Biomaterials 36 (2015) 66–79

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The ultrastructure of fibronectin fibers pulled from a protein monolayer at the air-liquid interface and the mechanism of the sheet-to-fiber transition

Maria Mitsi [a, 1], Stephan Handschin [b], Isabel Gerber [a], Ruth Schwartländer [a, 2], Enrico Klotzsch [a, 3], Roger Wepf [b], Viola Vogel [a, *]

[a] Laboratory of Applied Mechanobiology, Vladimir-Prelog-Weg 4, ETH Zurich, Switzerland
[b] SCOPEM – Scientific Center for Optical and Electron Microscopy, ETH Zurich, Switzerland

ARTICLE INFO

Article history:
Received 18 July 2014
Accepted 8 August 2014
Available online 13 October 2014

Keywords:
Fibronectin
Fibrillogenesis
Sheet-to-fiber transition
Monolayer-to-lamella transition
Electron microscopy
Kinetically trapped supramolecular system

ABSTRACT

Fibronectin is a globular protein that circulates in the blood and undergoes fibrillogenesis if stretched or under other partially denaturing conditions, even in the absence of cells. Stretch assays made by pulling fibers from droplets of solutions containing high concentrations of fibronectin have previously been introduced in mechanobiology, particularly to ask how bacteria and cells exploit the stretching of fibronectin fibers within extracellular matrix to mechano-regulate its chemical display. Our electron microscopy analysis of their ultrastructure now reveals that the manually pulled fibronectin fibers are composed of densely packed lamellar spirals, whose interlamellar distances are dictated by ion-tunable electrostatic interactions. Our findings suggest that fibrillogenesis proceeds via an irreversible sheet-to-fiber transition as the fibronectin sheet formed at the air-liquid interface of the droplet is pulled off by a sharp tip. This far from equilibrium process is driven by the externally applied force, interfacial surface tension, shear-induced fibronectin self-association, and capillary force-induced buffer drainage. The ultrastructural characterization is then contrasted with previous FRET studies that characterized the molecular strain within these manually pulled fibers. Particularly relevant for stretch-dependent binding studies is the finding that the interior fiber surfaces are accessible to nanoparticles smaller than 10 nm. In summary, our study discovers the underpinning mechanism by which highly hierarchically structured fibers can be generated with unique mechanical and mechano-chemical properties, a concept that might be extended to other bio- or biomimetic polymers.

1. Introduction

Fibronectin is a large (~500 kDa) glycoprotein found in a fibrillar state within the extracellular matrix of a great variety of tissues and guides multiple cell physiological processes, from cell adhesion and proliferation to differentiation [1–3]. A prominent feature of fibronectin is the ability to undergo fibrillogenesis through a variety of different induction methods, all of which have in common the ability to promote fibronectin self-interactions. Cells assemble fibronectin into a fibrillar meshwork [4], via interactions with integrins and the application of mechanical forces through the acto-myosin cytoskeleton [5,6]. However, fibronectin fibers form also in cell-free systems. Chemical agents, such as polyamines [7] and guanidinium hydrochloride [8], or mechanical perturbations [9–12] can initiate fibronectin fibrillogenesis. One of these techniques of artificial fibrillogenesis, whereby fibers are generated by pulling at the air-liquid interface of fibronectin solutions [9], has been adopted and used to study the mechano-regulated properties of fibronectin fibers in various stretch assays at the molecular and cellular level [13–15]. The stretch-dependent molecular conformation of fibronectin within these fibers has been investigated previously using FRET-based probes [14]. We have shown that the tunable conformational range within such fibers is similar to the

Abbreviations: FRET, Förster resonance energy transfer; MEMS, micro-electro-mechanical system; PBS, phosphate buffer solution; PDMS, polydimethylsiloxane; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

* Corresponding author.
E-mail address: viola.vogel@hest.ethz.ch (V. Vogel).
[1] Current address: Paul Scherrer Institute, Switzerland.
[2] Current address: KIT, Karlsruhe Institute of Technology.
[3] Current address: UNSW, Australia.

http://dx.doi.org/10.1016/j.biomaterials.2014.08.012

conformations fibronectin adopts within cell-derived extracellular matrix [14], and sensitive to the application of mechanical force [16]. Since we still lack information about the ultrastructure of these fibers and their 3D architecture, as well as the physical mechanisms that lead to their assembly, the present study aims at investigating the ultrastructure of the manually pulled fibronectin fibers with electron microscopy, thus completing the structural characterization of this biomaterial at different length scales, from the molecular to the macroscopic.

