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Self-stabilized fibronectin films at the air/water interface

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ABSTRACT

Fibronectin (FN) is a mediator molecule, which can connect cell receptors to the extracellular matrix (ECM) in tissues. This function is highly desirable for biomaterial surfaces in order to support cell adhesion. Controlling the fibronectin adsorption profile on substrates is challenging because of possible conformational changes after deposition, or due to displacement by secondary proteins from the culture medium. Here, we aim to develop a method to realize self-stabilized ECM glycoprotein layers with preserved native secondary structure on substrates. Our concept is the assembly of FN layers at the air-water (A-W) interface by spreading FN solution as droplets on the interface and transfer of the layer by the Langmuir-Schäfer (LS) method onto a substrate. It is hypothesized that 2D confinement and high local concentration at A-W interface supports FN self-interlinking to form cohesive films. Rising surface pressure with time, plateauing at 10.5 $mN \cdot m^{-1}$ (after 10 hrs), indicated that FN was self-assembling at the A-W interface. *In situ* polarization-modulation infrared reflection absorption spectroscopy of the layer revealed that FN maintained its native anti-parallel β-sheet structure after adsorption at the A-W interface. FN self-interlinking and elasticity was shown by the increase in elastic modulus and loss modulus with time using interfacial rheology. A network-like structure of FN films formed at the A-W interface was confirmed by atomic force microscopy after LS transfer onto Si-wafer. FN films consisted of native, globular FN molecules self-stabilized by intermolecular interactions at the A-W interface. Therefore, the facile FN self-stabilized network-like films with native anti-parallel *β*-sheet structure produced here, could serve as stable ECM protein coatings to enhance cell attachment on *in vitro* cell culture substrates and planar implant materials.

INTRODUCTION

The biological response to biomaterial implants e.g. polymeric substrates is decided by the inherent material characteristics such as elasticity, topography and by the glycoprotein layer rapidly adsorbed on the materials exposed to body fluids *in vivo* or to the culture media *in vitro*[1, 2]. Cells respond to the bioactive motifs presented by the adsorbed glycoprotein layer rather than material surface. Most artificial materials need extracellular matrix (ECM) glycoprotein coatings or covalently attached peptide motifs to support optimal cellular adhesion essential for cell culture applications [3]. However, depending on the surface characteristics such as wettability and surface chemistry, adsorption of ECM glycoproteins on material substrates can result in insufficient coverage or protein conformational changes [4]. Protein-substrate and protein-protein interactions determine the formation of glycoprotein layers by adsorption from solution and these layers are subject to displacement by other proteins from the cell culture medium [4]. Proteins on adsorption from solution on most hydrophobic surfaces lose their native structure and this can adversely affect cell behavior [5]. Thus, it is our aim to realize cohesive, stable glycoprotein films with native secondary structure on substrates as coatings in order to achieve reliable cell responses. These protein coatings should be self-interlinked to resist displacement by other proteins and the influence of substrate should be minimized on the formation of glycoprotein layers at the interface by enhancing protein self-interactions.

Fibronectin (FN) is an adhesive glycoprotein of importance in the development of vertebrates including embryogenesis, wound healing and regeneration [6]. It is a large dimeric protein (488kDa) with an isoelectric point of (5.5-5.7) and with a secondary structure consisting mainly of anti-parallel β-sheets [7]. A large number of integrins α3β1, α4β1, α4β7, α5β1, α8β1, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, αIIββ3 have been reported to be associated with binding to the adhesive motifs on FN [8]. This glycoprotein mediates cell attachment via multiple peptide sequence domains (RGD, RGDS, LDV, REDV) [9]. FN also has binding sites for other ECM components such as collagens, proteoglycans and other biomolecules like fibrinogen and fibrin [8]. FN self-assembly was achieved *in vitro* either by hydrophobic forces on micro-patterned pillars, or by electrostatic interactions by adsorbing FN onto polystyrene sulfonate (PSS) substrates [10, 11]. These approaches are limited to substrates with specific surface chemistries or morphologies. Here, we aim for a method that can be applied independent of substrate morphology or chemistry.

An established method to achieve defined glycoprotein layers on substrates is the Langmuir technique. ECM glycoproteins such as FN and collagens, due to their inherent amphiphilicity and large molecular weight (> 1000 kDa), can adsorb at the air-water (A-W) interface upon spreading from concentrated solutions and form films of monomolecular thickness [12, 13].

