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Langmuir-Schaefer films of fibronectin as designed biointerfaces for culturing stem cells

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ABSTRACT:

Glycoproteins adsorbing on an implant upon contact with body fluids can affect the biological response *in vitro* and *in vivo* depending on the type and conformation of the adsorbed biomacromolecules. However, this process is poorly characterized and so far not controllable. Here, protein monolayers of high molecular cohesion with defined density are transferred onto polymeric substrates by the Langmuir-Schaefer (LS) technique and were compared with solution deposition (SO) method. It is hypothesized that on polydimethylsiloxane (PDMS), a substrate with poor cell adhesion capacity, the fibronectin (FN) layers generated by the LS and SO methods will differ in their organization, subsequently facilitating differential stem cell adhesion behavior. Indeed, atomic force microscopy visualization and immunofluorescence images indicated that organization of the FN layer immobilized on PDMS was uniform and homogeneous. In contrast, FN deposited by SO method was rather heterogeneous with appearance of structures resembling protein aggregates. Human mesenchymal stem cells showed reduced absolute numbers of adherent cells, and the vinculin expression seemed to be higher and more homogenously distributed after seeding on PDMS equipped with FN by LS in comparison with PDMS equipped with FN by SO. These divergent responses could be attributed to differences in the availability of adhesion molecule ligands such as the Arg-Gly-Asp (RGD) peptide sequence presented at the interface. The LS method allows to control the protein layer characteristics, including the thickness and the

protein orientation or conformation, which can be harnessed to direct stem cell responses to defined outcomes, including migration and differentiation.

Keywords: Langmuir-Schaefer method, protein adsorption, stem cell adhesion, cell culture, fibronectin

INTRODUCTION

The surfaces of biomaterials are instantaneously covered by a layer of (glyco)proteins when exposed to body fluids *in vivo* or to (glyco)protein containing culture media *in vitro*.[1] Cells, therefore interact with the bioactive patterns presented by this adsorbed (glyco)protein layer rather than with the substrate interfaces.[2] Since the (glyco)protein layer formed at the interface is difficult to characterize and its formation is almost uncontrollable, predicting the cellular responses to implant materials and the subsequent tissue integration and regeneration is rather difficult.[3] Immobilization of defined (glyco)protein layers at the substrate´s surface could facilitate the understanding of protein/substrate/cell interactions, which is necessary to modulate cell responses to desired outcomes.

Langmuir-Blodgett technology offers a platform to generate mono- and multi- layers of protein films on solid substrates.[4] When the deposition of the protein monolayer from the air-water interface onto the substrate is carried out by touching the surface horizontally, the technique is referred as Langmuir-Schaefer (LS) deposition (Fig. 1).[5] The characteristics of the protein layer and the individual protein can be manipulated by this method in a very controlled fashion. For example, the LS films of several proteins like glutathione-S-transferase and alkaline phosphatase, have been shown to have enhanced long term stability of secondary structure and functional activity as compared with proteins in solution.[6] This was attributed to the closer molecular packing and two-dimensional order of the LS films. Biosensors having a layered structure of immunoglobulin G with a sublayer of protein A, both prepared by LS method, showed enhanced antigen binding capability due to significant molecular orientation when compared with single IgG LS layer on silanized aluminium surface.[7] Overall, one can presume that protein biointerfaces with designed features such as orientation, organization and secondary structure could be produced by LS method on substrates.

Polydimethylsiloxane (PDMS) is widely applied as soft implant material, for example as breast implants and artificial larynx. The clinical outcome suggests significant issues concerning the *in vivo* compatibility of PDMS.[8] Detrimental outcomes arise due to undesired activation of immune cells like macrophages.[9] Mesenchymal stem cells (MSCs) can directly participate in tissue regeneration either by their self-renewing and multipotent differentiation capacity or by their immunomodulatory properties directing macrophages towards a wound healing phenotype.[10, 11] Therefore, combination of biomaterials with MSCs, is a promising approach to direct systemic tissue and cell responses towards beneficial clinical outcomes. However, this requires the adhesion

of MSCs onto polymeric substrates like PDMS, which does not support cell adherence and often needs additional coatings of growth factors or extracellular matrix (ECM) glycoproteins.[12] Fibronectin (FN), an important ECM glycoprotein is widely used for coating substrates. It contains a relevant sequence controlling cellular adhesion Arg-Gly-Asp (RGD) and other regions, such as Pro-His-Ser-Arg-Asn (PHSRN) acting in synergy to RGD, promoting cell attachment via integrin binding.[13]

