Control of fibronectin displacement on polymer substrates to influence endothelial cell behaviour

L. RENNER, B. JØRGENSEN, M. MARKOWSKI, K. SALCHERT, C. WERNER, T. POMPE*
Institute of Polymer Research Dresden and The Max Bergmann Center of Biomaterials Dresden, Hohe Str. 6, 01069 Dresden, Germany
E-mail: pompe-tilo@ipfdd.de

Thin layered biomaterial surfaces of maleic anhydride copolymers are provided as a versatile platform for biomaterial applications. The provided comonomers define the character of the surface and its behaviour towards biomolecules and biosystems, such as proteins and cells. The kinetics of adsorption, desorption, and exchange of fibronectin and human serum albumin were investigated on different copolymer surfaces. Two different species of adsorbed proteins were found, a fast and a slow desorbing one. Furthermore, the exchange process depends on the kind of pre-adsorbed protein and the kind of exchange protein, as well as of the hydrophobicity of the copolymer surface. In this context adhesion, proliferation, and differentiation of endothelial cells from the umbilical cord vein onto fibronectin pre-coated surfaces were studied. Strong correlation between fibronectin exchange characteristics and the formation of focal adhesions, reorganisation of fibronectin, and generation of vascular-like structures by the cells was observed.

Introduction

The understanding of protein adsorption phenomena and protein interaction on biomaterial surfaces are fundamental for numerous applications in medicine, biotechnology, and biochemistry. Cell behaviour on biomaterial surfaces is predominantly influenced by the characteristics of the adsorbed protein layer. It is well known, that cell adhesion, proliferation, and differentiation can be controlled by the state of extracellular matrix proteins immobilised on the substrate surface [1, 2].

Fibronectin (FN), a blood plasma protein, was found to be one of the most important extracellular matrix proteins providing adhesion sites for cell attachment. By that, not only simple anchorage of cells to artificial substrates could be improved, but even control of intracellular signals for proliferation and differentiation could be achieved depending on the characteristics of the adsorbed protein and its lateral distribution [1–3]. In the context of the differentiation behaviour of endothelial cells the state of the immobilised extracellular matrix was further shown to be crucial for the fate of cellular development [4].

Displacement of proteins at solid/liquid interfaces are an important step in the interaction of the cells with the adsorbed proteins. Furthermore, it provides a tool for the characterisation of protein interaction with the underlying substrate. Thus, facts are claiming for a deeper understanding in the mechanisms and kinetics of protein displacement in the connection with cell behaviour on artificial substrates. Although a lot of research on protein adsorption was published [5, 6] providing fundamental interaction mechanisms, the knowledge of desorption and exchange of proteins is still puzzling. Protein interaction phenomena on solid substrates are described as irreversible processes [7] as well as dynamic desorption and exchange processes by buffer solutions [8–10] or other protein molecules [11], respectively.

To avoid simple fundamental studies on a singular protein–substrate system and to reveal the direct connection of protein–substrate interaction and cellular behaviour a more complex system of controlled modulation of substrate surface characteristics, investigation of the exchange kinetics of FN and human serum albumin (HSA) on these surfaces, and study of adhesion and differentiation of endothelial cells were used. Besides the importance of FN for cellular adhesion and proliferation HSA was chosen, because it is a major component of blood plasma and by this contributes to a great portion to protein exchange processes.

Materials and methods

Preparation of copolymer surfaces

Poly(octadecene-*alt*-maleic anhydride) (POMA) (Polysciences Inc., Warrington, PA), poly(styrene-*alt*-maleic anhydride) (PSMA), and poly(propene-*alt*-maleic anhydride) (PPMA) (both are special products of Leuna-Werke AG, Germany) films were produced by spin

*Author to whom all correspondence should be addressed.

coating (RC5, Suess Microtec, Garching, Germany) of 0.08%, 0.15%, and 0.1%, respectively, copolymer solutions in tetrahydrofuran (Fluka, Deisenhofen, Germany) on top of coverslips which had been freshly oxidised in a mixture of aqueous solutions of ammonia (Acros Organics, Geel, Belgium) and hydrogen peroxide (Merck, Darmstadt, Germany) and thereafter surface-modified with 3-aminopropyl-dimethylethoxysilane (ABCR, Karlsruhe, Germany). Poly(ethylene-*alt*-maleic anhydride) (PEMA) (Aldrich, Munich, Germany) films were prepared similarly out of 0.15% solution in acetone (Acros Organics, Geel, Belgium). Stable covalent binding of the polymer films to the glass carriers was achieved by annealing at 120 °C for 2 h. The polymer films were thoroughly characterised in respect to water contact angle, film thickness, surface roughness, and chemical composition as published recently [12]. Autoclaving induced hydrolysis of the anhydride moieties to provide a surface exclusively bearing carboxylic acid groups.

