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Molecular assembly and biological activity of a recombinant fragment of fibronectin ($FNIII_{7-10}$) on poly(ethyl acrylate)

Patricia Rico[a,b], Cristina González-García[a], Timothy A. Petrie[d], Andrés J. García[d,*], Manuel Salmerón-Sánchez[a,b,c,**]

[a] Center for Biomaterials and Tissue Engineering, Universidad Politécnica de Valencia, Spain
[b] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Valencia, Spain
[c] Regenerative Medicine Unit, Centro de Investigación Príncipe Felipe, Valencia, Spain
[d] Woodruff School of Mechanical Engineering and Petit Institute for Bioengineering and Bioscience, Georgia Insitute of Technology, Atlanta, USA

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ABSTRACT

Fibronectin (FN) fibrillogenesis is a cell-mediated process involving integrin activation that results in conformational changes of FN molecules and the organization of actin cytoskeleton. A similar process can be induced by some particular chemistries in the absence of cells, e.g., poly(ethyl acrylate) (PEA), which enhance FN–FN interactions leading to the formation of a biologically active network on the material surface. We have investigated the organization of a recombinant fragment of fibronectin ($FNIII_{7-10}$) upon adsorption on this particular chemistry, PEA. Atomic force microscopy (AFM) was used to identify individual molecules of the fragment after adsorption, as well as the evolution of the distribution of adsorbed molecules on the surface of the material as the concentration of the adsorbing solution increased. Globular molecules that turn into small aggregates were found as a function of solution concentration. Above a threshold concentration of the adsorbing solution (50 μg/mL) an interconnected network of the $FNIII_{7-10}$ fragment is obtained on the material surface. The bioavailability of specific cell adhesion domains, including RGD, within the molecules was higher on PEA than on the control glass. The biological activity of the fragment was further investigated by evaluating focal adhesion formation and actin cytoskeleton for MC3T3-E1 osteoblast-like cells. Well-developed focal adhesion complexes and insertions of actin stress fibers were found on PEA in a similar way as it happens in the control SAM-OH. Moreover, increasing the hydrophilicity of the surface by incorporating –OH groups led to globular molecules of the fragment homogeneously distributed throughout the surface; and the cell–material interaction is reduced as depicted by the lack of well-developed focal plaques and actin cytoskeleton.

1. Introduction

Plasma fibronectin is a glycoprotein which forms dimers consisting of two subunits of 220 kDa, linked by a single disulfide bond near the carboxyl termini [1,2]. Each subunit contains three types of repeating modules (types I, II and III) which mediate interactions with other FN molecules, other extracellular matrix (ECM) proteins, and cell surface receptors [3]. Cells interact with FN via integrins, a family of transmembrane receptors that govern interactions of cells with the ECM. Integrin mediated adhesion is a complex process which involves integrin association with the actin cytoskeleton and clustering into supramolecular complexes – focal adhesions – that contain structural proteins (vinculin, talin, tensin, etc.) and signaling molecules [4,5].

Integrin–FN interactions, governed mainly by the $\alpha_5\beta_1$ receptor, lead to the formation of extracellular matrix fibrils from the newly secreted FN [6] and even arrangement of those protein molecules adsorbed on the substrate [7,8]. The thickness of FN matrix fibrils ranges from 10 to 1000 nm in diameter and these fibrils consist of a few to hundred of FN molecules across [9]. FN binding to integrins induces reorganization of the actin cytoskeleton and activates intracellular signaling complexes. Cell contractility facilitates FN conformational changes and allows the unfolding of the native globular FN structure, thus exposing cryptic domains that were not available in the compact form of soluble FN [6]. Finally, fibrils are formed through FN–FN interactions, usually through binding of I_{1-5} either to III_{1-2} or III_{12-14} domains [10].

We have recently shown that FN is able to self-assemble into a network on hydrophobic poly(ethyl acrylate), PEA, in the absence

* Corresponding author.
** Corresponding author at: Center for Biomaterials and Tissue Engineering, Universidad Politécnica de Valencia, Spain.
E-mail addresses: andres.garcia@me.gatech.edu (A.J. García), masalsan@fis.upv.es (M. Salmerón-Sánchez).

