Creating Nanoscopic Collagen Matrices Using Atomic Force Microscopy

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ABSTRACT The atomic force microscope (AFM) is introduced as a biomolecular manipulation machine capable of assembling biological molecules into well-defined molecular structures. Native collagen molecules were mechanically directed into well-defined, two-dimensional templates exhibiting patterns with feature sizes ranging from a few nanometers to several hundreds of micrometers. The resulting nanostructured collagen matrices were only ~3-nm thick, exhibited an extreme mechanical stability, and maintained their properties over the time range of several months. Our results directly demonstrate the plasticity of biological assemblies and provide insight into the physical mechanisms by which biological structures may be organized by cells in vivo. These nanoscopic templates may serve as platforms on non-biological surfaces to direct molecular and cellular processes. Microsc. Res. Tech. 64:435–440, 2004. © 2004 Wiley-Liss, Inc.

INTRODUCTION

The nanotechnological revolution was initiated by the invention of scanning probe microscopes that allow the observation of single atoms and molecules and their manipulation in a controlled manner (Gimzewski and Joachim, 1999; Moresco et al., 2001). With this tool, nanodevices have been created from the micrometer to nanometer scale. By selection, however, biological evolution has created the smallest and most effective molecular machines, which work perfectly on the nanometer scale. In bionanotechnology, more than 100,000 variations of these machines, naturally optimized for biological systems, could build a machine park allowing, in principle, the design of molecular factories on the nanometer scale. To use these machines in future biotechnological, cell biological, and medical applications, they must be assembled into new functional systems.

The functionalization of surfaces with proteins is a key issue in several biomedical and biotechnological applications. Currently, many of these systems are used in bioanalytical sensors. Biosensors usually use proteins or nucleic acids to selectively recognize sample molecules of interest. The list of biological molecules for potential use is continuously growing, in part due to the newly described proteins and nucleic acid sequences emerging from the characterization of entire genomes and proteomes. Secondary processes, such as a colorimetric, a fluorescent indicator reaction, or an amplified bioelectric or biomagnetic signal, flag the primary molecular recognition event for analytical processing. Thus, many methods evolved from this approach such as antibody engineering (Malmborg and Borrebaeck, 1995), antigen detection (Van Regenmortel et al., 1997), artificial noses (Baller et al., 2000), biosensor mass spectroscopy (Nelson et al., 2000), DNA binding assays (Lin et al., 1997; McKendry et al., 2002), protein arrays (Malmqvist and Karlsson, 1997; Zhou et al., 2001), immunoassay platforms (Swanson et al., 2002), as well as the detection of biomolecular interactions by surface plasmon resonance (van der Merwe and Barclay, 1996).

Patterning of functionalized surfaces provides the potential to simultaneously carry out thousands of individual experiments to characterize how proteins interact with a given molecule, nucleic acid, drug, or lipids. Microarray printing of purified proteins has proven useful in the investigation of protein function on a genome-wide scale (MacBeath, 2002). The size of the patterned features has been recently reduced to a few tens of nanometers by dip-pen nanolithography (Demers et al., 2002; Lee et al., 2002), which increases its applicability for even ambitious proteomic approaches. The limitation is that the technique must be used in air such that fragile proteins could, potentially, denature. A fundamental part of the biofunctionalization of surfaces is the incorporation of a biological entity. It is vital that the native conformation and functionality of a protein is maintained by mimicking its native environment. Of similar importance is the protein orientation to prevent spatial constraints of molecular interactions. Thus, instead of printing biological molecules directly onto non-biological surfaces, a naturally embedding biological matrix is helpful to tether biological entities to surfaces.

Here, we have developed strategies to coat non-biological surfaces with an ultrathin, extremely stable,
and well-oriented collagen layer. Collagen, constituting up to 25% of the total protein mass in mammals, is the primary protein of the extracellular matrix, where it forms fibrils that resist tensile forces (Kadler, 1994). Collagen molecules, of which collagen type I found in bone, skin, and tendons is the most common representative, are fibrous proteins composed of three peptide chains winding into a triple-helix of about 300 nm in length and 1.5 nm in diameter. In tissues, these collagen molecules then self-assemble into 10–200-nm-diameter fibrils whose characteristic 67-nm banding is due to the staggering of the constituent collagen molecules (Kadler et al., 1996). Collagen has been the subject of intensive research, not just because of its role in human diseases (Kadler, 1993; Kunicki, 2002; Myllyharju and Kivirikko, 2001; Prockop, 1998, 1999) but also because synthetic collagen matrices (Bishop, 2000; Coombes et al., 2002; Guidry and Grinnell, 1985; Lee et al., 2001) serve as platforms for cell biological and tissue engineering applications. In tissues, cells are thought to organize the collagen fibrils by exerting tension on the matrix (Harris et al., 1981; Stopak and Harris, 1982), though the underlying molecular mechanisms are still not clear.