Proteins, labelling and displacement process

For fluorescence analysis proteins had to be labelled with a fluorescent dye. Therefore, tetramethylrhodamine isothiocyanate (TRITC) was used. Human serum albumin–TRITC (HSA–TRITC) was used as commercially available (Rockland, Gilbertsville, PA, USA). FN (Roche, Basel, Switzerland) was conjugated by a TRITC-labelling kit (Molecular Probes, Eugene, OR, USA). The amount of labelling was estimated to 3.8 ($\pm$ 1.1) mol dye/mol protein.

The following procedure was performed for desorption and exchange processes: Solutions of TRITC-labelled protein (FN, HSA) at a concentration of 50 µg/ml were exposed to the substrate surface. After an incubation time of 1 h the surface was rinsed three times by phosphate buffer saline (PBS) (Sigma, Steinheim, Germany) (pH = 7.4) and the exchange solution (FN, HSA at 50 µg/ml or pure buffer (PBS)) was added afterwards. The exchange process was monitored by fluorescence confocal laser scanning microscopy (cLSM) (TCS SP, Leica, Bensheim, Germany) for 48 h and high pressure liquid chromatography (HPLC) (Series 1100, Agilent Technologies, Böblingen, Germany) to quantify qualitatively obtained data. For details on the HPLC quantification procedure see elsewhere [13].

Cell culture

Copolymer surfaces were immersed in FN solutions (50 µg/ml) in PBS (Biochrom, Berlin, Germany) for 1 h at room temperature. After FN-incubation the coverslips were washed with PBS prior cell seeding. Human endothelial cells have been gained from umbilical cord vein and grown to confluence in endothelial cell growth medium (ECGM, Promocell, Heidelberg, Germany) containing 2% fetal calf serum. Detailed information on the preparation and analysis of cells have been published recently [14, 15].

Kinetic models for protein exchange

One of the most common approaches [7] for protein adsorption kinetics is a simple first order coverage dependent kinetic known as Langmuir isotherm. The differential equation can be applied to explain the behaviour of observed desorption and exchange processes

$$\frac{\partial \Gamma}{\partial t} = -k\Gamma \tag{1}$$

with Γ and k are surface coverage and desorption time constant, respectively. One possible solution is a sum of two exponential function, involving two types of immobilised proteins, a fast (index A) and a slow desorbing (index B) one, described by

$$\Gamma(t) = \Gamma_A \exp(-k_A t) + \Gamma_B \exp(-k_B t) \tag{2}$$

Results and discussion

The utilised thin film copolymer coatings have been intensively characterised in their surface properties as presented elsewhere [12]. Due to the different comonomers a defined variation of the substrate surface energy is achieved together with otherwise constant surface parameters. The different advancing water contact angles are noted in Table I. Another feature of the thin films is the possibility of an adjustable immobilisation mechanism. Switching between covalent or non-covalent anchorage of proteins was utilised by the reversible hydrolysis of the anhydride moieties.

Adsorption of FN from solutions of 50 µg/ml onto the different copolymer surfaces was found to reveal a similar surface coverage of ca. 400 ng/cm^2 together with no differences in the protein conformation as probed by immunoreactions with monoclonal antibodies to specific FN domains as described elsewhere [15].

The FN interaction strength to the different copolymer surface was analysed by displacement experiments with HSA. In general, protein exchange between FN and HSA could be observed on all surfaces. Depending on the hydrophobicity of the surface, the pre-adsorbed protein, and the exchange protein different degrees of exchange could be measured. Although different amounts of proteins have been exchanged the general behaviour of the displacement process seemed to be similar. This statement is confirmed by Fig. 1 as the displacement kinetics of FN vs. different displacing solutions is shown on a PEMA surface. Two different gradients in the same general scheme could be observed as displayed in Fig. 1. The first gradient is a steep decay, followed by a slow attenuating decay. As explained in the Materials and methods section the desorbed proteins could be accounted to a fast (A) and a slow desorbing species (B), as already introduced by Huetz *et al.* [9]. On the basis of this theoretical approach, we were able to describe the process of desorption and exchange with two