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of cells; that is to say, as a consequence of the protein–material interaction, leading to a so-called substrate-induced fibronectin fibrillogenesis [11–13]. Since cell–FN–material interaction involves the RGD sequence which is located on the 10th subunit of the III domain, we have investigated the conformation and distribution of a recombinant fragment of FN ($FNIII_{7-10}$) that incorporates both the PHSRN synergy sequence and the RGD binding motifs on this particular chemistry by atomic force microscopy (AFM).

$FNIII_{7-10}$ has been employed to engineer materials that direct $\alpha_5\beta_1$ integrin [14–16] since this receptor will only bind to RGD motif in the presence of the synergy domain on the 9th type III repeat [20]. This particular integrin controls proliferation and differentiation of different mesenchymal cells as well as FN matrix assembly [17–19]. $FNIII_{7-10}$ adsorption was previously investigated on self-assembled monolayers (SAMs) and significant surface-chemistry dependent structural changes and adhesive activity were found, suggesting that changes in protein structure were not dominated only by surface hydrophobicity [21]. Moreover, molecular modeling of $FNIII_{7-10}$ adsorption on SAMs revealed details on the unfolding of the protein upon adsorption to these different chemistries [22,23].

This work investigates the adsorption of a recombinant FN fragment ($FNIII_{7-10}$) on PEA by atomic force microscopy and its role on the biological activity of the fragment on this substrate. Moreover, because this recombinant fragment does not contain FN domains involved in FN–FN interactions (I_{1-5} and III_{1-2} or III_{12-14} domains), the present study allows further analysis of the PEA-driven FN fibrillogenesis process.

2. Materials and methods

2.1. Substrates preparation

Polymer sheets were obtained by polymerization of a solution of ethyl acrylate (EA, 99% pure, Sigma–Aldrich, Steinheim, Germany) using 0.1 wt% benzoin (98% pure, Scharlau, Barcelona, Spain) as a photoinitiator. Copolymer sheets were obtained by polymerization of a solution of both monomers ethyl acrylate, EA and hydroxyethyl acrylate, HEA (Aldrich 96% pure, Sigma–Aldrich, Steinheim, Germany), with the desired proportion using 0.1 wt% of benzoin (Scharlau, 98% pure) as photoinitiator and 2 wt% ethylene glycol dimethacrylate EGDMA (Sigma–Aldrich, 98% pure) as crosslinking agent. Five monomer feed compositions were chosen, given by the weight fraction of HEA in the initial mixture of 1, 0.7, 0.5, 0.3, and 0 (x_{OH} refers to the sample with fraction x of HEA in the copolymer). The polymerization was carried out up to limiting conversion. After polymerization, low molecular-mass substances were extracted from the material by boiling in ethanol for 24 h and then drying in vacuo to a constant weight. Small disks (approximately 5 mm diameter) were cut from the polymerized sheets in order to be used in the protein adsorption and cell adhesion studies. The samples were sterilized with gamma radiation (25 kGy) before the experiments.

SAM surfaces were prepared and characterized as described elsewhere [24]. Alkanethiols 1-dodecanethiol ($HS–(CH_2)_{11}–CH_3$), 11-mercapto-1-undecanol ($HS–(CH_2)_{11}–OH$) were purchased from Sigma–Aldrich Chemical (Steinheim, Germany) and used as received. The assembled SAMs of their respective alkanethiols are referred to hereafter as CH_3, OH. Au-coated glass coverslips (10 mm diameter) were used as SAM substrates that were cleaned with 70% H_2SO_4 and 30% H_2O_2 at room temperature for 1 h, rinsed with deionized H_2O, rinsed with 95% ethanol, and dried under a stream of N_2 prior to metal deposition. Au-coated substrates were prepared by deposition of thin films of Ti (150 Å) followed by Au (150 Å) using a high vacuum evaporator (Polaron E6100, Watford, Hertfordshire) at a deposition rate of 2 Å/s and a chamber base-pressure of approximately 2×10^{-6} Torr. Freshly prepared Au surfaces were immersed in alkanethiol solutions (1 mM in absolute ethanol), and SAMs were allowed to assemble overnight. SAMs were rinsed in 95% ethanol, dried under N_2. Surfaces were validated by contact angle measurements. The equilibrium water content (mass of water absorbed referred to the dry mass of the substrate) and the water contact angle (using a Dataphysics OCA) were measured for the different substrates.