Further, by controlling the pH and ionic concentration of salts, protein adsorption at A-W interface is promoted [13]. This technique is suggested as a method for fabricating defined and stabilized FN layers and transferring them to different substrates is suggested. In this approach, 2D confinement and high local concentration at the A-W interface shall enhance the FN-FN association. We assemble FN on a sub-phase with pH = 2, to promote the possible unfolding of FN and to aid the self-assembly. FN unfolding from the native compact form is an important activating step for the *in vitro* self-assembly [10]. The conformation of FN was shown to be extended from the compact native form when the pH of FN adsorbing solution on Au-edge coated silver nano-plates was altered from pH = 8 to pH = 4 [14]. It is hypothesized that FN can self-assemble to form interconnected films at the A-W interface. This cohesive layer can be effectively transferred onto solid substrates. To realize the concept, the adsorption of FN layers on a sub-phase with pH = 2 was followed using surface pressure measurements. The evolution of the morphology of the layers on the micrometer scale was followed by Brewster angle microscopy (BAM). In the next step, adsorption experiments in combination with *in situ* polarization-modulation infrared reflection absorption spectroscopy (PM-IRRAS) were carried out to understand the conformation of the FN molecules in the adsorbed layer. Further adsorption experiments were conducted in an interfacial rheology cell. The time-dependent resistance of the adsorption layers to shear deformation indicated the increasing strength of the cohesive interactions between the molecules. When the mechanical strength reached its final value, the self-stabilized FN layers were transferred to solid substrates using the Langmuir-Schäfer method. Films transferred to Si-wafers were used to investigate the film thickness and nano-scale morphology via atomic force microscopy (AFM). The realization of a laterally homogeneous, self-stabilized FN layer with preserved native, secondary structure on a common cell culture substrate was proven by immunofluorescence imaging of FN films transferred to PET.

MATERIALS AND METHODS

Preparation of FN Langmuir and LS layer

Langmuir films of FN were prepared by spreading the protein directly on the surface of the water sub-phase with pH = 2 at room temperature. FN from human plasma was procured from EMD Millipore (Taufkirchen, Germany), with 1 $mg \cdot mL^{-1}$ stock concentration in phosphate buffered saline (pH 7.3). The sub-phase was prepared by adjusting the pH of Millipore water (pH~5.7) using 1 M HCl prepared in-house. The experiments were carried out using a Langmuir-trough (KSV-NIMA, Helsinki, Finland) with an area of 120 cm^2 with a level compensation tool to minimize evaporation and support the reliable measurement of surface pressure and interfacial mechanical properties, which are sensitive to the changes in the water level. Unless

specified otherwise, the amount of protein used was always 120 µL of 1 mg·mL^{-1} FN. For the LS transfers, PET substrates (Nalge Nunc International, NewYork, USA) and p-type Si-wafer (IMS, Stuttgart, Germany) were used. After overnight FN assembly at the A-W interface, the substrates were brought in contact with A-W interface horizontally and were lifted gently. Before LS transfer, Si-wafers and PET were rinsed with acetone and ethanol respectively and air-dried. The LS layers were kept swollen in water to avoid de-wetting effects before characterization.

Brewster angle microscopy (BAM)

BAM images of a region of interest with a maximum area of 720 x 400 µm^2 were obtained using the Nanofilm Ultrabam microscope (Accurion, Göttingen, Germany). A 658 nm class IIb laser source with a lens and a CCD camera (1360 x 1024 pixel) were used to take all micrographs, with a resulting lateral resolution of ~2 µm. The experiment was carried out in a trough with dimensions (120 cm^2) using 60 mL of sub-phase with level compensation. 240 µL of 1 mg·mL^{-1} of FN was spread on the sub-phase and surface pressure and BAM images were obtained in parallel. The images were processed using ImageJ plug-ins (National Institutes of Health, Bethesda, USA; http://rsb.info.nih.gov/ij/) to evaluate the mean gray value.

