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Competitive protein adsorption on micro patterned polymeric biomaterials, and viscoelastic properties of tailor made extracellular matrices

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Abstract

Cell adhesion on biomaterial surfaces and the vitality of anchorage dependent cells is affected by several parameters of an adsorbate layer which is intentionally or spontaneously formed. Surface pre-treatments and several conditioning steps prior and during to the cell/biomaterial contact affect the composition, orientation, quantity and viscoelasticity of the interfacing layer between cells and biomaterial. This work was performed to elucidate the response of cells on two modified biomaterial surfaces based on protein or carbohydrate adsorbates:

(a) Masked UV irradiations opened a simple route to obtain chemically patterned substrates controlling serum protein adsorption and cell adhesion. It is possible to achieve structures of subcellular size and to produce immobilized gradients. In order to examine the protein matrix deposited on these substrates we applied a quartz microbalance technique (QCM-D) capable to extract viscoelastic data in addition to the mass uptake during plasma protein deposition. It was found that the quantity and viscosity of surface bound albumin is lowered when the surface is modified (patterned) by UV exposure. Hence, the UV modification promotes the competitive adsorption of cell adhesion proteins from the media or upon secretion by the cells and yields to the observed cell patterns.
(b) Another tissue engineering technique, using immobilized, modified and/or cross linked hyaluronic acid (HA), an important extra cellular matrix component in vivo, is also examined by QCM-D. Our data demonstrate that HA can be modified by an activation with a carbodiimide, followed by the application of an α,ω-bisamino polyethyleneglycol. The QCM-D data can be interpreted as a stiffening of the HA layer combined with the release of hydration water. Further, the hydration state and the viscoelastic behaviour of surface bound ultrathin HA hydrogels was examined.

Quantification of viscoelastic parameters of thin films of ECM by QCM-D is valuable for the interpretation of durotaxis, describing effects of mechanical substrate parameters on the adhesion and motility of cells.

Keywords: Quartz crystal microgravimetry; QCM-D; Serum protein adsorption; Hyaluronic acid; Viscoelasticity

1. Introduction

The application of a resist free photo patterning technique, described in Welle and Gottwald (2002), allows to create chemical patterns in the micrometer scale on some different polymers, e.g. polystyrene (PS), poly(methylmethacrylate), poly(carbonate)s and others. These modifications of the polymer surfaces exhibit a pronounced effect on cell adhesion. Cell adhesion on native PS is usually negligible, whereas irradiated PS showed an enhanced adhesion of hepatoma cells, fibroblasts (Fig. 1), and some other cell lines. Previous experiments indicated already the important influence of plasma protein adsorption under cell culture conditions on the observed cell adhesion effects. This report describes the application of an advanced quartz crystal microbalance (QCM) method to study the of formation of protein films and the implications for subsequent cell adhesion.

Proteins other than collagen are widely applied in forms of physisorbed or covalently bound (mono) layers to control in vitro and in vivo cell adhesion on biomaterial surfaces, however most of them a not suitable to build three-dimensional scaffolds for tissue engineering. For these applications foams, sheets,

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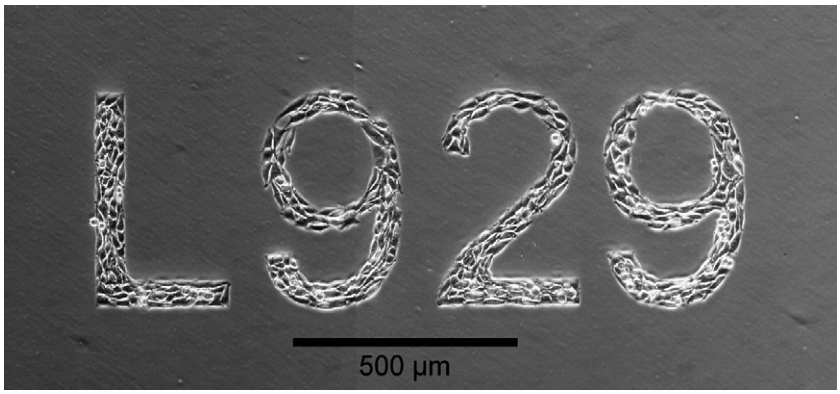

Fig. 1. Phase contrast micrograph of patterned L929 fibroblasts on polystyrene. Thirty minutes UV exposure followed by 4 days standard cell culture.

tubes or other structures from biodegradable polymers, either biomimetic or naturally occurring like hyaluronic acid are the materials of choice and therefore studied by several groups. An example for the application of quartz crystal microgravimetry with dissipation monitoring (QCM-D) to study intrinsic viscoelastic properties of important extra cellular matrix component, hyaluronic acid (HA) is provided.

