPO_4/14.5 mM KH_2PO_4, pH = 6.2, Sigma-Aldrich Co.). Cell pellets (10^7 cells) were stored on ice and used within 8 h.

These cells were attached to a horizontal disc (coated titanium and silicon discs, for plasma sterilization parameters, see ''Characterization of static cell expression of human fibroblasts'' section) (Figure 2A). A second, upper disc (stainless steel) with a center hole (1.5 mm in diameter) was mounted in distance e of 0.1 mm for Ti, TiN, and DLC and 0.25 mm for Ti(C,N) films. The larger distance is as a result of the high hydrophobicity of Ti(C,N). Liquid (21 ± 1°C) was supplied through the hole, forming a radial hydrodynamic flow of Sörensen buffer. Thus, the shear flow depends on the distance from the center hole (see finite element simulation in Figure 2B). When the exerted forces are sufficient, cells are removed from the solid surface and taken away in the laminar bulk flow. After testing, the samples are observed in fluorescence microscopy to characterize and count the detached cells (for detailed description, see Ref. [13], shown, e.g., for DLC coating on silicon wafer in Figure 2C). The percentage of detached cells was then redrawn as a function of the wall shear stress [$\sigma(r)$ dependent on the radius r] calculated using the relation

$$\sigma(r) = 3\times D\times \eta/(\pi\times r\times e^2) \quad (1)$$

where D is the flow rate (set to 40 ml/min) and η is the dynamic viscosity of the Sörensen buffer fluid. A quantity of 50% detached cells was assumed for the critical shear stress.

Results and discussion

Growth of functional coatings with PLD at room temperature – the nanoscience approach for optimized properties

The PLD process at room temperature possesses several unique deposition mechanisms, strongly and positively influ-

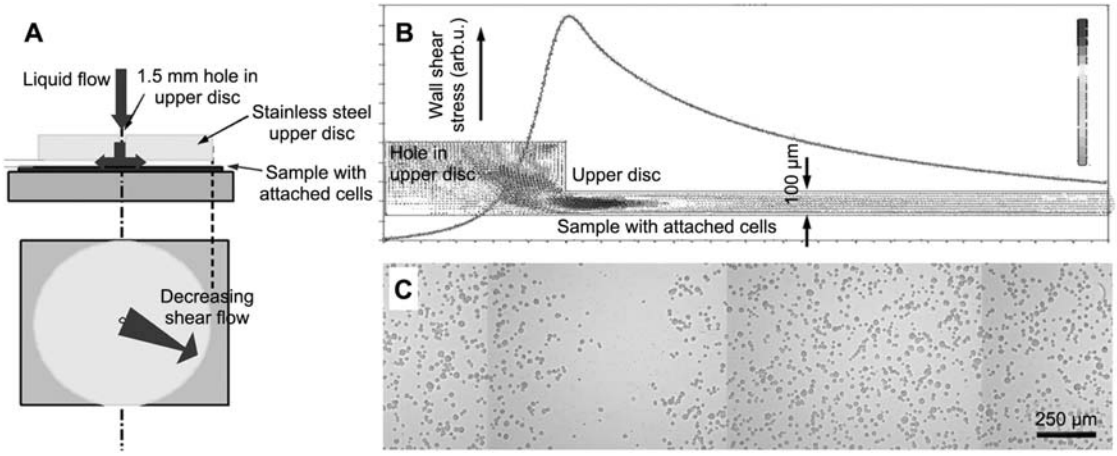

Figure 2 (A) Experimental set-up of the applied shear flow-induced cell motility test (based on Ref. [10]): a radial hydrodynamic flow is generated between the upper stainless steel disc and the lower sample (coated) plate on which cells adhere. (B) Finite element simulation of the shear flow in the gap between the upper and lower disc and the wall shear stress on the (coated) sample plate. The shear stress induced by the flow on the plate decreases as $1/r$. (C) Dark field light microscopy images of the cell attachment on a DLC coated silicon wafer sample disc dependent on the distance from the middle axis of the SFICM test device (correlated to Figure 2B), indicating no cell adhesion in the regions of very high wall shear stresses.

