Detachment Kinetics of Eukaryote Cells From Biocompatible PVD Coatings

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ABSTRACT

Biocompatible coatings gather more and more interest. However, depositing them at low temperatures is a big challenge, which is necessary to use polymers and other bio-materials as substrates.

In the current work, the PVD techniques pulsed laser deposition and magnetron sputtering were used to deposit highly adherent TiN, TiCN and diamond-like carbon (DLC) coatings on titanium substrates at room temperature in order to provide substrate materials for studying the detachment kinetics of cells from these biocompatible coating materials.

These kinetic mechanisms of eukaryote cells (type Dictyostelium discoideum) were tested in a two-plate cell detachment assay with the coated substrates as test plates and stainless steel discs as counter plates. The suspension of the cells in Sörensen buffer rested between the two plates, retained by the balance between gravity and capillary forces. All coatings influenced the cells leading to the observation of high cell attachment. The highest amount of detached cells was observed for the carbon-free and low-carbon containing coatings. Additionally, the agglomeration tendency of the *D. discoideum* cells increases on these coatings under medium shear stresses.

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. [1]. 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) may not always be achieved. Thrombus formation can occur, if blood is exposed to something foreign, such as the biomaterial. The first clinically manifested process in this contact is the activation of hemostasis (clotting). The hemostasis involves the adsorption of blood proteins, followed by platelet adhesion and activation. Many types of eukaryotic cells (to which all blood cells belong to) exhibit force sensibility, especially in the context of cell adhesion. Shear stresses triggered by flowing blood strongly influence all cell adhesion processes on vessel walls in human body or the biomaterials 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, whose number and surface increase with the mechanical tension applied to the cell [2,3]. 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 [4].

Dictyostelium discoideum is often used as a model organism to investigate cellular response to force, so called shear-flow-induced cell motility (SFICM). This simple unicellular eukaryote cell is a genetically and biochemically tractable model organism that is used extensively to study cytoskeleton organization, chemotaxis, cell differentiation and development [5]. The lifestyle of *D. discoideum* consists of two phases: During the vegetative phase, the small (8 μm diameter) ameba feeds upon bacteria and yeast by phagocytosis. Plasma membrane adhesion is therefore directly related to the phagocytic properties of the cell. In the development phase, cell-cell adhesion is more important, and specific contact proteins are expressed [6]. 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 kinds of adhesion proteins [4,7,8]. Mainly the vegetative phase including the phagocytic process is a good model to describe adhesion of human fibroblasts to vessel and biomaterial surfaces.

For *D. discoideum* the detachment is not affected by depolymerisation of the actin or tubulin cytoskeleton [9]. The detachment rate is strongly affected by the presence of an intact actin cytoskeleton and by the cell-substrate adhesion energy [9]. An interpretation of the detachment process is partly possible by an adhesive belt model describing the passive behaviour of the cell edge under external or internal forces [10].

The current work shows the first time application of the SFICM characterization for physical vapour deposited coatings (PVD) grown at room temperature by magnetron sputtering and pulsed

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laser deposition (PLD). Such coatings will be used in future to cover polymer surfaces (e.g. polyurethane) in implants in order to control cell adhesion and to prevent inflammatory reactions (e.g. described in [11]). For this study, titanium nitride (TiN), titanium carbonitride (TiC_xN_y) and diamond-like carbon (DLC) coatings were chosen for the characterization in the radial flow detachment assay.

EXPERIMENTAL

Coating Deposition

Before starting deposition the vacuum chamber was evacuated to pressures below $2x10^{-3}$ Pa. Titanium and carbon targets were used to deposit the about 300 nm thick films on electrolytically polished titanium substrates (grade 2 titanium) at room temperature in argon atmosphere (DLC), nitrogen-argon atmosphere (TiN), or nitrogen-acetylene atmosphere (TiC_xN_y). DLC and TiN coatings were deposited by using DC unbalanced magnetron sputtering, TiC_xN_y by using PLD by a pulsed Nd:YAG laser system operating at 1064 nm [12]. The different carbon content in these coatings was achieved by using 0.5 sccm acetylene ($TiC_xN_y^{(low\ C)}$) and 2.5 sccm acetylene gas flow ($TiC_xN_y^{(high\ C)}$) during the deposition. To provide homogenous film thicknesses over the whole coated surfaces, the substrates were moved with a relative speed of 5.4 cm s^{-1} through the plasma plumes during deposition.