Polarization-Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS)

The PM-IRRAS device (KSV-NIMA, Helsinki, Finland) allows the measuring of surface specific FT-IR spectra of materials because of the differences in the reflection of p- and s-polarized light from interfaces. This device was coupled to a Langmuir trough with surface area of (120 cm^2). A photo-elastic modulator (Hinds Instruments, Hillsboro, USA) was used to modulate the polarization of the light in addition to the intensity modulation of the spectrometer. The photoelastic modulator was set to achieve a phase shift between p and s-polarized light of λ/2 at 2900 cm^{-1}. The angle of incidence was set to 74°. The integration time was 500s. The maximum of the differential reflected intensity, calculated as $R' = \frac{\Delta R}{R} = \frac{R_p - R_S}{R_p + R_S}$, was at about 1750 cm$^{-1.}$ The spectrum of the sample (S) was calculated by normalizing R' from the sample (R's) with respect to R' from the bare air-water interface, (R'$_R$), according to $S = \frac{R'_S}{R'_p} - 1$. A sample spectrum calculated in this way is very sensitive to fluctuations of the reference spectrum. Therefore, N reference spectra were recorded until the normalized differential intensity spectrum between the last two spectra ($\frac{R'_N}{R'_{N-1}}$) was a flat line between 3000 cm^{-1} and 1200 cm^{-1}. For the FN Langmuir experiments, a PM-IRRAS spectrum was recorded

at different time points (0, 30, 60, 90, 120 and 240 min) after FN spreading at the A-W interface.

Interfacial Rheology

A rotational rheometer (MCR502, AntonPaar GmbH, Graz, Austria) with bi-conical bob geometry was used. The setup consists of the bi-conical bob disk, which can be rotated or oscillated and was connected to a motor, which can detect torque τ, displacement and rotational angle γ, while the sample was held stationary in a measuring cell. The circular measuring cell consisted of a cup fixed to the bottom part of the rheometer with a flange. The measuring position of the bi-conical disk at the interface was detected using a normal force sensor and the system can be easily aligned to ensure reproducible measurements. After the cell was filled with sub-phase of 35 mL, the bi-conical bob was positioned at the interface and 120 µL of 1 $mg \cdot mL^{-1}$ FN was spread at the edge of the vessel. For the oscillatory experiments, the rotational angle was oscillated with given amplitude γ_a and frequency ν. A maximum shear stress of $\gamma_a = 0.1\%$ and a frequency of $\nu = 0.1$ Hz were used. These parameters assured that the layer was within the linear viscoelastic regime as determined by amplitude sweeps with a $\nu = 0.1$ Hz. The interfacial shear and loss moduli G', G'' and the interfacial shear viscosity were calculated using the algorithm provided with the rheocompass software package.

Atomic Force Microscopy (AFM)

Imaging of surface topography of the FN films on the Si-wafer were performed using AFM MFP-3D (Asylum Research, Santa Barbara, CA) in Tapping mode. The micrographs were recorded in Millipore water or air at ambient conditions (25 °C) using AC240 TSA-R3 gold-coated Si cantilever tips (Oxford instruments, Santa Barbara, CA) with a spring constant of 2 N/m and a driving frequency around 70 kHz. The thickness of the FN films was determined by a classical scratch method. Briefly, a part of the film was gently removed from the supporting Si-wafer by a scalpel. The border between filled and bare substrate areas was imaged by AFM in air, and the corresponding height differences equated to the layer thickness.

Immunostaining

For FN detection on PET, the samples were first fixed with 4% (w/v) paraformaldehyde (Merck, Germany) diluted in PBS and non-specific sites were blocked by using a blocking buffer composed of 3% (w/v) bovine serum albumin (Sigma Aldrich, Germany) in PBS. FN was visualized by using a mouse anti-human FN antibody (Abcam, Germany; 1:200 dilution in blocking buffer) and a goat anti-mouse immunoglobulin G conjugated with

AlexaFluor® 633 (Invitrogen, Germany; 1:400 dilution in blocking buffer). A confocal laser scanning microscope (510, META, CarlZeiss, Germany) was used to observe the stained samples.

RESULTS AND DISCUSSION

In a first step, the adsorption behavior of FN at the A-W interface was studied by following the surface pressure after spreading of FN solution. The increase in surface pressure with time can be divided into 3 regions: a rapid initial increase (25 min), a gradual increase (500 min) and a plateau region (longer time scales). (Fig. 1a) Typically, if a protein only adsorbs at the A-W interface, the surface pressure plateaus after an initial rapid increase after spreading [13]. The increase profile observed here implies that FN adsorption is only one of the processes occurring to FN at the interface. Further, the FN assembly visualized by BAM showed that FN forms a homogeneous layer (absence of µm structures) and there were minimal changes in the BAM intensity between the surface pressures 10.5 $mN \cdot m^{-1}$ and 1 $mN \cdot m^{-1}$ (≈ 0.5%) during the prolonged increase of surface pressure with time (Fig. 1b). There is 1.5 times higher intensity observed for the FN films in comparison to bare water surface. This signifies that after the initial layer formation, no further molecules were adsorbed at the interface.