encing the room temperature growth and properties of the deposited film. The excellent film adhesion in PLD coating [22] is a consequence of the high energetic ion fraction (100–1000 eV) leading to an implantation of atoms in the substrate surface zone. The thickness of the implanted ''pseudodiffusion zone'' (Figure 3A) is particularly dependent on the material: here, it was found in 50 (to 120 nm) distance to the PU surface implanted titanium atoms (Figure 3B). This implantation allows a much better anchoring of the film compared with abrupt interfaces (Figure 3A) by the formation of a gradient layer of chemical composition, structural, and mechanical properties [20]. The gradient layer bridges the very different mechanical properties of polymers and metal/ceramic films. In polymers, such atoms can furthermore trigger chemical changes in the binding – e.g., chain scissoring or crosslinking. Moreover, we found strong chemical binding of titanium to oxygen atoms [23]. Abrupt interfaces are mainly found in coatings deposited by conventional techniques (e.g., sputtering) at low temperatures [24].

The very low film porosity (<1%) is a further feature, rarely found for room temperature deposited coatings by

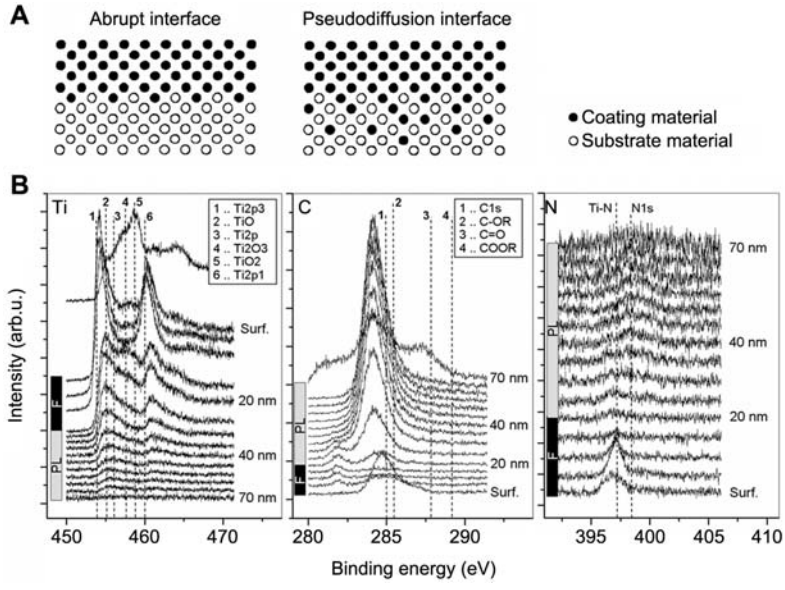

Figure 3 (A) Difference between abrupt and pseudodiffusion interface. (B) XPS characterization of binding in the 20 nm thick PLD Ti film (F) pseudodiffusion zone (PL) on the thermoplastic polyurethane surface, revealing a minimum depth of implantation of 50 nm as well as strong binding of implanted Ti atoms to oxygen of the polymer chains.

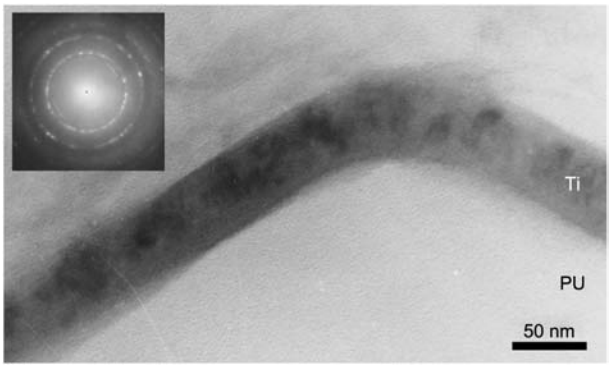

Figure 4 Elastic, crack-free bending of a thin, nanocrystalline PLD titanium (Ti) film on a polyurethane (PU) substrate in HR-TEM examination.

competing techniques [21]. Furthermore, the PLD at room temperature results in a fully dense covering of the smooth substrate surface without island growth after just a few plasma pulses at film thicknesses of even <10 nm, as proven for Ti and reactively deposited TiN coatings [20].