Since about 25 years ago, QCM found increasing application in the analysis of surface processes in aqueous systems. During the last decade the applications of QCM technology were further expanded by studies of cell adhesion and biofilm formation (Zhou et al., 2000). QCM measurements with additional monitoring of the energy dissipation of the oscillating quartz and adlayers allow time resolved, non-destructive observations of the surface attached mass together with *viscoelastic properties* of, e.g. adsorbed protein films (Höök et al., 1998) or adherent cells (Fredriksson et al., 1998). Especially for applications in biotechnology QCM-D is advantageous as compared to conventional QCM since most of the analysed adsorbates are not sufficiently rigid to be treated according to Sauerbreys equation (Sauerbrey, 1959). Instead, a viscoelastic adsorbate on the quartz crystal has to be regarded as a coupled oscillator for which Δf is not directly proportional to Δm, see Höök et al. (2001).

QCM-D is applied to monitor cell attachment, spreading and subsequent events (Cans et al., 2001). However, due to the complexity of those cellular systems no general approach for the evaluation of QCM-D data exists. The mass of cells attached to the sensor surface is extremely high and cannot be treated as a continuous viscoelastic layer. Therefore, the models used for molecular adsorbates (proteins, lipids, etc.) cannot be applied and measured frequency and dissipation data of cell adhesion experiments can be only empirically related to cell adhesion or spreading.

2. Experimental

Substrate preparation: For QCM-D experiments PS surfaces were freshly prepared by spin coating of the polymer solution (polystyrene, 100 kDa, obtained from Polymer Standards Service, Germany, 1.5% in toluene) onto the quartz crystals supplied by Q-Sense AB, Sweden, resulting in PS layers of 50–75 nm thickness, as verified by ellipsometry. The surface modifications of PS, and other polymers were performed by UV irradiation of the polymer samples in air using a low pressure mercury lamp (Heraeus Noblelight, Germany, NNQ lamp, $\lambda = 185$ nm, quartz tube, 15 W) at 10 cm distance. Conventional Hg high pressure lamps emitting $\lambda > 250$ nm were found to be ineffective.

Cell patterns are produced by using masked irradiations (30 min) of sterile bacteria petri dishes (untreated PS) followed by an inoculation with a cell suspension in FCS containing standard media. Cell suspension was exchanged after 1 h incubation, standard cell culture was continued for several days. This protocol is described in detail in Welle and Gottwald (2002).

A mixture of hyaluronic acid (HA) and chondroitin sulfate (Ref. H1876) from human umbilical cord, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and *N*-hydroxysuccinimide (NHS) were provided by Sigma. α,ω-bisamino polyethyleneglycol, 3.4 kDa, was obtained from Shearwater. Photosensitive derivatives of hyaluronic acid (PhotoHA) were synthesized according to Magnani et al. (2003). Briefly, the introduction of azidoaniline groups to the polysaccharide was performed by EDAC activation. This photosensitive derivative was be applied by spin coating and irradiated with a conventional high pressure mercury lamp ($\lambda > 250$ nm) for cross linking.

Quartz crystal microbalance with dissipation monitoring: in the described experiments the protein adsorption was measured under static conditions consistent with the usual cell culture procedure. The QCM-D instrument supplied by Q-Sense, Sweden, uses the ac output of the damped oscillation of the AT-cut quartz crystal at 5, 15, 25 and 35 MHz to extract the resonance frequencies and energy dissipations. This allows high time resolution and avoids the influences of parasitic contact resistance and capacitance. For data analysis we modelled the protein adsorbate on top of the polymer coated quartz crystal as a viscoelastic solid with a frequency dependent complex shear modulus according to the Voight model, for details see Höök et al. (2001). Using a two layer model with the polymer being the first layer and the protein adsorbate the second was not necessary since all polymer films used in this study can be regarded as totally rigid with high shear moduli as compared to hydrated protein films. Densities and viscosities of all solutions were set equal to the corresponding values of pure water. Incremental fitting of all three parameters d, η, and G, was performed to find a possible time dependence of the viscosity and the shear modulus. Starting values for the curve fitting were taken from Höök et al. (2001): $\eta = 1.5 \times 10^{-3}$ Ns/m^2 and $G = 200 \times 10^3$ Pa, the density, ρ, of all protein adsorbates was fixed to 1.15×10^6 g/m^3 corresponding to a densely packed low hydrated protein film. Film thickness values, d, scales nearly linear with $1/\rho$. Results are presented as average ± standard error of the mean.

