Giant Bent-Core Mesogens in the Thread Forming Process of Marine Mussels

Tue Hassenkam,[†] Thomas Gutsmann,[†] Paul Hansma,[†] Jason Sagert,[‡] and J. Herbert Waite*[,‡]

Department of Physics, University of California at Santa Barbara, Santa Barbara, California 93106, and Department of Molecular, Cell, Developmental Biology, University of California at Santa Barbara, Santa Barbara, California 93106

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In marine mussels (*Mytilus*), byssal threads are made in minutes from prefabricated smectic polymer liquid crystals by a process resembling reaction injection molding. The mesogens in these arrays are known to be natural block copolymers with rodlike collagen cores. Using atomic force microscopy, it was shown that these collagenous mesogens are bent-core or banana-shaped in a manner that is consistent with and predictable from their amino acid sequence. The overall bend angle in preCOL-NG in *Mytilus galloprovincialis* is about 130°. The mesogens have a center-to-center separation of approximately 22 nm and a length of 200 nm. It is evident that the smectic structure of the prefabricated mesophases remains largely intact over 1−3 μm distances in the molded fibers and is presumably locked in place during molding by cross-linking. Like the smectic liquid crystals of many synthetic banana mesogens, the collagenous mesogens of the byssal threads exhibit SmC_2 symmetry with a characteristic tilt of 24.6°. At about 100% extension, this tilt is considerably reduced and the globular end domains are no longer visible presumably because they have been unraveled.

Introduction

Synthetic bent core or banana shaped mesogens are liquid crystal forming molecules that have recently come under much scrutiny. Some important reasons for the interest include the fact that chiral mesophases can be made from achiral mesogens,[1] that significant ferroelectric behavior is present,[2] and that banana mesogens appear to have excellent and diverse fiber forming properties.[3,4] Although it is assumed that bent-core mesogens are solely the product of human ingenuity, they have also been reported in nature, namely in dogfish egg capsules[5,6] and mussel byssal threads.[7] The purpose of this report is to better describe the gigantic banana mesogens used by marine mussels to make their byssal threads.

Mussels rely on byssal threads to attach to rocks and oppose dislodgement by waves. Background information on byssal thread structure comes principally from two technical approaches: extensive scanning and transmission electron microscopy[8,9] and biochemical and molecular characterization.[7,10] The important structural facts determined by earlier studies and relevant to this study will be briefly summarized. The mussel foot produces and secretes micrometer-sized membrane-bound smectic liquid crystals (LC) from which new threads are molded. These LCs consist of alternating layers of amorphous and rodlike arrays. The denser rodlike regions of the LCs have dimensions in transmission electron microscopy (TEM) of 8−9 nm (diameter) by ∼125 nm (length). Lateral center-to-center distances are 21−23 nm. In cross-section, the rods exhibit a 6+1 pattern (Figure 1), that is, a hexagonal array of elements with one central element, each having a diameter of 1.5 nm. Upon secretion, the LCs are molded into continuous fibers which no longer exhibit prominent smectic layering, at least to scrutiny by TEM. Fiber anisotropy is preserved, however, as is the center-to-center distance of 22 nm.[11]

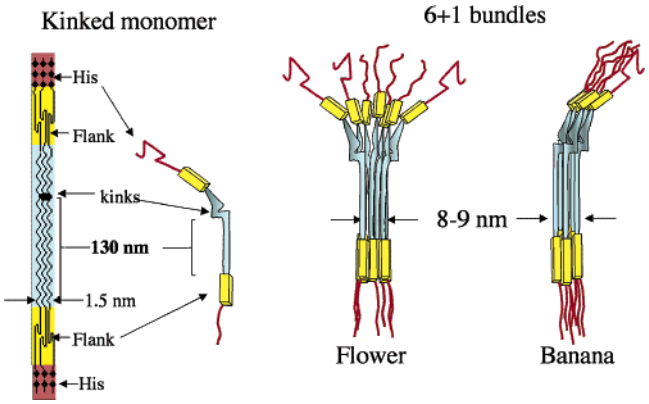

Figure 1. Proposed structure of the giant bent-core mesogens known as preCOLs that make up the bulk of mussel byssal threads. The block domain structure in 2D of a trimer (a); the bent-core analogue of a trimer (b); the proposed hexagonal (6+1) bundles of bent-core trimers in the flower and banana configurations. Amino to carboxy terminal orientation is left-to-right and top-to-bottom.