In the PLD film growth at room temperature, we found a further interesting feature of such thin metal films (e.g., titanium), being extremely mechanically elastic. Deposited on polymers, they follow the elastic deformation of the polymer substrate without forming cracks, visible in HR-TEM investigations. Even cyclic bending does not lead to film delamination. Figure 4 shows such an elastically deformed Ti film on a PU surface.

Static cell expression of human fibroblasts

Initially to the cell expression investigations, we ensured that the number of adhering species ($\sim$600 cells/cm^2) was equal on glass reference substrates and Ti, TiN, and Ti(C,N)^h PLD coated PU substrate surfaces. Subsequently, the expression of internal and external fibronectin was investigated by confocal microscopy. Fibronectin is a glycoprotein existing extracellularly and on the cell surfaces in blood, body fluids, and connective tissue. This protein associates with the other proteins of the extracellular matrix such as fibrinogen, collagen, glycosaminoglycans, and with suitable receptors of the cell membrane. The analyses in confocal microscopy (Figure 5A–D) reveal the creation of bands with dense fibers laying on the axis of the adhered cells. On the control glass substrates and – a bit less developed – on Ti(C,N)^h coated PU the net of the fibronectin create irregular connections in all directions.

Vinculin is a polypeptide connecting the proteins of the cell membrane with an active cytoskeleton of the cytoplasm cortex at the connection between cell and extracellular matrix – the place of adhesive plate formation. A huge expression proves strong adhesion of the cells. By analyzing the vinculin expression in confocal microscopy, such huge expression is evident for Ti and Ti(C,N)^h, whereas the expression is lower on the glass substrate and the TiN film on PU (Figure 5E–H). The cell viability investigation revealed 99% alive cells and only individual dead cells. Additionally, the missing IL-1β showed no evidence of its formation in the 48-h cultivation liquid gathered from the tested surfaces. Testing silver for comparison resulted in an immediate IL-1β expression. This strongest immunostimulator is one of the basic factors for examining the immunogenicity of a biomaterial being formed in the cultivated cells, if the biocompatibility is low. Its missing expression reveals the high acceptance of all investigated materials by fibroblasts, which is in high accordance to Breme et al. [4].

Dynamic cell expression of *Dictyostelium discoideum* cells

As our first step, we tested the static attachment of *Dictyostelium discoideum* cells from Sörensen phosphate buffer solution for referencing the subsequent dynamic testing. *D. discoideum* is a simple unicellular eukaryote cell and is a genetically and biochemically tractable model organism that is used extensively to study cytoskeleton organization, che-