Purified collagen can be polymerized in vitro to form several different structures including polar and bipolar native-type fibrils (Kadler et al., 1996), segmental-long-spacing crystallites (Paige and Goh, 2001; Schmitt et al., 1953), and fibrous-long-spacing collagen (Gross et al., 1954; Lin and Goh, 2002). Here we report the in vitro self-assembly of a novel fibrillar collagen structure, namely an ultrathin, extremely stable, and well-oriented collagen monolayer that forms adjacent to a solid support. The structure has the unexpected property that during a critical, initial period it is highly malleable and the collagen molecules can be reoriented by forces applied by the stylus of an atomic force microscope (AFM). After this critical period, the resulting collagen coatings harden and remain stable for several months without loss of fiber orientation or mechanical strength. We propose that collagen fiber formation in vivo may also have a critical period during which cellular forces can remodel the collagen network. Our results directly demonstrate the plasticity of collagen assemblies, and give insight into the physical mechanisms by which the collagen matrix may be organized by cells in vivo (Guidry and Grinnell, 1985; Harris et al., 1981; Lee et al., 1994). These collagen sheets may also serve as platforms on non-biological surfaces to direct molecular and cellular processes.

MATERIALS AND METHODS

Sample Preparation

Solubilized bovine dermal collagen was prepared and purified as described (Bell et al., 1979), concentrated to ~2.5 mg/ml, and stored at low pH of ~2. The final composition of the sample was 97% collagen type I and 3% collagen type III. Chemicals used were grade p.a. and the water was ultrapure (~18 MΩ/cm). For preparation, the collagen was diluted to 0.3 μg/ml (PBS, pH 7.8). This solution was flushed over a freshly cleaved mica surface by creating a hydrodynamic flow. The flow direction of the buffer solution determined the alignment of collagen molecules on the support. After an adsorption time of 10 minutes, the sample was rinsed with the same buffer solution to remove loosely bound molecules.

Atomic Force Microscopy

The AFM (Nanoscope, Digital Instruments, Santa Barbara, CA; NanoWizard, JPK Instruments, Berlin, Germany) was operated in buffer solution using the standard fluid cell, without an O-ring. Imaging was performed in the contact mode (Müller et al., 1999) or tapping mode (Möller et al., 1999) at an imaging force ≤ 100 pN. To orient individual collagen fibers in the area of interest, the applied force was enhanced to ~ 200 pN while the cantilever was drawn over the sample in a controlled manner (Pottiadis et al., 2002). After this, the sample was re-imaged at imaging forces ≤ 100 pN. The oxide sharpened Si₃N₄ cantilevers (OMCL TR400PS) employed were purchased from Olympus Ltd. (Tokyo) and had a nominal force constant of 0.09 N/m. All samples were prepared, imaged, and manipulated in buffer solution at 21°C.

RESULTS AND DISCUSSION

Self-Assembly of Collagen Microfibrils on a Surface

To assemble collagen on a supporting surface, collagen molecules were adsorbed under hydrodynamic flow. When the pH of the buffer was between 2.5 and 5.5, the supporting surface was covered by globular particles (Fig. 1A) that presumably correspond to individual collagen molecules (Mertig et al., 1997). This situation changed dramatically when the pH of the buffer was 6.5 and higher. In this case, the collagen molecules were assembled into fibrillar structures (Fig. 1B,C). As the pH was increased, the spacing between the structures increased and above pH 10.5 no fibrillar structures were observed.