The choice of DLC in this investigation is based on promising results from own investigations and from haemocompatibility tests in literature [13,14]. Delayed clotting time, a trend to inhibition of the platelets and to complementation of convertase activation was found for DLC, standing out against silicon based coatings [13]. In contrast, TiN and TiC_xN_y coatings were selected due to their higher mechanical toughness than DLC. Furthermore, preliminary results [11,12] outlined the application of highly biocompatible titanium and its ceramic alloys as possible new biomaterials for blood contact.

Cell Preparation

Ax-2 cells were grown in axenic medium [15] in agitated suspension (180 rpm). The vegetative cells were harvested during exponential phase at a density of $2 - 4x10^6$ 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). Cell pellets (10^7 cells) were stored on ice and used within 8 h.

Shear Flow Testing

Several techniques are used as setup for studying SFICM [16]. Among them, the applied radial flow detachment assay based on the setup used by Lauffenburger et al. is often used for studying cell adhesion to solid substrates [17-21]. Cells are attached to a horizontal disc manufactured from the biomaterials (coated titanium discs). A second dish (stainless steel) with a centre hole (1.5 mm diameter) is mounted in distance e of 0.1 mm above. Through the hole liquid (21°C) is supplied forming a radial hydrodynamic flow of Sörensen buffer. Thus, the shear flow depends on the distance from the centre hole. When the forces exerted are sufficient, cells are removed from the solid surface and taken away in the laminar bulk flow. After testing (5 and 10 min) the samples are observed in a fluorescence microscope in order to characterize and count the detached cells (detailed description see [20]). 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 (Eq. 1)

$$\sigma(r) = 3*D*\eta / (\pi * r * e^2) \qquad \text{Eq. 1}$$

where D is the flow rate and η is the dynamic viscosity of the Sörensen buffer fluid. For the critical shear stress an amount of 50% detached cells was assumed.

RESULTS AND DISCUSSION

The shear flow tests revealed increasing cell detachment in dependency on the shear stress and time, although the percentage of detached cells after 5 and 10 minutes testing time seems to become similar at high shear stresses (stagnation). Comparing the results for all coatings and the uncoated substrate (Figure 1) reveals an increasing tendency to *D. discoideum* cell adhesion from $TiC_xN_y^{(low\ C)}$ films over the titanium substrate, TiN, $TiC_xN_y^{(high\ C)}$ to DLC films. The critical shear stress values for 50% cell detachment were found to be – as indicated in Figure 1 – 15, 18, 20, 26, and >60 Pa for $TiC_xN_y^{(low\ C)}$, the titanium substrate, TiN, $TiC_xN_y^{(high\ C)}$, and DLC, respectively. Thus, the influence of the carbon content in the films is clearly visible: As higher the carbon content as better the adhesion of the cells to the surface is. Due to feeding of *D. discoideum* cells (in their vegetative phase) by phagocytosis, the high critical shear stress could be in close context to the biocompatibility of carbon due to the lack of damage to the cells during the interaction.

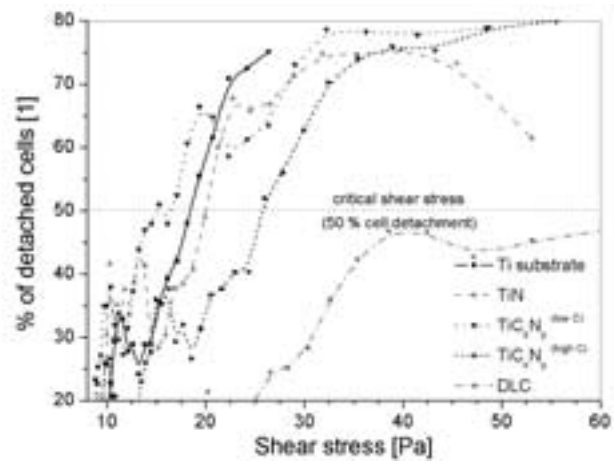

Figure 1: Analyses of the results of the radial shear flow test (10 min. test duration): Amount of detached D. discoideum *cells in dependency on the shear stress and the coating material.*

