Journal of Structural Biology 170 (2010) 50–59

Contents lists available at ScienceDirect

Journal of Structural Biology

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

# Periodic beaded-filament assembly of fibronectin on negatively charged surface

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ARTICLE INFO

*Article history:*
Received 22 September 2009
Received in revised form 4 January 2010
Accepted 20 January 2010
Available online 28 January 2010

*Keywords:*
Fibronectin
Protein assembly
Extracellular matrix
Surfaces
Nanoscale
Atomic force microscopy

ABSTRACT

Fibronectin (FN) is an extracellular glycoprotein with critical roles in many fundamental biological processes. A hallmark of FN function is its characteristic assembly into filaments and fibers to form an insoluble matrix which functions as a scaffolding onto which cells attach, migrate, and deposit other matrix constituents. In this study, we have investigated the effects of differently charged and functionalized surfaces on FN conformations using atomic force microscopy. We demonstrate that a negatively charged polysulfonated surface promotes the formation of highly periodic, micrometer-long FN filaments having a "bead-on-a-string" structure with a bead periodicity of about 60 nm. Beaded filaments were observed when FN was adsorbed to polysulfonate surface in water; higher ionic strength allowed formation of filamentous structures but altered the regularity of the beads. FN did not form filaments when adsorbed onto the polysulfonate surface in the presence of soluble polysulfonates emphasizing the role of negatively charged, solid-phase elements on FN assembly. This charge-driven assembly likely derives from the negative surface promoting extension and opening of the protein, and we suggest a model where this assembly pattern is further stabilized by known self-assembly regions. Our results give insight into how FN fibrillogenesis might be promoted in vivo at cell surfaces by the negatively charged and sulfonated environment created by cell-surface, transmembrane proteoglycans.



## 1. Introduction

Fibronectin (FN) is a large, multi-module glycoprotein ubiquitously found in the cells, tissues, and bodily fluids of all vertebrates (Hynes and Yamada, 1982; Peters and Mosher, 1989; Potts and Campbell, 1994; Pankov and Yamada, 2002). FN plays a critical role in many fundamental biological processes such as embryogenesis, angiogenesis, wound healing, and tissue repair, where it regulates basic cell adhesion, growth, proliferation, migration, and differentiation (Hynes and Yamada, 1982; Peters and Mosher, 1989; Potts and Campbell, 1994; Moursi et al., 1997; Magnusson and Mosher, 1998; Pankov and Yamada, 2002; Zhou et al., 2008; Dzamba et al., 2009). Although FN exist as a soluble, disulfide-linked dimer in tissue fluids such as plasma, cerebrospinal, and amniotic fluid (Pankov and Yamada, 2002), in extracellular matrix FN has a specific characteristic to self-assemble and to produce filaments and fibrils that give rise to an insoluble matrix which provides a scaffold important for cell attachment, migration, and further matrix deposition (Pankov and Yamada, 2002; Sottile and Hocking, 2002; Wierzbicka-Patynowski and Schwarzbauer, 2003; Mao and Schwarzbauer, 2005; Al-Jallad et al., 2006; Sabatier et al., 2009). FN fibrillogenesis is a cell and force driven process that requires an $\alpha 5\beta 1$ integrin interaction and clustering in coordination with alignment of cytoskeletal stress fibers (Pankov and Yamada, 2002; Berrier and Yamada, 2007). The process involves opening and extending of the compacted FN molecule and cellular contraction which results in tension on the FN molecules. This further stretches FN and reveals cryptic self-assembly sites that subsequently allow proper molecular alignment and fibril formation (Zamir et al., 2000; Baneyx et al., 2002; Smith et al., 2007). Upon cellular differentiation and tissue formation, these fibrils are further stabilized into an insoluble, permanent matrix via disulfide isomerase activity (Langenbach and Sottile, 1999) and also likely via interactions with other matrix constituents such as collagen type I (Dzamba et al., 1993; Al-Jallad et al., 2006).