3. Results

3.1. Protein adsorbates

The adhesion of L929 fibroblasts from suspensions in cell culture medium is hindered on native PS (background in Fig. 1) and favoured on UV irradiated PS (letters in Fig. 1). This has to be attributed to different compositions of the protein adsorbates

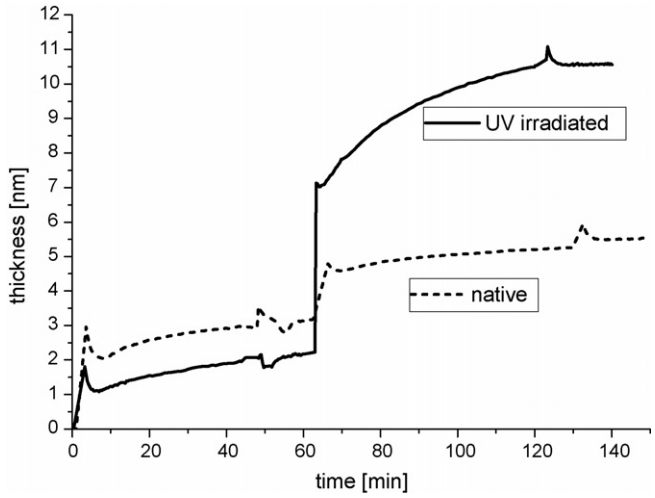

Fig. 2. Protein adsorbate thickness vs. time. Experiment: albumin/FCS adsorption on native PS (dashed line) and UV-modified PS (solid line). Polymer coated quartz crystals were exposed to the following solutions: $t < 15$ min: PBS; $15 < t < 60$ min: 50 μg/ml BSA in PBS; $60 < t < 75$ min: PBS; $75 < t < \sim 135$ min: 1 vol.% FCS in PBS; $t > 135$ min: PBS. Spikes are due to temperature and pressure fluctuations during medium exchange.

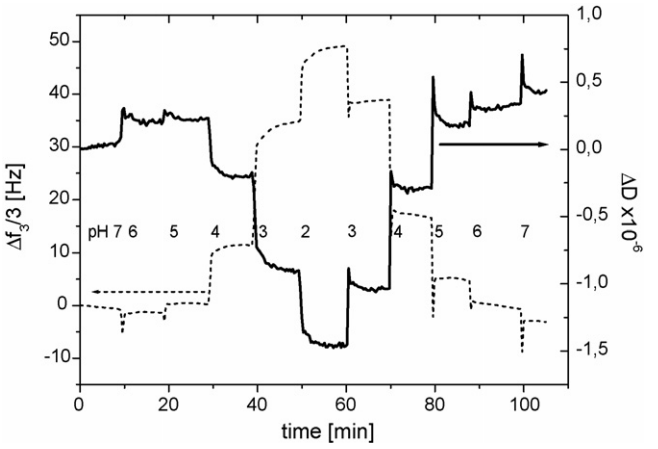

Fig. 3. Changes in the normalized resonance frequency and the dissipation at 15 MHz during the exposure of a PhotoHA layer to different buffer solutions in 10 min intervals.

which are formed on the materials surfaces during incubation in serum containing media. As shown in Fig. 2, protein adsorption on PS is altered by UV modifications of the polymer surfaces. The suppression of cell adhesion on native PS has to be attributed to the high binding of albumin with only slight displacement by other serum proteins as presented in Fig. 2, dashed line. In case of UV irradiated PS the protein adsorption patterns change significantly (Fig. 2, solid line). Bovine serum albumin (BSA) adsorption decreases, whereas the subsequent deposition of proteins from FCS increases. Thus, we conclude that the albumin rich protein adsorbate passivates the surface concerning the deposition of adhesion proteins from cell culture media like fibrinogen or fibronectin and others secreted by anchorage dependent cells themselves. The applied protocol does not allow to select specific cell attractive proteins on irradiated regions. This can be achieved by applying defined cell adhesive proteins like fibronectin or laminin (Welle et al., 2005) instead of FCS. The observed protein adsorption and displacement patterns are in accordance with ELISA experiments (data not shown).