2.2. Recombinant fibronectin fragment

$FNIII_{7-10}$ was produced and purified as described previously [14]. *Escherichia coli* transformed with cDNA coding for human $FNIII_{7-10}$ and ampicillin resistance were streaked onto an LB agar plate containing 100 μg/mL ampicillin and incubated overnight at 37 °C. A single colony was isolated and expanded in 5 mL of LB broth with 50 μg/mL ampicillin for 3–5 h at 37 °C. This starter culture was added to 500 mL of LB + 50 μg/mL ampicillin + 0.4 mM IPTG to induce $FNIII_{7-10}$ expression. The culture was grown overnight at 28 °C. Bacteria pellets were collected at 25,000 × *g* for 10 min and frozen at −80 °C. Upon thawing, bacteria were lysed and cytosolic proteins were exposed by addition of CellLytic B-Clear II (5 mL/g) and DNAse I (5 μg/mL) for 30 min. The lysate was centrifuged at 25,000 × *g* for 15 min. The protein supernatant was sterile-filtered, and purified by affinity chromatography using a 5 mL column of Ultralink Immobilized Monomeric Avidin (Pierce) connected to a gradient pump, UV monitor, and fraction collector (BioRad, Hercules, CA). Briefly, after sequential column washes with regeneration and elution buffers, the protein solution was allowed to bind to the column for 1 h at a 0.4 mL/min flow rate. After washing with DPBS, elution buffer (0.5 mg/mL d-biotin in DPBS) was flowed through (1 mL/min) and the eluted fractions monitored for protein. Protein fractions were filtered using 30 kDa Microcon centrifugal filter devices (Millipore, Bedford, MA) to remove d-biotin, and verified as >98% pure $FNIII_{7-10}$ by Western blotting and SDS-PAGE. Purified samples were flash frozen for storage (−80 °C).

2.3. Atomic force microscopy

AFM was performed in a NanoScope III from Digital Instruments (Santa Barbara, CA) operating in the tapping mode in air; the Nanoscope 5.30r2 software version was used. Si-cantilevers from Veeco (Manchester, UK) were used with force constant of 2.8 N/m and resonance frequency of 75 kHz. The phase signal was set to zero at a frequency 5–10% lower than the resonance one. Drive amplitude was 200 mV and the amplitude setpoint Asp was 1.4 V. The ratio between the amplitude setpoint and the free amplitude A_{sp}/A_0 was kept equal to 0.7.

2.4. Antibody assay for $FNIII_{7-10}$ conformation

Samples were incubated in 2-fold serial dilutions of $FNIII_{7-10}$. Surfaces were then rinsed in PBS and blocked against nonspecific antibody binding using blocking buffer (1% BSA/DPBS) for 30 min at 37 °C. Primary monoclonal antibody HFN7.1 (Developmental Hybridoma, Inc., Iowa City, IA) directed against the flexible linker between the 9th and 10th type III repeat and mAb1937 (Chemicon, Temecula, CA) directed against the 8th type III repeat were used [25]. Substrates were incubated in primary antibody (1:4000 for HFN7.1 and 1 μg/mL mAb1937 in blocking buffer) for 1 h at 37 °C. After washing (0.1% Tween 20/DPBS), substrates were incubated in alkaline phosphatase conjugated anti-mouse IgG (1:5000) for 1 h at 37 °C, washed again, and incubated in 4-methylumbelliferyl phosphate (4-MUP) liquid substrate system for 45 min at 37 °C. Reaction products were quantified using a fluorescence plate reader (Victor 3, PerkinElmer, Foster City, CA) at 360 nm excitation/465 nm emission.