Although many cell supporting materials have been created from FN (King et al., 2003), FN assembly into organized fibrils in in vitro conditions has been difficult to replicate and most successful of these approaches have used applied force (Ulmer et al., 2008). Many biophysical and biochemical characterization studies have been performed to investigate opening, extending, and unfolding of FN, its physical interactions and conformational patterns, shapes and sizes in solution, or when interacting with

*Abbreviations:* AFM, atomic force microscopy; APTES, 3-aminopropyl-triethoxysilane; ECM, extracellular matrix; EDA, extra-domain A; EDB, extra-domain B; FN, fibronectin; PAA, poly(allylamine hydrochloride); PLL, poly(L-lysine) hydrochloride; PSS, poly(sodium 4-styrene sulfonate); TRIS, tris-hydroxymethylmethylamine.

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doi:10.1016/j.jsb.2010.01.009

surfaces at molecular and mesoscopic scales and these include static and dynamic light scattering measurements (Williams et al., 1982; Rocco et al., 1983; Lai et al., 1993; Nelea et al., 2008), and imaging techniques such as transmission electron microscopy (TEM) (Koteliansky et al., 1980; Engel et al., 1981; Erickson et al., 1981; Price et al., 1982; Tooney et al., 1983; Erickson and Carrell, 1983), scanning electron cryo-microscopy (Chen et al., 1997; Peters et al., 1998), and atomic force microscopy (AFM) (Emch et al., 1992; Lin et al., 2002; Bergkvist et al., 2003; Ngankam et al., 2004). From these studies, it has become evident that FN shows various conformations that depend both on solution characteristics and on the physical and chemical properties of the surface/substratum with which it interacts.

In this study, we have investigated by AFM the effects of differently charged surfaces on FN conformations. We demonstrate for the first time that FN aligns into periodic beaded filaments on a polysulfonated, negatively charged surface in a process where no external force was applied. Our results provide insight into how solid-phase factors affect FN unfolding and assembly, and how molecules such as polysulfonated proteoglycans could participate in the assembly process on the cell surface by providing negatively charged environment.

## 2. Materials and methods

### 2.1. Protein and functionalization agents

Bovine plasma FN was purchased from Sigma (St. Louis, MO, USA) and received as a 0.1% solution (1 g/L) in 0.5 M NaCl, 50 mM tris-hydroxymethylmethylamine (TRIS) buffer at pH 7.5. Stock solutions with FN at different concentrations were prepared by serial dilution with double-distilled water (ddH2O) and with solutions of high ionic strength NaCl and PSS of various molarities. The surface functionalization reagents 3-aminopropyl-triethoxysilane (APTES, $\geqslant$98%), and poly(L-lysine) hydrochloride (PLL, $M_w$ >30,000 Da), poly(allylamine hydrochloride) (PAA, $M_w$ ~70,000 Da), and poly(sodium 4-styrene sulfonate) (PSS, $M_w$ ~70,000 Da) polyelectrolytes were also from Sigma and received either as solution or powder. APTES, PLL, and PAA are positive-charge agents, while PSS was used as a negative-charge agent. The ddH2O used for dilution of FN and reagents was first filtered through 0.22 μm filter papers and further filtered through 0.02 μm Anodisc™ ultra-thin membrane filters (Whatman International Ltd., Kent, England) to remove contaminants that potentially includes dust, residues or non-miscible impurities from the water.

### 2.2. Substrates and surface functionalization

Fresh-cleaved Muscovite mica sheets (Cedarlane, Montreal, QC, Canada) and square-diced (1 cm × 1 cm surface area) pieces of silicon (Si) wafers (semiconductor grade, polished, n-type, cut on 100 surface plane, El-Cat Inc., Waldwick, NJ, USA) were used as substrates for FN adsorption. Surface-functionalized specimens were prepared by surface conditioning for 1 h with a 100-μL drop of either APTES, PLL, PAA, or PSS on the Si substrate in a humidified chamber. Samples were then briefly rinsed with ddH2O, and dried with a lightly applied jet of compressed air or pure argon. The concentrations of the agents used were 1% (v/v) for APTES, 1% (w/v) for PAA and PSS, and 0.1% (w/v) for PLL. Table 1 summarizes the substrates and functionalizing agents used with some of the characteristics of surfaces used for FN adsorption.