QCM-D is also able to differentiate viscoelastic properties of the albumin adsorbates on native and UV modified PS substrates. Whereas the shear moduli, G, of albumin adsorbates on both substrates are equal ($1100 \pm 140 \times 10^3$ Pa) the shear viscosities, η, apart from the thickness, d, as shown in Fig. 2, differ significantly. On native PS $\eta_{BSA} = 7.6 \pm 1.3 \times 10^{-3}$ Ns/m^2 was found, on irradiated PS $\eta_{BSA} = 4.5 \pm 0.4 \times 10^{-3}$ Ns/m^2 was found.

The hypothesis of changes in BSA film viscosity and shear modulus during adsorption caused by packing effects was verified by incremental fits of all three parameters d, η, and G, permitting time dependent values. After elimination of temperature drift affected data points the incremental fitting resulted in no time dependency of viscosity and shear modulus of the albumin layer during adsorption on irradiated PS. Within 3 min after injection of the albumin solution in PBS 0.7 nm albumin adsorbate thickness (~50% of saturation thickness) is detected having no significant deviations of η and G from the final values after rinsing with phosphate buffered saline.

3.2. Hyaluronic acid layers

We produced coatings of defined thickness from the photo sensitive derivative PhotoHA which is applied by spin coating to the sensor crystal surface and cross linked by exposure to UV of $\lambda > 250$ nm. The irradiation process can be followed by detection of the decrease of N_3-peaks in FT-IR spectroscopy. Using this technique together with photomasks we are able to produce micrometer scales patterns of immobilized PhotoHA hydrogels and sulphonated derivatives (Barbucci et al., 2003). Fig. 3 shows the frequency and dissipation changes of a photo cross linked hyaluronic acid film being exposed to different buffers of identical ionic strength and pH values ranging from 2 to 7. The QCM-D data confirm on the nanometer scale previous rheological experiments.

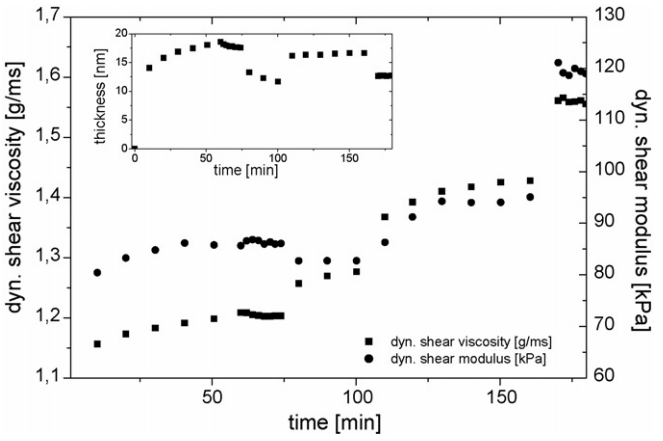

Fig. 4. QCM-D data of in situ cross linking of a hyaluronic acid/chondroitin sulfate coadsorbate after carbodiimide/NHS activation with α,ω-bisamino polyethyleneglycol. For details see text.

Fig. 4 shows the time course of the shear modulus, G, viscosity, η, and thickness, d, (insert) of a hyaluronic acid/chondroitin sulfate coadsorbate on the QCM-D sensor surface: during the first 60 min adsorption takes place, during $60 < t < 75$ min the adsorbate was rinsed with pure phosphate buffered saline, during $75 < t < 100$ min the carboxylic acid groups of the adsorbate were activated by carbodiimide/NHS exposure, directly followed by the application of the α,ω-bisamino polyethyleneglycol ($100 < t < 160$ min), finally the system was rinsed again. The most important observation from suchlike experiments is the increase of the dynamic shear viscosity (Fig. 4, left scale) and the shear modulus (right scale), indicating the stiffening of the film.