In less densely packed regions, the height of these structures above the mica surface was 2.9 ± 0.6 nm (mean ± SD; n = 700). In contrast, the width of the fibrillar structures varied considerably ranging from 5 to >35 nm (measured at half of maximum height). The smallest fibrillar structures exhibited a height of ~3 nm and a width of ~5 nm, which was consistent with the dimensions of recent models of a single collagen microfibril (Hulmes 2002; Orgel et al., 2001; Ottani et al., 2002). This model was developed from ultrastructural insights revealed by electron microscopy and X-ray diffraction. The experiments indicated that tendon fibers consist of building blocks separated by average periodicities around 4–5 nm (Hulmes et al., 1981; Hulmes and Miller, 1981; Orgel et al., 2001; Prockop and Fertala, 1998; Squire and Freundlich, 1980). It was suggested, that five collagen molecules assemble into one microfibril (Hulmes, 2002; Orgel et al., 2001; Ottani et al., 2002). The microfibril cross-section of five stranded microfibrils exhibit a dimension of ~3 × 5 nm, which is consistent with current models suggesting them as building blocks of larger fibrils. However, fibrillar structures exhibiting widths of more than 20 nm suggest that the collagen microfibrils predominantly assembled in two dimensions forming flat bands. We, therefore, call them microribbons (Ottani et al., 2002) and refer to the surface coating as a monolayer, because the average height corresponds to that of a single collagen microfibril.
Collagen microfibrils were aligned parallel to each other over distances of more than 100 μm. The orientation corresponded to the direction in which the collagen-containing solution was guided over the supporting surface. To determine the spatial extent of alignment, large mica surfaces of ~20 mm in diameter were covered by the same preparation procedure. AFM topographs showed that the microfibrils were aligned in the same direction over the entire surface.

**Mechanical Realignment of Microfibrils**

After the microfibrils were assembled on the supporting surface, their orientation could be manipulated in a controlled manner using the stylus of an AFM (Fotiadis et al. 2002). First, the collagen-coated surface was imaged in the buffer solution with the AFM stylus exerting a relatively low force of 100 pN on the sample. The images in Figure 1 were taken at this force. The force was then increased to 300 pN while the stylus was scanned over an operator-defined region. The surface was then re-imaged at low force.

The AFM topograph shown in Figure 2A contains a rectangular region that was scanned at high force. The collagen microfibrils in the region are now aligned with the fast-scanning direction of the AFM stylus and are approximately perpendicular with the original orientation. The boundaries of the manipulated area are well defined as the individual microfibrils bend through approximately 90 degrees. This shows that collagen molecules, although exceptionally well suited to withstand tensile stresses, can adopt large curvatures (smallest radius of curvature observed was <10 nm) and hence demonstrate high elastic resilience. Because the individual microfibrils cross the boundaries, the material remains molecularly continuous. If the re-orientation of the microfibrils by the AFM stylus breaks bonds between collagen molecules, then the bonds evidently reform. The microfibrils can be re-oriented in any direction relative to the orientation of the initial array, and the angle between the boundaries and the initial array is also arbitrary (see, e.g., Fig. 2B for different angles). The average height of these re-oriented microfibrils was 3.0 ± 0.4 nm (average ± SD; n = 50), which indicated that they did not change their height after manipulation.

Providing that the high-force scanning is performed within the critical time period described below, the surface can be reoriented multiple times. For example, after creating two rectangular areas (Fig. 2B), the microfibrils between the rectangles could be assembled to form a “connection” (Fig. 2C,D).

The AFM stylus can be used to assemble collagen molecules into long fibrils. Figure 2E shows well-defined horizontal fibrils of width ~20 nm and Figure 2F and G show fibrils of width ~10 nm that are more closely woven into the original matrix. It was possible to create collagen fibrils of length up to 100 μm (data not shown). Some of the manipulated collagen fibrils exhibited widths that were comparable to the diameter of native collagen fibrils observed in tendon (Kadler, 1995). However, because the manipulated fibrils exhibited a height of only ~3 nm, they had a completely different cross-section. Additionally, we did not resolve the characteristic D-band periodicity of ~67 nm, which is typical for native collagen fibrils (Squire and Freundlich, 1980).

Sometimes the AFM topographs showed (Fig. 2E) that the density of the collagen has changed after manipulation. Currently, we cannot answer unambiguously were the material has gone. One possibility may be that some of the collagen adhered to the AFM tip. Alternatively, one may assume that some material has been released back to the surrounding buffer solution.