Here, the surface of medical grade PDMS was equipped with FN by two different methods. FN was immobilized on to PDMS either by LS or by solution deposition (SO). From the two protein deposition techniques, one could expect largely different (glyco)protein layer characteristics at the interface. It is common knowledge that SO of (glyco)proteins onto substrates often leads to inhomogeneous coatings including the possibility of protein aggregation or multilayers depending on the experimental conditions.[14] Whereas in the case of LS method, a protein monolayer of defined density is transferred onto the substrate. It is hypothesized that the stem cell adhesion on PDMS immobilized with FN by LS and SO methods will be different due to differences in the (glyco)protein layer presented at the interface. The protein monolayer behavior of FN at the air-water interface was investigated. The conditions for successful FN monolayer transfer were determined. Finally, adipose derived human mesenchymal stem cells (hAD-MSCs) were seeded for 24h on PDMS equipped with FN by LS and SO immobilization methods. The stem cell adhesion behavior on these surfaces was observed by visualizing the focal adhesion and cytoskeleton formation. Bare PDMS served as negative control.

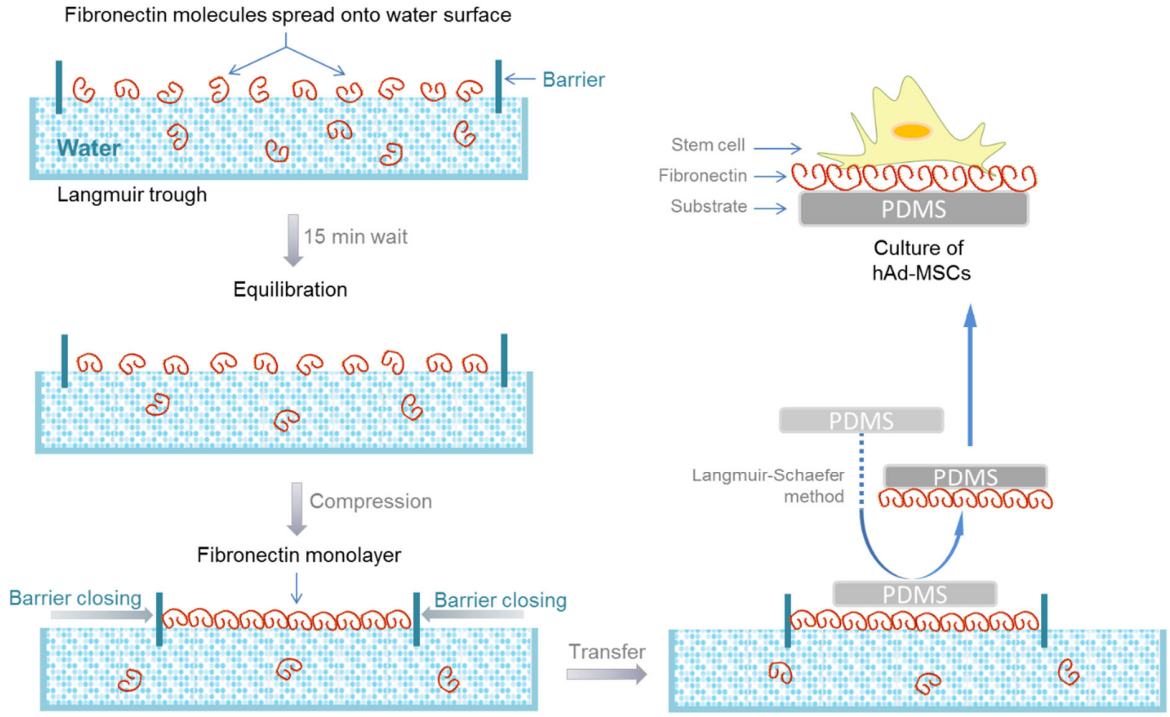

Figure 1. Steps involved in Langmuir-Schaefer method of immobilizing (glyco)proteins on substrates for the investigation of cell behavior.

EXPERIMENTAL SECTION

Materials

For the protein immobilization experiments, medical grade PDMS foil with 1mm thickness (REF ID: BM 121010; Bess Medizintechnik GmbH, Germany), which was synthesized by chemical crosslinking via addition reaction using a platinum catalyst at high temperature (manufacturer's information) was used as substrate. The PDMS foil showed a dynamic contact angle $\theta_{advancing}$ of 103.9 ° ± 0.6 ° (captive bubble method, DSA 100; Krüss, Germany) and a roughness R_q of 14 ± 2 nm determined by optical profilometry.[9] The samples were cut to fit into standard 24-well cell culture plates from Corning® (Dow Corning GmbH, Germany). FN from human plasma (Sigma Aldrich, Germany or BIOPUR-AG, Switzerland) was obtained as a lyophilized powder and was dissolved in phosphate buffer saline (PBS; Biochrom AG, Germany) to get the desired stock concentrations. The concentration of protein solution prepared was quantified using a UV-Vis spectrophotometer Cary 50 (Agilient systems, Netherlands) by measuring absorbance at 280 nm.