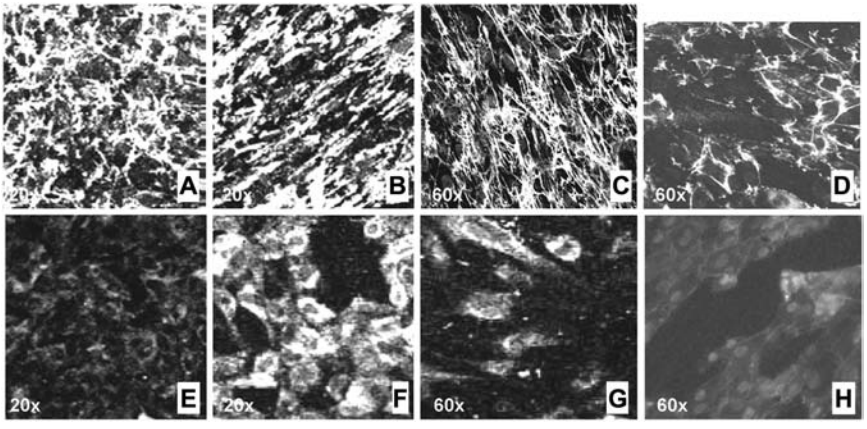

Figure 5 Selected results of the biocompatibility tests.
(A–D) Expression of internal and external fibronectin in the fibroblast cultivation. (E–H) Vinculin expression in the fibroblasts. Substrate material: (A, E) control glass; (B, F) 50 nm Ti coating on PU; (C, G) 50 nm TiN coating on PU; (D, H) 50 nm Ti(C,N)^h coating on PU.

motaxis, cell differentiation, and development [17]. The lifestyle of *D. discoideum* consists of two phases: (1) during the vegetative phase, the small (8 μm in diameter) ameba feeds upon bacteria and yeast by phagocytosis. Plasma membrane adhesion is therefore directly related to the phagocytic properties of the cell. (2) In the development phase, cell-cell adhesion is more important, and specific contact proteins are expressed [3]. Axenic strains showing an enhanced fluid-phase endocytosis were obtained and are able to grow in suspension in nutritive medium. Several mutant studies suggested the existence of three types of adhesion proteins [6, 9, 29]. Only the vegetative phase including the phagocytic process is a good model to describe adhesion of human fibroblasts to vessel and biomaterial surfaces. The detachment rate is strongly affected by the presence of an intact actin cytoskeleton and by the cell-substrate adhesion energy. An interpretation of the detachment process is partly possible by an adhesive belt model describing the passive behavior of the cell edge under external or internal forces [15].

The static attachment of *D. discoideum* was found to be initially time-dependent, but leading to stable, time-independent cell densities on the surfaces after ~5 min for $Ti(C,N)^l$ and $Ti(C,N)^h$, ~10 min for DLC, and ~15–20 min for TiN and Ti films. The higher the carbon content in the coating, the higher density was found: the maximum was reached with ~1400 *D. discoideum* cells/mm^2 for $Ti(C,N)^h$, followed by DLC, whereas Ti and TiN surfaces lead to only ~600 adhering cells/mm^2. For all coatings an agglomeration of cells is evident, which develops during the static cell attachment, mainly at lower adhering cell densities.

Performing SFICM tests generally leads to dependencies of the percentage of detached cells and the hydrodynamic stress as a result of the forced flow of Sörensen buffer solution as shown in Figure 6A. At low shear stress, close to the edge of the disc, nearly all cells are still attached after testing. Increasing the stress by investigating regions closer to the disc center leads to a linearly to slightly exponential increase of the detached cell percentage, which logarithmically approximates to 100% close to the center hole, through which the buffer solution is pumped.

In addition to the static attachment, the dynamic detachment is strongly dependent on time. Thus, reaching stable detachment rates is decisive for pointing out differences of the surfaces. If such stable conditions are found, the detachment rate (detached cells/unity of time) describes the detachment kinetics rather well for up to 50% detached cells. As shown in Figure 7B, the detachment rates increase linearly or exponentially with increasing shear stress [except $Ti(C,N)^l$ as a result of the very high rates]. Ti and DLC surfaces allow good binding/sticking of *D. discoideum* cells, whereas TiN and Ti(C,N) coatings apparently promote cell detachment. As a result, the hydrodynamic stress for 50% detached cells (''50% threshold stress'') is lower than for the other coatings on the silicon substrates. The similar threshold stress found