TABLE I Advancing water contact angles

	POMA	PSMA	PEMA	PPMA
Contact angle ($\pm$3°)	100°	75°	41°	38°

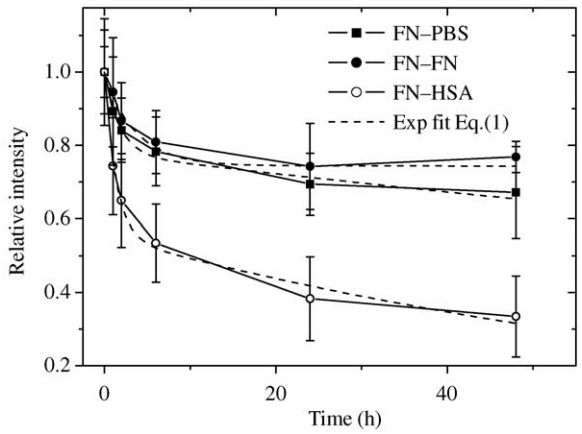

Figure 1 Kinetics of desorption and displacement processes of FN over 48 h on PEMA surface. FN was replaced by solutions of PBS, FN, and HSA.

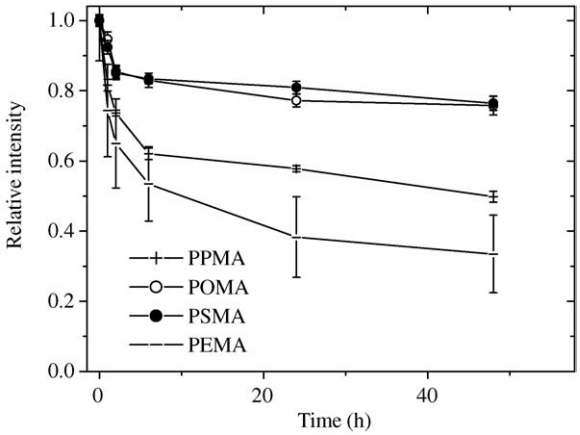

Figure 2 Kinetics of exchange from FN by HSA on different copolymer surfaces.

exponential functions. In a more detailed multivariate regression analysis it could be demonstrated that the calculated kinetic parameters are able to explain protein exchange behaviour on distinct surfaces regarding to their surface properties, which will be published in a forthcoming paper.

We found in general, that the more hydrophilic a surface has been, the faster proteins desorbed or could be exchanged as illustrated in Fig. 2. Another fact is that the calculated desorption rates for the fast desorption step (A) depend on the pre-adsorbed protein and the added exchange solution. For example, if FN is adsorbed, the desorption step is faster for hydrophilic substrates compared to hydrophobic ones if the displacing agents are applied (Fig. 2). Furthermore, by utilising FN as an exchange protein the desorption step is faster in comparison to the displacement with HSA. All this points to a higher interaction strength of FN to hydrophobic surfaces, as established before.

A further parameter of the displacement process is the amount of the two protein species, a fast and slow desorbing one. In nearly all experiments, the hydrophilic surfaces lost more protein than the hydrophobic ones. There is a higher affinity of the utilised proteins towards the hydrophobic coatings, wherefore more protein is bound more strongly. Hence, the surface energy of the substrates is found to be an important parameter concerning the interaction of proteins at solid surfaces.

In progress of the analysis of the displacement processes, we studied adhesion, proliferation, and differentiation of endothelial cells from the umbilical cord vein, in order to reveal their dependence on the different interaction strength of the immobilised FN. The cell behaviour was investigated on pre-coated FN layers, immobilised on the different copolymer surfaces. Formation of focal adhesions by means of phosphotyrosine antibody staining was investigated by fluorescence confocal laser scanning microscopy for time periods up to 3 h. Enhanced formation of focal adhesions was found on the hydrophilic substrates in comparison to hydrophobic substrates. Fig. 3 illustrates this fact. More details of this study and the varying degree of reorganisation of the pre-adsorbed FN are published elsewhere [15].