Langmuir monolayer experiments of FN

A medium size Langmuir trough (KSV NIMA, Finland) was placed in a cleanroom facility with monitoring system (BRIEM, Germany). The trough was placed on an active vibration isolation system (Halcyonics Variobasic 40, Accurion, Germany). Surface pressure π was measured with a calibrated sensor located equidistant between the barriers using the Wilhelmy technique. The trough and barriers were thoroughly cleaned with sterile deionized water prior to each experiment. The FN was spread at a subphase with a pH of 5.7, which is in the range of isoelectric point of FN (5.5 and 6.2). This condition reduces the solubility to the subphase.[15] The FN spreading solutions had concentrations of 1 or 0.5 mg·mL^{-1} diluted in PBS. The spreading volumes were 100 or 200 or 500 µL applied dropwise on the air-water interface using a microsyringe (Hamilton Co., USA). After an equilibration period of 15 minutes, the Langmuir monolayers were compressed at a constant compression rate of 10 mm·min^{-1}. The surface pressure was recorded as a function of the mean molecular area at room temperature 25 ± 0.5 ºC.

Immobilization of FN on PDMS by LS and SO methods

In order to identify the surface pressure, at which successful transfer can be performed, a quantity called compressibility modulus (K) is calculated using the following equation:

$$K = -a \left[\frac{\delta\pi}{\delta a}\right]_T$$

where a is the molecular area (Å^2·molecule^{-1}), π is the surface pressure (mN·m^{-1}), and T is the temperature (ºC).

For the LS film preparation, the protein film was compressed to a defined surface pressure at the air-water interface, and then the layer was transferred by horizontally touching the protein film

with the substrate. In this work, the FN LS transfer was carried out at a surface pressure of 5 $mN \cdot m^{-1}$ as will be discussed in the results section.
For the solution deposition, the PDMS films were placed in 24-well plate and were incubated overnight at 37 °C with 300 µL of 5 $\mu g \cdot mL^{-1}$ FN solutions.
The protein films deposited on PDMS were generally prepared a day before cell seeding and were dried at 4 ºC overnight.

Atomic force microscopy (AFM) of FN films on PDMS

Imaging of surface topography was carried out by an atomic force microscopy (AFM; MFP-3D, Asylum Research, USA) in air. The scanning was conducted with OMCL-AC200TS-R3 silicon cantilevers (Olympus, Japan) having a typical spring constant of 9 $N \cdot m^{-1}$.

Immunofluorescence (IF) staining of FN immobilized on PDMS

For FN detection on PDMS, the samples were first fixed with 4% (w/v) paraformaldehyde (Merck, Germany) diluted in PBS and non-specific sites were blocked using blocking buffer composed of 3% (w/v) bovine serum albumin (Sigma Aldrich, Germany) in PBS. FN was visualized using a mouse anti-human fibronectin antibody (Abcam, Germany; 1 : 400 dilution in blocking buffer) and a goat anti-mouse immunoglobulin G conjugated with Alexa Fluor® 488 (Invitrogen, Germany; 1 : 1000 dilution in blocking buffer). A confocal laser scanning microscope (LSM 510, META, Carl Zeiss, Germany) was used to observe the stained samples.

Human adipose derived mesenchymal stem cells (hAD-MSCs) culture

The hAD-MSCs were isolated from human adipose tissue obtained by abdominal liposuction from the donor after informed consent (No.: EA2/127/07; Ethics Committee of the Charité-Universitätsmedizin Berlin, approval from 17.10.2008) as previously described.[16] In brief, the fat tissue was first enzymatically dissociated for 60 minutes at 37 °C using the collagenase NB4® (Serva GmbH, Germany). The digested solution was passed through a 100 µm cell strainer to remove the undissociated tissue. The obtained cells were washed and cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. After 2 days, the non-attached cells were aspirated and the attached cells were maintained in the stem cell culture medium (MSCGM™, Lonza, USA). The medium was changed every three days and cell passage was performed at a ratio of 1:3 when the cells reached ~ 90% confluence.