### 2.3. Preparation of FN specimens for AFM

Specimens with adsorbed FN at very low ionic strength (0.5 mM NaCl, by ddH2O dilution) and at high ionic strength of 150 mM, 1 M and 4 M NaCl, and from solutions of 1 μM, 10 μM and 100 μM PSS were prepared. Application to the surface of FN at very low ionic was made by injecting a FN solution into a droplet of ddH2O previously placed on the surface substrate with a volume fraction of 1:10 (10 μL of FN solution) such that the FN concentration after injection equals 1 μg/mL. For all the PSS solutions and FN solutions of 150 mM and 1 M NaCl, the same injected in a ddH2O drop method as above was applied with volume fractions of 1:10 or 1:5 such that the FN concentration after injection stands always to 1 μg/mL. In the case of the 4 M NaCl FN solution specimen was prepared by directly placing a small volume drop onto the surface. For all specimens, the incubation was for 20 min at ambient temperature to allow for protein adsorption, and this was followed by two washes of ddH2O to remove loosely bound protein, and the samples were then dried using a lightly applied jet of compressed air or pure argon. The adsorbed protein preparation procedure using the injection-into-a-drop method was derived from the work of Bergkvist et al. (2003), and it is thought that this procedure allows a more gradual and gentle protein adsorption process to occur, with protein not directly and instantly contacting the surface immediately after adsorption as is the case for the traditional placement of a drop-on-the-surface (with the protein of interest) (Lin et al., 2002). The adsorption of FN onto the functionalized surfaces was performed immediately after the functionalization step was finished (within 1 h), and AFM scanning started no later than 2 min after the FN specimen was prepared (i.e., the time necessary to place the specimen in the AFM sample holder and set the measurement parameters).

**Table 1**
Chemical characteristics of surfaces used for FN adsorption.

| Surface | Charge | Hydrophilicity |
|---|---|---|
| Mica | Slightly negative immediately after cleaving,[a] from hydroxyl and oxygen of aluminosilicate groups | High |
| Silicon[b] | Neutral | Low |
| Aminopropyl-triethoxysilane | Positive, from primary amines | Medium |
| Poly-L-lysine | Positive, from primary amines | Medium |
| Poly-allylamine | Positive, from primary amines | Medium |
| Poly-styrene sulfonate | Negative, from sulfonates | Medium–low[c] |

[a] Neutralizes quickly in air.
[b] With a superficial, native $SiO_2$ layer.
[c] Higher than silicon.

### 2.4. AFM imaging and post-processing

AFM imaging of FN molecules adsorbed on differently functionalized surfaces was performed using a multi-mode scanning probe microscope (Digital Instruments Nano-Scope IIIa, MultiMode® SPM, Santa Barbara, CA, USA) operating in the tapping mode in air at ambient temperature. AFM probes were PointProbe® Plus (NANO*SENSORS*™ STM, Neuchatel, Switzerland) with 10–130 N/m force constant and a typical radius of curvature of the tip at its apex less than 7 nm. All AFM scans presented here and those that have been used for analysis were performed with a 0.5-Hz scan rate on specimen areas of 5 μm × 5 μm or smaller with 512 lines and 512 sampling per line providing images with 262,144 pixels. Drive frequency was ~300 kHz. Amplitude setpoint was in the 1–2 V range with drive amplitude of 50–500 mV. Ranges of feedback control parameters are as follows: integral gain 1–2, proportional gain 2–4, look-ahead gain, 0–0.8. Post-processing of AFM obtained images was performed using the WSxM 5.0 Develop 1.1 software that has been developed to process AFM raw data images (Horcas et al., 2007). Post-processing of images presented here included planeing, flattening and adjustments of brightness, contrast, *Z*-axis