As a final read-out the differentiation of the endothelial cells into vascular-like structures was analysed on the

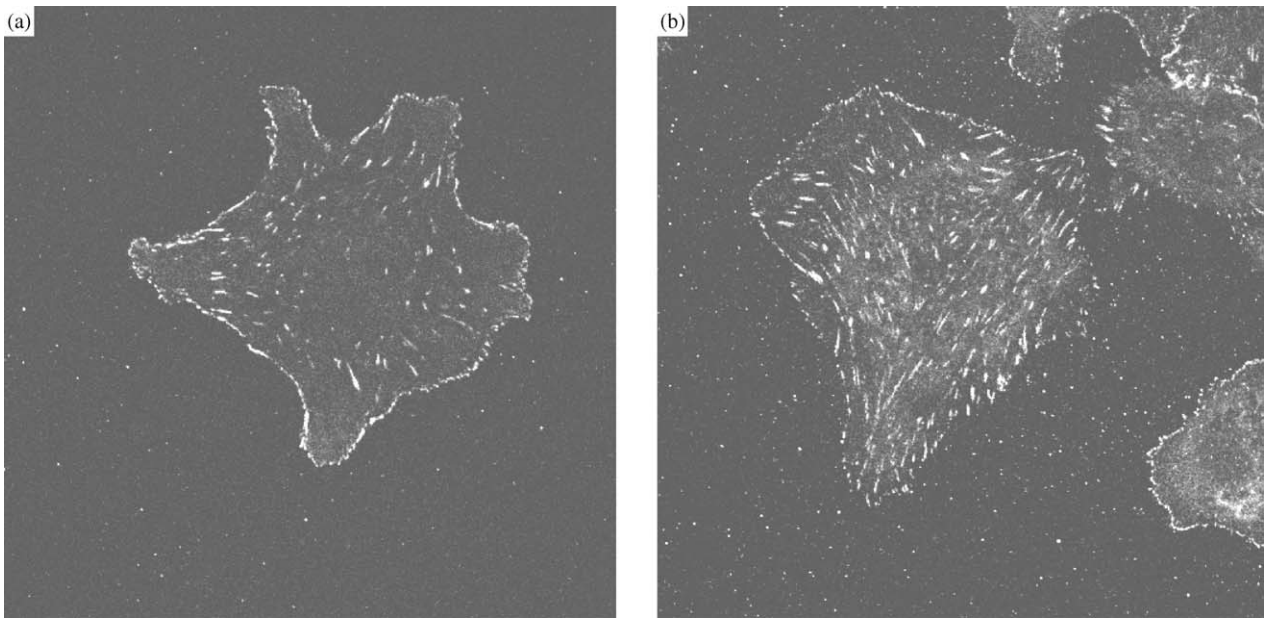

Figure 3 Focal adhesion formation on POMA (a) and PPMA (b) visualised by anti-phosphotyrosine and fluorescence laser scanning microscopy. Image sizes: 125 μm.

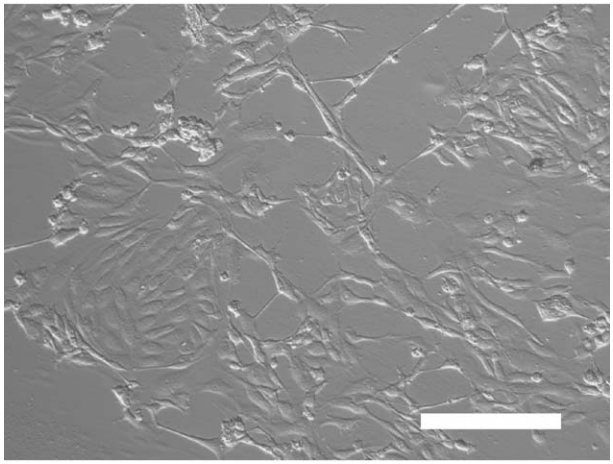

Figure 4 Formation of vascular-like structures of endothelial cell on a PPMA surface. Scale bar: 150 µm.

different copolymer substrates. During the initial phase, the cells grew in a dense monolayer. After a time period of 5 days the cells still grew as monolayers on the hydrophobic surface (POMA). On the hydrophilic surfaces they started a build-up of a vascular-like network after the applied time scale. A typical image is presented in Fig. 4. This could be correlated to an intense 3-D reorganisation of FN on the hydrophilic surfaces in contrast to surface bound less restructured FN network on the hydrophobic surfaces (for more details see [14]).

Conclusions

Desorption and displacement processes are guided by the physicochemical characteristics of the surfaces due to the chemical modulation of the surface energy (hydrophobicity). The study of physisorbed protein layers revealed that bio-functionalised copolymer surfaces are subject to dynamic exchange processes. The more hydrophobic a surface is the higher affinity and binding strength towards FN is developed. The modulated binding strength of FN towards the different maleic anhydride copolymers was found as a key parameter for influencing adhesion and differentiation of endothelial cells. The control of the protein exchange processes on artificial substrates by the modulation of the surface characteristics was revealed to be a crucial point for a successful implementation of various tissue-engineering strategies.

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