Visualization of focal adhesion and cytoskeleton formation of hAD-MSCs

The cells were seeded at a density of 1 x 10^4 cells/well on PDMS modified with FN by LS and SO methods and bare PDMS surface for 24 h.

To visualize the focal adhesion and F-actin cytoskeleton, the cells were fixed, permeabilized, and blocked by using Image-iT® Fixation/Permeabilization Kit (Life Technologies, Germany). Vinculin was detected with monoclonal anti-vinculin antibody (Millipore, Germany) and Alexa Fluor® 488 labeled anti-IgG antibody (Invitrogen, Germany). F-actin was detected with phalloidin

using ActinRed™ 555 ReadyProbes® (Invitrogen, Germany) and the nuclei were stained with 4',6-diamindino-2-phenylindole (DAPI) by using NucBlue® Fixed Cell ReadyProbes® (Invitrogen, Germany) using manufacturer's protocol, and stained cells were observed using the confocal microscope.

Statistics

Statistical analysis and graphical representation were performed using GraphPad Prism version 6.02 for Windows (GraphPad Software, La Jolla, USA). One-Way Anova followed by the Turkeys multiple comparisons test was used to statistically compare the mean of every experimental group with mean of every other group.

RESULTS AND DISCUSSION:

Surface pressure-molecular area isotherms of FN at the air-water interface

The spreading methodology and the amount spread are important factors in the generation of protein monolayers at the air-water interface because proteins have the propensity to go to the subphase as well. To determine the optimal spreading amounts, FN was spread dropwise on the air-water interface at different protein amounts by spreading 100 µL and 200 µL of 1 $mg \cdot mL^{-1}$ FN solutions (total protein amount: 0.1 mg and 0.2 mg, respectively) (Fig. 2a). As expected, the π-*A* isotherm is more expanded when the protein amount is increased, as a consequence of more molecules remaining at the interface.

The surface loading of proteins can be enhanced by increasing the concentration of the spreading solution, even though the total protein amount spread remains constant. This is shown in (Fig. 2b), where the isotherm is expanded for the 1 $mg \cdot mL^{-1}$ FN solution as compared with 0.5 $mg \cdot mL^{-1}$ FN solution. This effect is caused by the Marangoni effect described in detail for bovine serum albumin (BSA).[17] If the protein concentration is sufficient, due to spreading from the bulk of the droplets, then protein films can be formed. In the case of diluted protein solutions spreading, dissolution of the protein to the subphase dominates and only a textured adsorbed layer forms over time. For rather expensive proteins including fibronectin or vitronectin, the strategy of using higher concentration of protein solutions to enhance their delivery to the air-water interface could be useful.

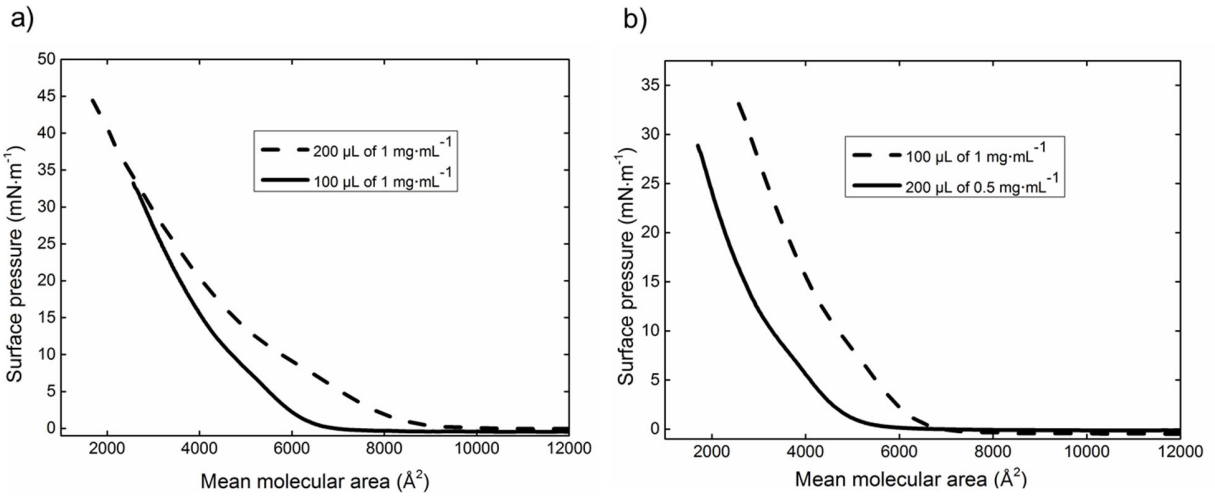

Figure 2. Surface pressure – molecular area isotherms of FN: a) influence of total protein amount spread; b) influence of protein concentration