4. Discussion

It was shown that the new chemical functionalities obtained by the exposure of polystyrene to deep UV, λ = 185 nm, being mainly carboxylic acid groups, affect the competitive adsorption of several plasma proteins under standard cell culture conditions which, in turn, is reflected in cell adhesion and organization at photo patterned polymer surfaces. If the protein adsorption is performed subsequentially, starting with the smallest, most abundant and cell repellent plasma protein, albumin, the thickness and stiffness of the formed albumin layer differs between native and UV modified PS. The albumin layer on irradiated PS allows the deposition of cell adhesion stimulating proteins present FCS or being applied from single protein solutions (not shown here). The comparison of the adsorbate thickness results obtained from QCM measurements with dissipation monitoring and the Voight model of viscoelasticity with the Sauerbrey equation excluding viscoelasticity of the adsorbates on the surface of the oscillator, demonstrates an underestimation of the thickness in the latter case. However, in the albumin and serum protein adsorption presented in Fig. 2 a nearly constant ratio between both thickness values is obtained.

The closer evaluation of QCM-D data did not indicate changes of viscoelastic properties of the albumin layer during adsorption. Since albumin is a non-spherical and rigid protein this observation can be explained by a reorientation of the adsorbed albumin film from a procumbent to a standing state. Going from a fragmentary to a densely packed film of albumin molecules on the polymer surface is likely to produce major changes in the apparent viscosity and shear modulus of the adsorbate layer.

An important feature of QCM based techniques as compared to optical techniques like surface plasmon resonance, ellipsometry and others is the inherent sensitivity of QCM for hydration, this means water entrapped in an adsorbate layer. Therefore the resonance frequency changes for HA films at different pH values. Decreasing pH from 7 to 2 decreases the resonance frequency since the protonation of the carboxylate functionalities along the hyaluronic acid backbone neutralizes net charge of the immobilized carbohydrate polymer and hence reduces hydration (swelling). This process is reversible and exhibits a pronounced step around the pK_a of the acidic groups in HA. A preliminary data evaluation indicates a shear viscosity change from 1.2–1.6 g/ms at pH 2 to 2.7–3.0 g/ms at pH 7. As shown by Lo et al. (2000), who introduced the term durotaxis, (Richert et al., 2002) and several other groups viscoelastic properties of biomaterials are important for cell adhesion and motility. Lo et al. showed, by using collagen coated polyacrylamide gels of different cross linking densities, that 3T3 fibroblast movements can be controlled by purely physical interactions at the cell substrate interface and the cells preferred a stiffer substrate. Data of (Mendelsohn et al., 2003) also indicate that cross linked films with reduced hydration and swelling ability exhibit a more pronounced cellular adhesion on the films as compared to less cross linked derivatives of comparable composition. Hence, the presented model experiments on hyaluronic acid derivatives and existing knowledge of several biochemical and biophysical parameters influencing cell adhesion suggest several further approaches to control the interactions of cells with artificial surfaces and scaffold materials based on the combination of signalling peptide sequences immobilized in inert biomatrices like hyaluronic acid of defined structure and mechanical properties.

5. Conclusion

The adhesion, spreading and motility of cells on biomaterial surfaces is influenced by chemical and mechanical stimuli (Curtis and Riehle, 2001). Whereas (bio-) chemical information is obtained by some widely applied techniques, most important immunostaining microscopy, micromechanical and structural data of ultrathin adsorbates and biomaterial surfaces often lacks in-depth characterization. In respect thereof, QCM-D helps bridging the gap between materials science and biology and is a promising method to study material properties of cell culture substrates or medical implants, protein and carbohydrate adsorbates and cell adhesion phenomena. We have demonstrated that the QCM-D technology is able to give quantitative results not only on adsorbed mass but also on η and G, even for thin adsorbates of a small protein like albumin. Assigning viscosities and shear moduli to protein films with a few nanometers thickness demands for some imaginativeness. However, our QCM-D data explained to formation of cell patterns on photopatterned polystyrene by differences in the protein adsorbates which are spontaneously formed under cell culture conditions. The quantity and the viscoelasticity of albumin adsorbates formed in the early state of cell culture is decisive for cell attraction or rejection.

Further, the intrinsic sensitivity of QCM-D for entrapped water in adsorbate layers allowed to study hydration effects in hydrogels which are known to influence the response of cells via mechanical instead of chemical interactions.

Hence, quartz crystal microgravimetry with dissipation monitoring is an important complemental tool for the characterization of biomaterials.

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