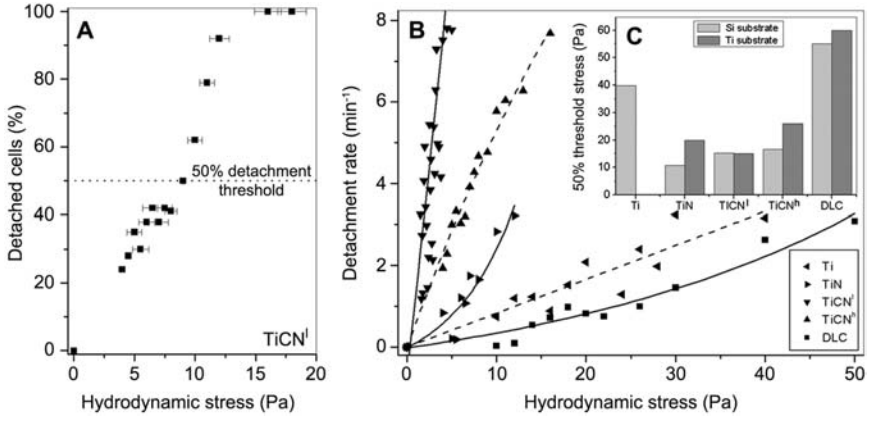

Figure 6 Results of SFICM studies with *Dictyostelium discoideum* cells on PLD coatings.
(A) Dependency of detached cells on the hydrodynamic stress for a $Ti(C,N)^l$ film on Si wafer substrate under stable conditions. Evaluation of the 50% threshold stress (50% cell detachment) is marked. (B) Detachment rate dependent on the hydrodynamic stress of all coatings on Si wafer substrate. (C) 50% threshold stress for all coatings on Si and Ti substrates.

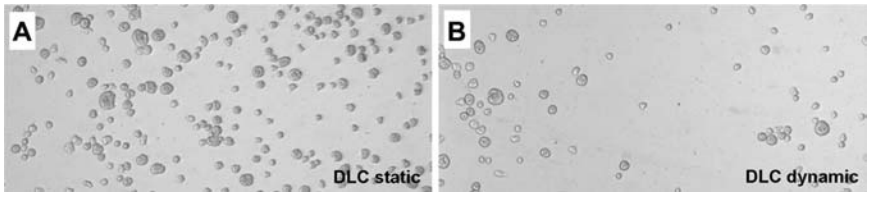

Figure 7 Fluorescence microscopy images of the cell distribution on a DLC coating in (A) static attachment and (B) dynamic detachment of *Dictyostelium discoideum* cells in the region of maximum shear stress in SFICM.

for Ti substrates [25] reveals the high influence by the nature of the coating/surface which is directly in contact with the cells and the diminishing influence of the substrate itself. Extremely high hydrodynamic stress (>60 Pa) is required to detach *D. discoideum* cells (in their vegetative phase) from DLC surfaces revealing the optimal behavior of DLC as a biomaterial shown in numerous studies in the literature in the past number of years ([6], [12], [16]). The comparison of the cell shape before and after shear flow testing (Figure 7A,B) reveals no visible damage of the adhering cells even after applying the highest shear flows on DLC.

Conclusion and outlook

The present study showed that the application of room temperature coating by the PLD process, industrially developed at Laser Center Leoben, allows high-adhesive deposition of Ti, TiN, Ti(C,N), and DLC coating on extremely soft polyurethane, but also on medical grade pure titanium and silicon wafers. Testing the cell expression revealed comparable adhesion of fibroblast cells and cell death for these coatings and control glass substrates and a missing expression of the IL-1β immunostimulator. Thus, the PLD coating allowed expected improvements in decreasing the tendency to thrombus formation in blood flowing on such surfaces. Additionally, the radial shear flow test, as a strong tool for investigating the influence of the shear stresses to cells, reveals high critical stresses for cell detachment for coating of high carbon content, the highest for pure DLC. These tests were performed with *D. discoideum* cells in their vegetative phase allowing high comparability of the adhesion phenomena to human fibroblasts. Agglomeration tendency of these cells was found to be indirectly proportional to the carbon content of the films, an indication for the stress response of the cells.

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