There is limited information available about the organization of FN at the air-water interface from literature. In a previous work, investigating the plasma FN monolayers at the air-water interface, it was shown that the surface densities at the highest pressures indicate extensive folding.[15] However, to have a rough estimate, the area occupied by a single FN molecule was calculated, assuming that the FN of cylindrical shape is lying with the diameter parallel to the plane of the spreading area (length of diameter = 300 Å, height = 20 Å).[18] The area occupied by one FN molecule at the air-water interface would be 90000 $Å^2$ (calculated value is the area occupied by a square of length 300 Å). Considering the isotherm, when 100 µL of 1 $mg \cdot mL^{-1}$ of FN was spread, the first onset of increase in surface pressure occurs at 7000 $Å^2$ (experimental value) (Fig. 2a) and this calculation is performed by assuming all the molecules are on the interface. One can estimate from the ratio of the calculated value and the experimental value that roughly only about 7.8% of the molecules remain at the air-water interface. Therefore, it is clear from this estimation that for each protein, the optimum amount to be spread for achieving a typical surface pressure – molecular area isotherm should be empirically determined. It will depend on trough volume, protein characteristics, and subphase conditions.

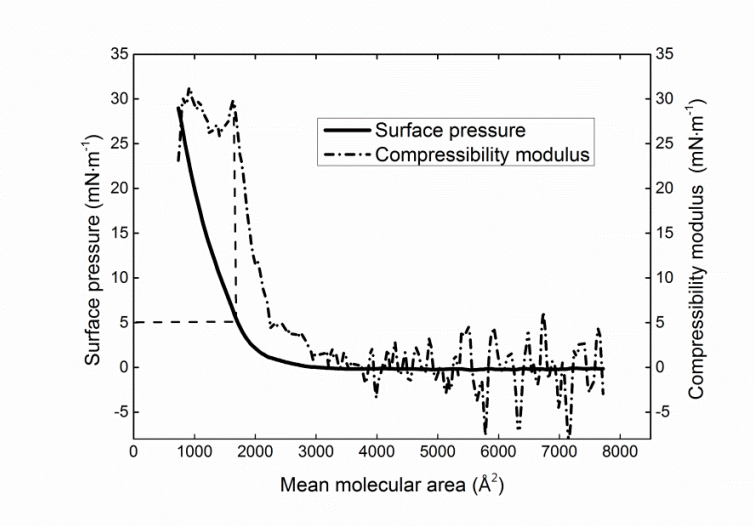

Figure 3. Surface pressure (solid line) and compressibility modulus (dotted line) as a function of molecular area for a fibronectin isotherm (500 µL of 0.46 mg·mL^{-1} FN). The dashed line is a guide to represent the surface pressure corresponding to the maximum of the compressibility modulus.

In a previous work, successful protein monolayer transfers onto solid substrates were shown to be possible at regions of surface pressure corresponding to the maxima of the compressibility modulus values, where the monolayer is highly condensed and packed.[19] The surface pressure and compressibility modulus with respect to the molecular area for a particular FN isotherm (500 µL of 0.46 mg·mL^{-1}) is depicted in Fig. 3. Two maxima can be observed in compressibility modulus - molecular area graph. Accordingly, transfer was performed at a surface pressure of 5 mN·m^{-1} corresponding to the first maximum. The second maximum occurs at limiting trough areas, at which transfer is not practical. However, by spreading higher amounts of fibronectin, it might be possible to shift this isotherm to larger trough areas, which can later be transferred. The protein presentation such as cell binding sequence RGD could be different in the surface pressures corresponding to the different maxima of compressibility modulus and could be of relevance to investigate cell responses.

Surface morphology and organization of FN immobilized on PDMS

In order to visualize the distribution of FN on PDMS, AFM investigations were performed in dry state and bare PDMS served as control (Fig. 4 a, b). In parallel, immunofluorescence (IF) stainings were also carried out. AFM micrographs and IF images indicate that the FN layer adsorbed on PDMS by LS method is rather uniform and homogeneous when compared with that prepared by the SO method (Fig. 4 c, d, g). Furthermore, one can observe structures that could indicate aggregates or multilayers on the PDMS immobilized with FN by SO method (Fig. 4 e, f, h).