**Collagen Network Stabilizes Over Time**

The collagen arrays stabilized over 4–5 hours, after which time they could not be further manipulated in a controlled manner. After the critical period, scanning at 300 pN had no effect on the orientation of the microfibrils. Increasing the scanning force to 2,000 pN resulted in the destruction of the network rather than the re-orientation and re-assembly of microfibrils.
Thus, the collagen network forms strong bonds after some hours. We speculate that, initially, the collagen molecules within the microfibrils are not properly registered to form strong bonds and can, therefore, slide with respect to each other; over time, the molecules rearrange into a more native-like stagger that confers an increased stiffness on the network. Interestingly, the maturation time of 4–5 hours during which our collagen monolayers lose their ability to be patterned by the AFM in vitro is similar to the time of 6–8 hours during which collagen gels begin to lose their ability to be reorganized by fibroblasts in cell culture (Sawhney and Howard, 2002). This suggests that the maturation process taking place in our 2-dimensional network of non-native microfibrils is similar to that in 3-dimensional networks of native collagen fibrils, and that a similar process may take place in tissues. Following the setting, the structured collagen sheets were extraordinarily stable. When the samples were re-investigated after 8 weeks of storage at room temperature in buffer solution, it was found that both the orientation and mechanical stability of the collagen fibers had not changed.

**Fig. 2.** Controlled nanomanipulation of collagen. A: The AFM stylus was used as a tool to align collagen fibers within a rectangular area. Individual fibers were aligned perpendicular to the surrounding collagen molecules. Alignment and connection of individual fibers to the surrounding assembly of collagen molecules are clearly visible. After creating two rectangular areas (B), the fibers between the rectangles could be assembled to form a “connection” (C,D). E: Alternatively, it was possible to adjust the separation between the fibers until they were completely separated from each other. F: Collection of fibers reassembled into different structures. Top: A single fiber extending over a length of ~4 μm. Center: Region of two fibers reassembled in the horizontal direction and of individual fibers forming bridges between both fibers. Bottom: Two close fibers aligned horizontally. G: Different structures of reassembled fiber created by the AFM stylus. All experiments were performed on native collagen molecules in buffer solution at pH 7.5. Topographs exhibit a gray scale corresponding to a vertical range of 4 nm.

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**Force Required to Re-Assemble Collagen Correlates to Forces of Many Biological Processes**

The forces required to realign the collagen molecules in our experiments, between 100 and 300 pN, lie within the upper range of forces measured for other molecular interactions in cell biology. Thus, the realignment forces are larger than those generated by single motor proteins (up to 10 pN) (Howard, 2001). They are similar to the intermolecular forces associated with ligand-receptor interactions (of P-selectin ligand complexes, up to 165 pN; Fritz et al., 1998; between biotin and streptavidin, up to 170 pN; Merkel, et al., 1999), and to the intramolecular forces required to unfold proteins (e.g., fibronectin up to 300 pN; Rief et al., 2000). The realignment forces are smaller than those associated with covalent bonds (up to 2000 pN; Grandbois et al., 1999).

The re-alignment forces are also within the range of forces that fibroblasts can generate. Moving fibroblasts exert forces on substrates of up to 10 nN/μm perpendicular to their leading edges (Harris et al., 1980). Stationary fibroblasts exert forces of similar magnitude parallel to the long axes of the focal adhesions that they make on synthetic substrates (Schwarz et al., 2002). Given that there are 5–20 microfibrils per micrometer in our monolayers (Fig. 1B,C), it is likely that a fibroblast could produce a sufficiently high force to reorient our monolayers, a possibility that we are presently testing.

Because of the similarity in the magnitudes of the forces and the similarity of the maturation times, we hypothesize that the mechanism underlying the patterning of the collagen monolayers by the AFM in vitro described here may be similar to the mechanism underlying the reorganization of collagen gels by fibroblasts in vivo. If this is the case, then application of force spectroscopy (Clausen-Schaumann et al., 2000) should provide molecular details of the dynamic interactions between collagen molecules that endow them with their extraordinary plasticity required for tissue formation.
**CONTROLLED MANIPULATION OF COLLAGEN MICROFIBRILS**

**Perspectives**

The collagen-coated surfaces described here may find biomedical or biotechnological applications. The orientation of the monolayers may be useful for directing molecular or cellular processes such as wound healing, and the ability to nanostructure the monolayers may provide a mechanism to tune the monolayers to particular cellular features or cell types (Chen et al., 1997). Finally, the extraordinary mechanical stability of the monolayers could even make them useful for information storage or nano/microarray printing (MacBeath, 2002) for application in molecular electronic or optical circuits.

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