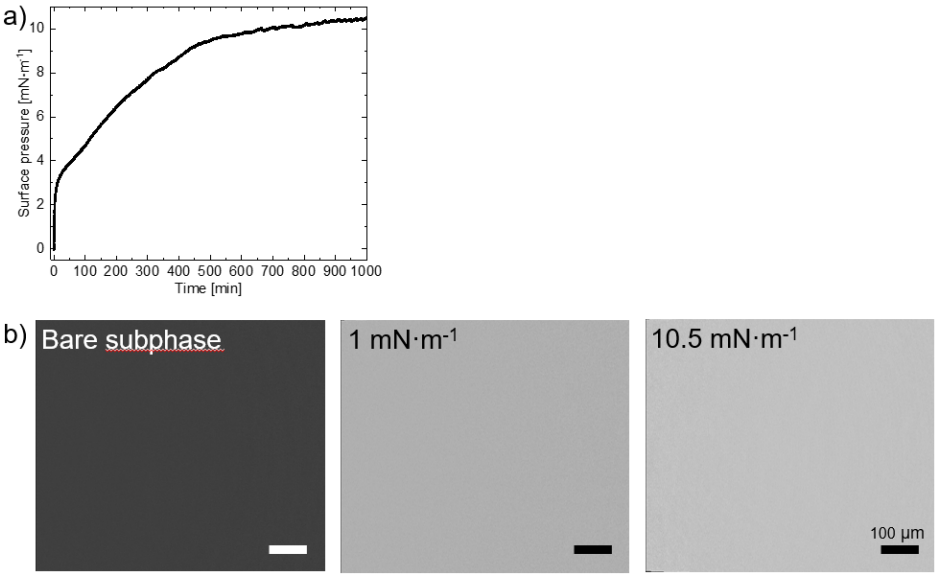

Fig 1. FN adsorption at the A-W interface. a) Surface pressure measurement using Wilhelmy balance. The reported surface pressure curves were the average curves from the 3 independent experiments and maximum deviation between the individual curves and the average curves was 5%. b) Brewster angle microscopy images during FN assembly (scale bar = 100 µm).

The internal molecular structure and the mechanical strength of the FN layer was evaluated using PM-IRRAS and interfacial rheology. By measuring the response to shear deformation with interfacial rheology, we gain insights into the cohesive interactions in the layer. PM-IRRAS is used to probe conformational changes by detecting the amide I (C=O stretching of the peptide backbone) and amide II (N-H bending and C-N stretching of the peptide bond) of the proteins at the A-W interface [15]. FN can be thought of as a molecule that resembles a string of beads with modular units and is mainly composed of anti-parallel β-sheets [16]. Depending on the secondary structure of FN, characteristic peaks and intensities can be assigned in the IR spectra: strong peak (1630-1640 cm^{-1}) and weak peak (1670-1690 cm^{-1}) - β-sheets and (1660-1670 cm^{-1}) - β-turns/random loops [17]. Secondary structure prediction using amide I peaks suggested that the structure of FN in water at RT (20 °C) is predominantly β-sheets (47%) and the rest is β-turns or random loops [7]. PM-IRRAS spectra of FN films adsorbed at the A-W interface clearly show the peaks corresponding to β-sheets and β-turns at all-time points (Fig. 2). The ratio of the peak intensities of β-sheets and β-turns did not change substantially with adsorption time, although the intensity corresponding to β-sheets increased slightly with time. Thus, we can state that the FN conformational structure is not changed after adsorption at the A-W interface on a sub-phase with pH = 2.