In the LS method, firstly a FN protein network assembles at the air-water interface and after compression to high density; this protein layer is transferred onto PDMS. In solution deposition, if the protein solution concentration is high enough to saturate the surface, finite number of molecules

can occupy the first layer due to steric jamming. Thereafter, depending on the substrate and solution conditions multilayers can be formed. Due to this it appears that the SO method is more random and has little control over the protein organization, which can explain the differences for the two protein deposition methods.

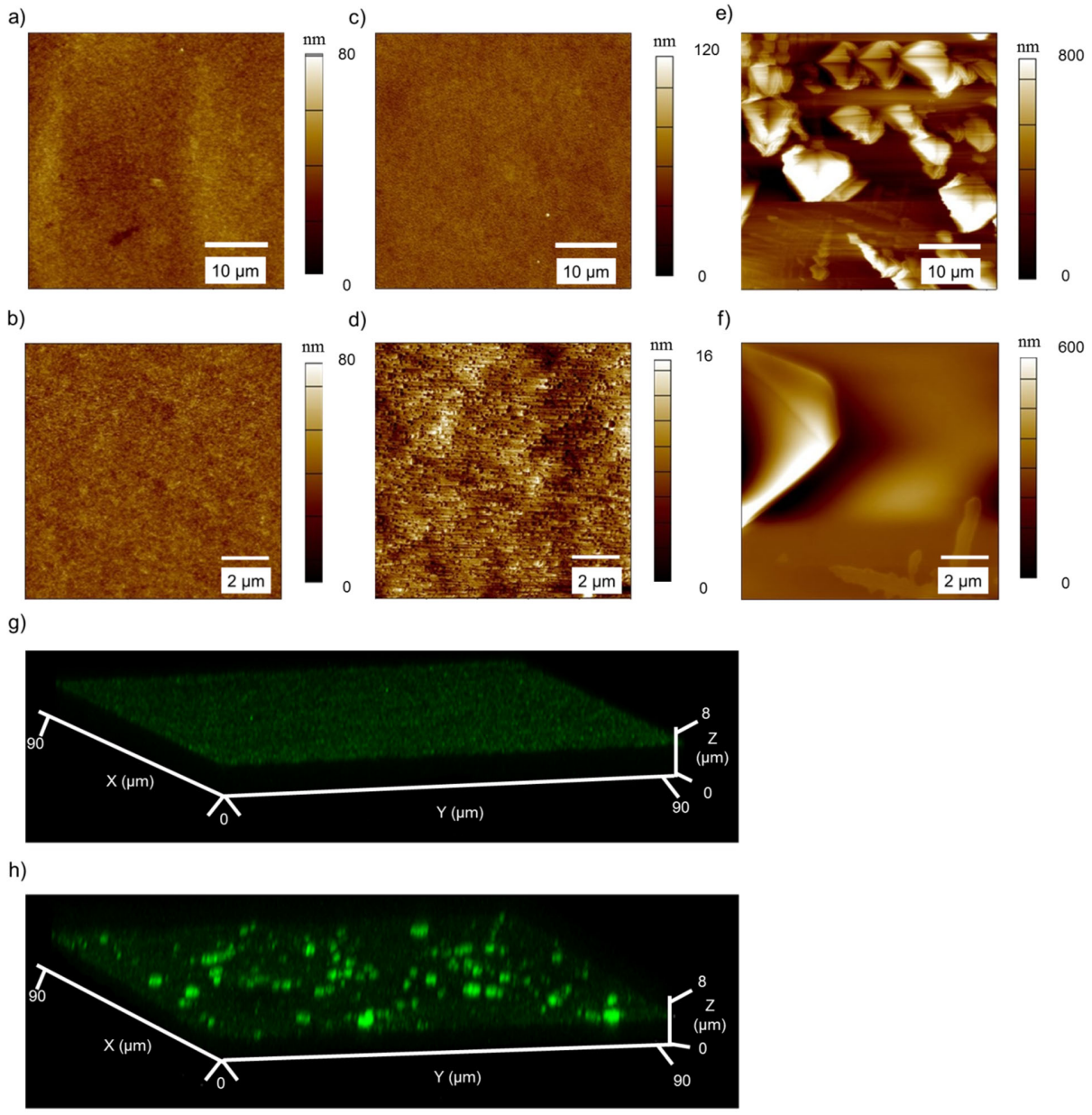

Figure 4. AFM micrographs and IF images of FN adsorbed on PDMS by LS and SO methods. AFM images: a) and b) - bare PDMS; c) and d) - FN LS on PDMS; e) and f) - FN SO on PDMS. The first row images represent scan sizes of 40 µm x 40 µm, while the second row images represent sizes of 10 µm × 10 µm. IF images: g) FN LS on PDMS and h) FN SO on PDMS.