The need for experimental model systems that can serve as stretch assays to explore fibronectin's mechano-regulated functions motivates the urgency of understanding the structure and properties of the manually pulled fibronectin fibers presented here. As an example, morphogenetic processes that require complex and highly coordinated cell behavior, such as embryonic development, wound healing, angiogenesis and carcinogenesis, depend on the presence of fibronectin [17,18], however, we only begin to understand which aspects of cell behavior are directly affected by fibronectin's mechano-regulated biochemical and/or biophysical factors [15]. One of the confounding factors in the study of such problems is the structural flexibility of fibronectin. Depending on the chemical and physical properties of its microenvironment, fibronectin (also in a surface-adsorbed state) assumes different tertiary/quaternary structures [19,20], which could lead to distinct functionalities [21]. The structural flexibility of fibronectin results from its molecular architecture: it consists of two almost identical monomeric chains linked by disulfide bridges on their C-termini [22] and each chain consists in turn of a series of structural repeats connected via linkers [23], which can participate in various interactions with one another, depending on the physicochemical properties of the environment, leading to different final structures of the protein [19]. A consequence of this structural flexibility is the differential exposure of the numerous binding sites on fibronectin, including sites for growth factors [24], cytokines [25], integrins [26], proteoglycans [27], heparin [28], collagens [29], fibrin [30] and bacterial adhesins [13,31]. Any factor, either chemical or mechanical, which can affect fibronectin conformation, has thus the potential to alter the availability of binding sites for a number of ligands [13,32]. The large number of fibronectin interactions that can be regulated in such a way leads to the hypothesis that fibronectin could be the extracellular equivalent of a mechano-tunable signaling node, which integrates various stimuli to guide an appropriate cell response [3]. One of the mechanisms employed by cells to regulate the availability of binding sites on fibronectin is unfolding of the protein by mechanical forces [3,5]. Experimental [33] and computational [34] studies have shown the ability of fibronectin molecules to unfold under mechanical strain, first by losing the tertiary structure and subsequently, by sequential unfolding of the individual domains. The unfolding is reversible and upon release of the mechanical strain the protein assumes its original configuration. However, in the extracellular matrix, fibronectin experiences the mechanical stress not as a single molecule but as part of fibrillar structures. Due to the complex interlaced architecture of fibrillar extracellular matrix, a large range of conformations is seen in native extracellular matrix and often, major conformational alterations are seen at distances along a extracellular matrix fibril of one micron or shorter [35]. To study correlations between mechanical unfolding of fibronectin, availability of binding sites and subsequent biological responses within fibrillar structures similar to the *in vivo* substrates, manually pulled fibronectin fibers, as characterized in this study, present a suitable model system with a much more narrow conformational heterogeneity, at least as concluded from FRET studies [14]. They can be produced at any desired orientation, deposited on flat (stretchable) substrates or microfabricated structures and the molecular conformation of fibronectin fine-tuned by the application of biomechanical forces. By manipulating these parameters, the mechanosensitive binding of various ligands to fibronectin and the subsequent cellular response has been studied in a controlled manner [13,15,36]. In addition to the applications in basic research on fibronectin biology, the manually pulled fibers can be used for tissue engineering applications [37].

Here, we thus characterized the internal structure of manually pulled fibronectin fibers by electron microscopy. Our findings revealed a lamellar structure, stabilized mainly by electrostatic interactions, and which originates from the insoluble monolayer that fibronectin is known to form at the air–liquid interface [38,39]. Knowledge of the ultrastructure of manually pulled fibers and how it is altered by mechanical stress would benefit not only the numerous applications for which these fibers are used, but it can also shed light on the mechanism for their assembly, as well as provide a basis for comparisons between this model system of fibronectin fibrillogenesis and the cell-derived fibronectin fibrils within the extracellular matrix.

2. Materials and methods

2.1. Fibronectin isolation from human plasma

Fibronectin was isolated from human plasma with two-step affinity chromatography as previously described [35]. Briefly, the plasma was passed through a sepharose 4B size exclusion chromatography column. The flow through was subsequently applied to a gelatin-sepharose column. The column was washed with PBS and 1 M NaCl, until no protein was detected (monitored by absorbance at 280 nm). Gelatin bound fibronectin was eluted from the column either under denaturing conditions with 6 M urea or under non-denaturing conditions with 1 M arginine. In the case of arginine elution, the gelatin column was washed additionally with 0.2 M arginine prior to elution. Typical yields ranged from 1 to 4 mg/ml. Fibronectin was stored at −80 °C as eluted from the column and was dialyzed against PBS prior to use. There was no difference in the ultrastructure of fibers produced from fibronectin purified under denaturing and non-denaturing conditions (data not shown).

2.2. Production of manually pulled fibers

Following previously published protocols [14], fibronectin was diluted in the appropriate buffer to a final concentration of 0.4 mg/ml. A droplet of this solution was deposited on a silicone sheet. A sharp tip was immersed in the droplet and, as it was withdrawn, it pulled a fiber from the surface of the droplet. The fiber could be pulled to 0.5–1 cm final length before it was deposited to the substrate. Following deposition onto the substrate, pressing the fiber down with the pulling tip severed the connection of the fiber to the droplet. From a typical droplet of 2.5 μl, an average of 35 fibers could be produced. Any remaining of the droplet was aspirated, the fibers were washed three times with the buffer and they were kept in buffer prior to any further processing. For the purpose of the present study, two types of pulling tips were used: plastic pipet tips (1–200 μl volume capacity), which were cut at the very front to produce a concave, sharp tip and microfabricated metal tips with a defined radius of 2 μm, produced from a solid tungsten wire with an electrochemically etched taper (American Probes & Technologies, model 72T-J3). As described in the results, the morphology and size of the tip did not affect the fiber ultrastructure, as seen by TEM.

2.3. Stretching of manually pulled fibers

Manually pulled fibronectin fibers were deposited onto a silicone sheet, mounted on a uniaxial stretching device and were either left as deposited or subjected to different amounts of strain. According to previous studies in our group [14], by relaxing or further stretching manually pulled fibronectin fibers, samples with strains ranging from 0% to 500% could be prepared. Briefly, the fibers just pulled out of the solution are mechanically strained due to the pulling process itself, and relaxing them to a third of their initial length is required to produce fully relaxed fibers (0% strain). The maximum strain that can be applied on fibers deposited on silicone sheets [14] before fibers start breaking amounts to 500% (stretch fully relaxed fibers six times their length).

2.4. Force-extension curves

Measurement of force–extension curves of fibronectin fibers in solutions of different ionic strength were conducted with a MEMS actuator as previously described [16]. Briefly, fibers were pulled on microfabricated PDMS trenches, out of a fibronectin solution in PBS. Through glutaraldehyde functionalization of the surface, the fibers were covalently linked to the top of the trenches, whereas they were freely suspended over the wells. Before the measurements, the fibers were fully relaxed,