Besides the tendency to *D. discoideum* adhesion on surfaces with higher density of carbon atoms, a second feature was found during the microscopical analysis of the adhered cells on the coated surfaces: The higher the carbon content on the substrates is the less number of aggregates of cells is formed. Figure 2 shows exemplary images of surfaces with detached cells taken at equal shear stress (4.7 Pa). For DLC a homogenous distribution of the cells is evident, on $TiC_xN_y^{(high\ C)}$ surfaces a very low number of aggregates is found. In contrast, the $TiC_xN_y^{(low\ C)}$ coating is covered by cell aggregates. For the Ti substrate and the TiN coating the probability for forming aggregates is less than for $TiC_xN_y^{(low\ C)}$. The highest tendency to aggregation is generally evident at shear stresses between 4 and 6 Pa. Higher shear stresses lead generally to the increasing tendency of detachment and, thus, only the carbon containing coatings show aggregates at stresses higher than 10 Pa on their surfaces. Especially for TiN and $TiC_xN_y^{(low\ C)}$, the regime of aggregation is also extending down to 2.5 Pa.

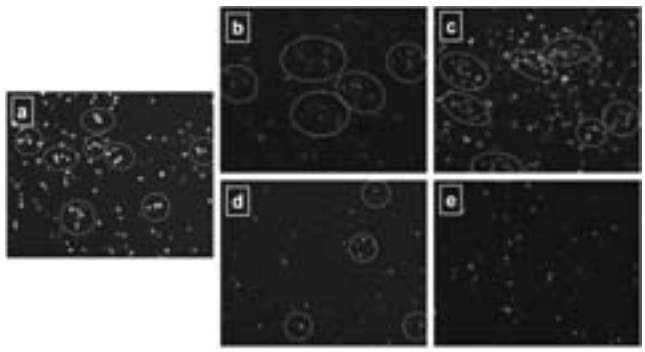

Figure 2: Fluorescence microscope images of the aggregate formation at a shear stress of 4.7 Pa: (a) Ti substrate, (b) TiN, (c) $TiC_xN_y^{(low\ C)}$, (d) $TiC_xN_y^{(high\ C)}$, (e) DL.

A closer investigation of the *D. discoideum* cell structure in the aggregates showed no symptoms of necrosis or apoptosis. Neither an "accidental" (poisoning) or a "programmed" death of the cells could be proved due to the missing of any modifications of the shape in the cells in the aggregates. Additionally, effects caused by the disc preparation and/or coating can be ruled out due to the differences in shear-stress dependency. Thus, the aggregation can only be caused by the normal evolution behaviour the *D. discoideum* cells. A possible theory occurring in the coating could be the following: It's well known that the mechanism to aggregation of the *D. discoideum* amoebae relies on cyclic adenosine monophosphate (cAMP) as a signal molecule. One cell, the founder of the colony, begins to secrete cAMP in response to stress, like the shear stress in the SFICM. Others detect this signal, and respond in two ways: The amoeba moves towards the signal and secretes more cAMP to boost the signal. The effect of this is to relay the signal throughout the nearby population of amoebae and cause inward movement to the area of highest cAMP concentration. Mainly the missing of agglomerates at shear stresses < 2.5 Pa gives a strong argument in favour for a stress-induced mechanism. For verifying this theory, the measurement of the activity of the cAMP signal molecule has to be done in future.

CONCLUSIONS

The radial shear flow test presents a strong tool to investigate the influence of the shear stresses to cells. A widely used model cell type for this test is the *Dictyostelium discoideum* cell in its vegetative phase due to the comparability of its adhesion to human fibroblasts. In the current work, this test was used the first time to investigate the response of *D. discoideum* cells on vacuum coated surfaces to applied shear strain. Magnetron sputtering as well as pulsed laser deposition were used to coat electrolytically polished titanium substrates at room temperature with TiN, DLC, and TiC_xN_y films. The tests showed high critical shear stresses for 50% detachment of cells for coatings with high content of carbon, the highest for pure DLC. Agglomeration of *D. discoideum* cells was found on the carbon-free and low-carbon coatings as well as on the Ti substrates, most probably caused by the stress response of the cells.

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