Stem cell responses to PDMS immobilized with FN

In order to evaluate the influence of the FN layers deposited on PDMS, on the focal adhesion and on cytoskeleton formation of hAD-MSCs, the cells were stained with vinculin and F-actin, respectively. DAPI was used to visualize the nuclei. Divergent hAD-MSCs adhesion behavior in response to the PDMS immobilized with FN by the LS and SO methods was observed (Fig. 5). The number of cells adherent on PDMS equipped with FN by SO method was significantly higher than on PDMS modified by LS method. As expected, on bare PDMS very few cells could adhere (Fig. 5 a, b). A similar effect was shown previously, when fibroblasts were cultured for 24h on LS and SO films of various extracellular matrix (ECM) proteins such as collagen, fibronectin and vitronectin. Cell adherence on SO films was found to be higher than on LS films as in the present work.[20]

Furthermore, stem cells exhibited elevated and more homogeneous vinculin expression on PDMS immobilized with FN by LS method than on the PDMS surface equipped with FN by SO method. Additionally, one could also observe slightly stronger and more oriented F-actin fibers in the latter case compared with the former. For bare PDMS, the few adherent cells had weak vinculin expression, but similar stress fiber formation as PDMS equipped with FN by LS method. (Fig. 5 c)

The FN layer immobilized on PDMS by LS is more uniform and therefore, one could expect a more homogeneous presentation of the FN modules containing RGD sequences to the cells. Due to the appearance of protein aggregates on PDMS equipped with FN by SO method, the FN modules containing RGD are expected to be more clustered. The molecular information is transmitted from the external environment to the cell interior by the dynamic interconnections between the transmembrane integrins and the cytoskeleton mediated by crucial focal adhesion proteins such as vinculin.[21] In the present work, the differences in the cell adhesion behavior for the PDMS equipped with FN by the two methods could be due to the differences of the RGD availability and distribution leading to differential integrin engagement on the cell membrane as well as different intracellular actin cytoskeleton organization. The elevated expression of vinculin has been implicated to cause stronger adhesion and lower migration in several cell types on two-dimensional planar substrates.[21] Vinculin expression on PDMS immobilized with FN by LS method was higher relative to SO method implying that the cellular migration might be different on PMDS equipped with FN by the two methods, which will be investigated in further studies.