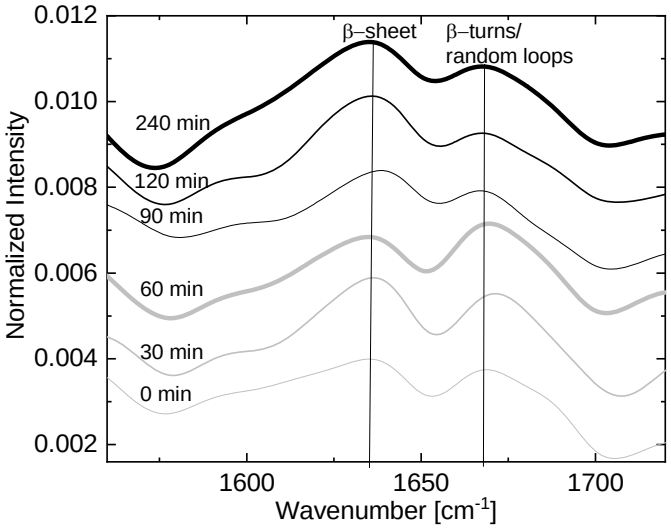

Fig 2. In situ PM-IRRAS of FN films adsorbed on the sub-phase pH = 2.

By means of interfacial shear rheology, it was confirmed that FN forms cohesive films at the A-W interface, indicated by the increase in storage and loss modulus with time (Fig. 3a). Most proteins in solution display a patch-like distribution with hydrophobic, hydrophilic, cationic and anionic local

patches [18]. Here, FN interlinking could be attributed to the intermolecular interactions, which are enhanced by the 2D confinement at the A-W interface. The FN films are elastic, since the elastic modulus (6 $mN \cdot m^{-1}$) is much higher than the loss modulus (1 $mN \cdot m^{-1}$). Further, the layer is elastic up to a strain of 1%, at which elastic modulus decreases and the loss modulus increases slightly in the amplitude sweep (Fig. 3b). This elasticity of FN films has biological significance, as native FN layers are highly extensible and this material is constructed to maximize how much they can be reversibly stretched by cell generated forces [19]. When extrapolating the bulk modulus of the FN layer by dividing by its thickness (~65 nm, see below), the result is 100 kPa. Such a high stiffness is also found for certain decellularized human ECM matrices [20]. It should be mentioned that the elastic modulus of other globular proteins such as lysozyme is two orders of magnitude higher than FN films at the A-W interface. The adsorbed lysozyme layers can be strained up to 5% strain displaying linear viscoelastic response and the higher elasticity could be attributed to multilayer adsorption and cohesion due to intermolecular interactions at the interface [21].

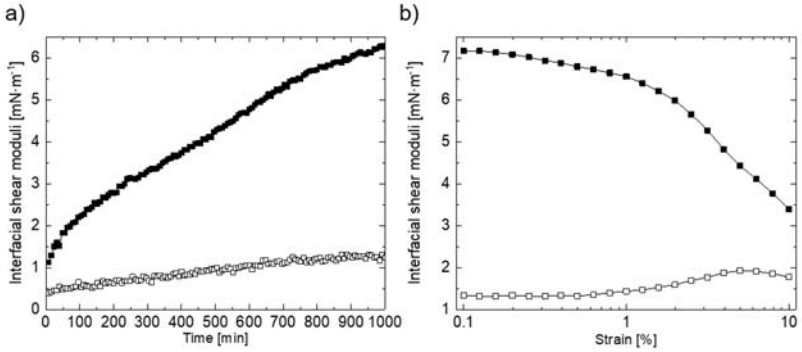

Fig 3. Interfacial rheology of spread FN on the sub-phase pH = 2. a) Time sweep with frequency at 0.1 Hz and strain 0.1 %. b) Strain sweep with frequency at 0.1 Hz, after 1000 min FN assembly at A-W interface. Storage modulus denoted by closed squares and loss modulus denoted by open squares. The reported interfacial rheology curves are the average curves from 3 independent experiments. The maximum deviation between the individual curves and the average curves was 13% throughout the experiment.

The Langmuir approach has the advantage that defined protein layers can be easily transferred onto substrates by LS transfer. Here, we show the successful transfer of FN onto PET and Si-wafer by IF imaging and AFM (Fig. 4). IF images show that the layer is uniform on the microscale (similar to BAM images). Network-like structures are evident on the LS layers deposited on Si-wafer. FN self-assembled films at the A-W interface consist of FN molecules in their native state, connected by intermolecular interactions. The thickness of the layer estimated using AFM was (67 ± 13) nm, which correlates well with FN native globular structure (diameter of 32-48 nm) [22]. Such defined inter-linked FN films can be transferred onto planar substrates with a water contact angle ≤ 80°, as shown previously for Col-IV LS layers [13]. In our

earlier study, FN LS layers having a thin-homogeneous layer on polydimethylsiloxane that supported MSC adhesion [12]. In this study, we can attribute the MSC compatibility of the FN LS layers to the cohesive, self-interlinked layer with native FN structure formed at the A-W interface. These layers can be produced to coat large areas of substrates by spreading FN solution on any vessel containing water subphase, followed by LS transfer. FN self-interactions would minimize the influence of substrate on the FN conformation during glycoprotein adsorption. The coatings produced here are less susceptible to be displaced by secondary proteins from cell culture medium or body fluids. Further, multilayer LS deposition on surfaces can be performed to achieve thicker films to support mesenchymal stem cell (MSC) adhesion and improve *in vivo* implant compatibility. Thus, FN self-stabilizing at the A-W interface with preserved native β-sheet structure is an important finding to realize cohesive ECM protein coatings on substrates that can also support cell attachment. Such stable defined FN films can be used as bioactive coatings for cell culture and for planar implant surfaces.

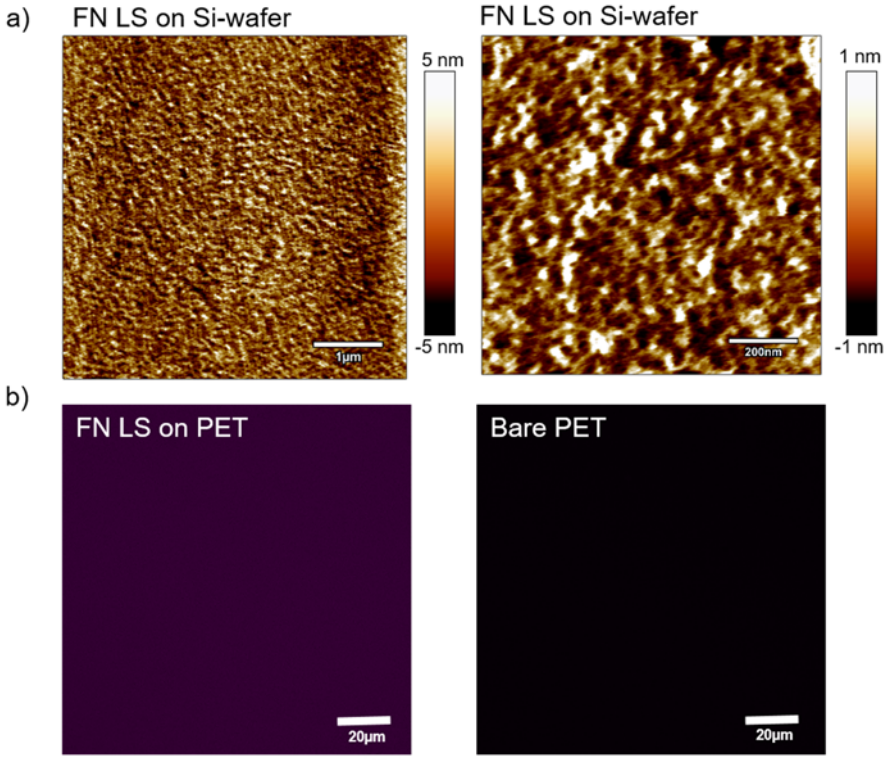

Fig 4. Surface characterization of FN LS layers on solid substrates Si-wafer and PET. a) AFM height image in water (left) and in air (right). b) Immunofluorescence of FN LS on PET and bare PET is depicted as control.

CONCLUSIONS

FN can form self-interlinked films in a cell-free environment using the A-W interface as a platform with preservation of secondary structure. At acidic conditions (pH = 2), FN showed a high propensity to adsorb. FN self-assembly, resulting in cohesive films, was supported by the 2D confinement and high local FN concentration in the layer. The FN films are connected by inter-molecular interactions of native, globular FN molecules. These films are elastic, which is of biological significance for the reversible stretching by cell-traction forces. Such films can be produced artificially in a facile manner on large-areas (12 cm^2) and the self-interactions would minimize the possibility of conformational changes after glycoprotein adsorption on substrates. Therefore, these interlinked cohesive network structured FN films are attractive candidates as stable ECM coatings for *in vitro* substrates and for implant surfaces. Further, the Langmuir-technique can be used to manipulate the native FN films using rapid barrier movements to impose compressive and expansive forces to induce FN unfolding. Such an approach will allow us to estimate the cell-traction forces leading to unfolding of the FN molecules in nature.

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