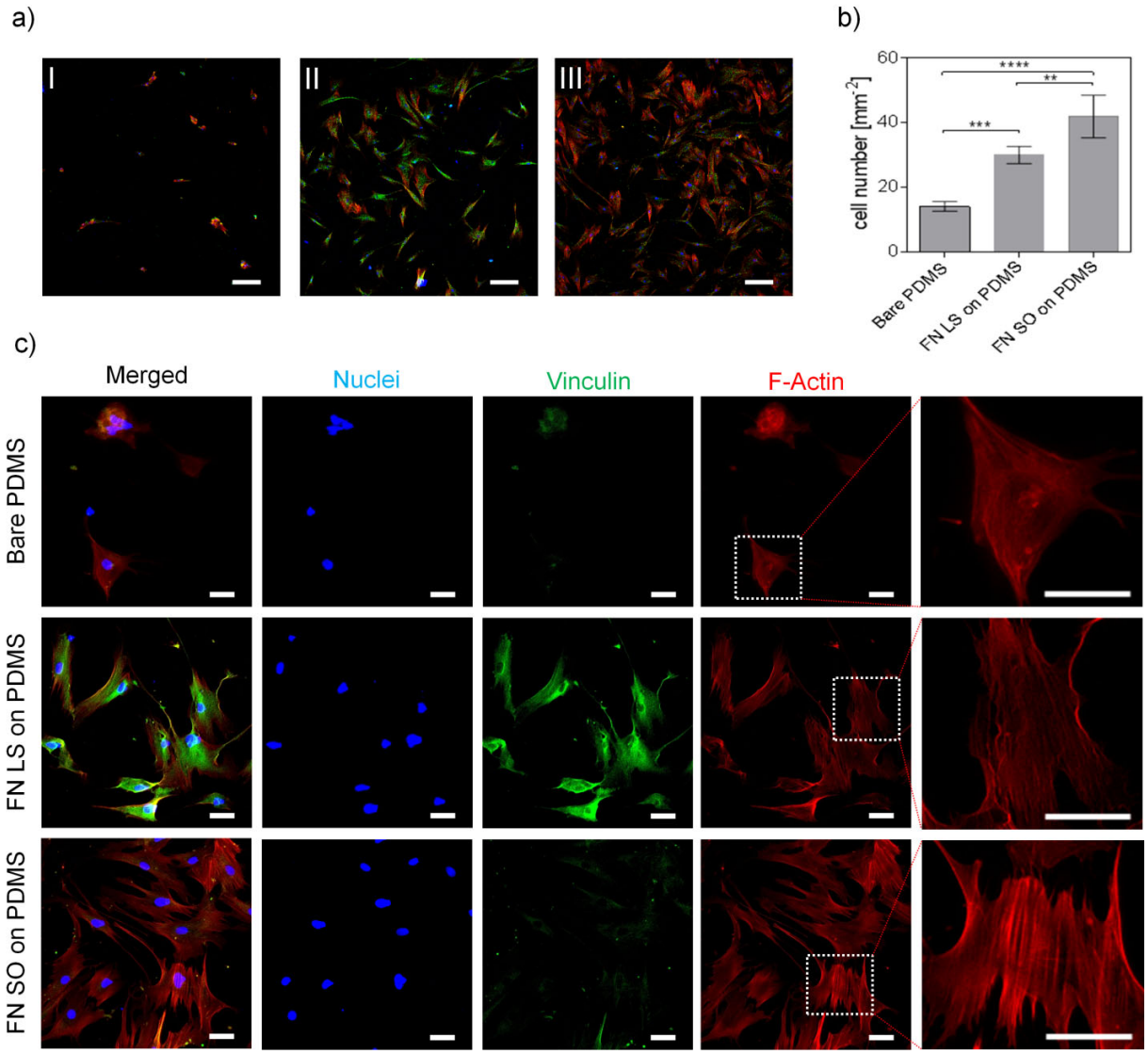

Figure 5. Focal adhesion and cytoskeleton structure of hAD-MSCs after 24 hour culture on PDMS with and without deposited FN. a) Representative confocal laser scanning images of cells seeded on bare PDMS (I), FN LS on PDMS (II) and FN SO on PDMS (III) using fivefold magnification objective. (green: vinculin; red: F-actin; blue: nuclei; bar = 200 µm). b) Quantification of the cell adherence on PDMS equipped with and without FN. Data represented are average cell numbers of four fields of view obtained in two independent experiments (mean ± standard deviation; **** for $p<0.0001$; *** for $p<0.001$). c) Confocal laser scanning images of cells seeded on bare PDMS (top row), FN LS on PDMS (middle row) and FN SO on PDMS (bottom row) using 20 fold objective. The images in each row are represented in the following order: merged channel, nuclei channel, vinculin channel, F-actin channel and the zoom of an area of the F-actin image indicated by the white box. (green: vinculin; red: F-actin; blue: nuclei; bar = 50 µm).

CONCLUSIONS

The bioactivity of (glyco)proteins presented at the biomaterial-cell interfaces is a key aspect regulating cellular fate and functions. Using the LS method, an inert PDMS substrate was equipped with a defined FN layer and for comparison; FN was deposited by SO method on PDMS. Prior to the LS deposition, FN monolayer behavior at the air-water interface was investigated and the optimal, experimental conditions for LS transfer were obtained. Additionally, it was found that efficient surface loading of proteins at the air-water interface was possible, when higher concentration solutions with equivalent total FN amount were spread.

AFM micrographs and IF images showed that a homogeneous coating of FN was achieved on PDMS by LS method, while those substrates modified by SO method were inhomogeneous with some structures that could be aggregates or multilayers. Lower number of adherent stem cells, more homogeneous and elevated vinculin expression and weaker stress fiber formation was found on PDMS equipped with FN by LS method in comparison to SO method. This result could be explained by the RGD accessibility and the subsequent integrin/RGD interaction. RGD ligands are most likely more uniformly distributed on PDMS equipped with FN by LS method, while high clustering of RGD ligands can be expected on PDMS on which FN was deposited by the SO method. This assumption will be investigated in future studies.

The data presented here indicate that stem cell functions such as adherence can be controlled by employing the LS method to immobilize ECM glycoproteins onto substrates. Conclusively, the LS method offers the possibility to control aspects of (glyco)protein features presented to the cells at the interface such as molecular density and conformation. Such biointerfaces could be transferred onto a variety of substrates and represent new ways to functionalize substrates and thereby, modulating the cell behavior in the desired direction.

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