The precursor mesogens of the smectic LCs are proteins with a series of blocklike domains. From the N- to C-terminus, these include a histidine-rich domain, flank A, the

* To whom correspondence should be addressed. Fax: (805) 893-2817. E-mail: waite@lifesci.ucsb.edu.
[†] Department of Physics.
[‡] Department of Molecular, Cell, Developmental Biology.

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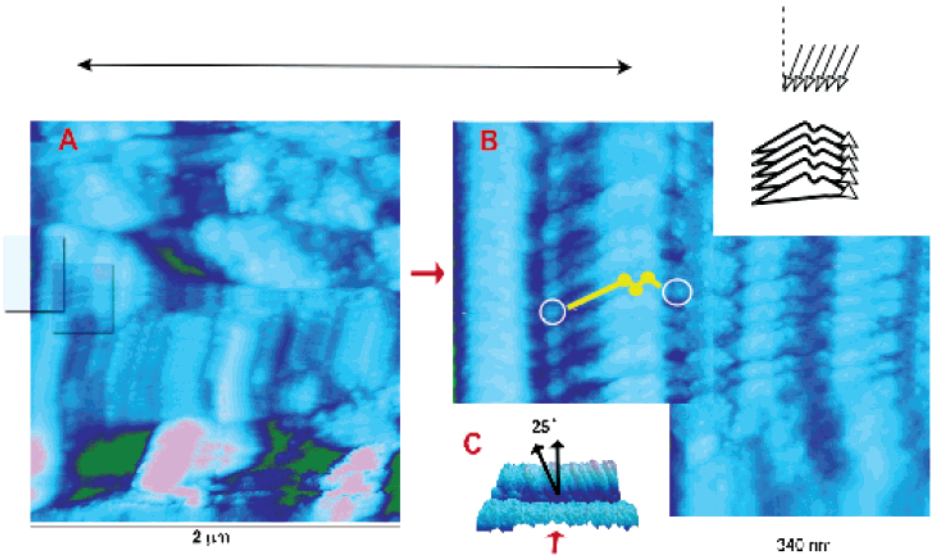

Figure 2. AFM image of a smectic array of preCOL-NG bundles in the proximal portion of a relaxed thread just inside the outer coating. (A) Low magnification; (B) close-up of two overlapping regions from A. The bends in the bent-core region are indicated by an overlaid model. White circles highlight terminal globular regions. (C) View of B from the left (red arrow) revealing the angle of tilt between the surface normal and the molecules at higher magnification. Double headed arrow shows fiber axis. Inset (upper right) shows a graphical representation of preCOLs in smectic array viewed from the side and from the N-terminus (arrowhead).

central collagen domain, the acidic patch, flank B, and finally another histidine-rich domain (Figure 1). The collagen domain imposes a trimeric rodlike structure overall with a mass of about 230 kD. From the highly conserved features of collagen, this predicts a core rod diameter of 1.5 nm and rod length of ~135 nm. The most unique features of these collagens are the obvious aberrations in the canonical Gly-X−Y repeats present in the first quarter of the core domain. In two variants (preCol-P and -NG) of *Mytilus edulis*, the aberration consists of a single missing Gly, whereas in the third variant (preCol-D), there are three locations with canonical aberrations.[10] Molecular models of these aberrant sequences predict that they lead to bends in the rods.[7] A consequence of combining seven bent core molecules into a bundle is that the bundle can be modeled like a flower (Figure 1), a banana, or some intermediate configuration.

Despite the wealth of accumulated information about the formation and structure of byssal threads, there are many unanswered questions. The chief among these has to do with the specific shape of the mesogenic units, the nature of their ordered assembly in the byssus, and how they deform under tension. This report thus addresses the following specific topics: (1) bend geometry, (2) symmetry in axial and lateral interactions, and (3) strain induced deformation. By examining byssal thread fractures with atomic force microscopy (AFM), we now can cast some light on preCOL containing assemblies in the thread.

Methods and Experiments

Individual byssal threads (50 μm × 3 cm) were dissected from intact byssus shed by marine mussels (*Mytilus galloprovincialis*) maintained in a laboratory mariculture system in the Marine Biotechnology Laboratory (UCSB). Threads were kept moist with deionized water during all sample preparations. Under an optical stereomicroscope, each thread was split at one end. Splitting was done in a drop of distilled water on a circular glass coverslip using a scalpel to intiate the split and pulling with microforceps to perpetuate the split to the other end of the thread. The split thread was left to dry on the coverslip for about five minutes at room temperature. Subsequently, the sample coverslip was transferred to an atomic force microscope (Digital Instruments Nanoscope IIIa, Digital Instruments, Santa Barbara, CA), on which images were recorded at a scan rate of 1 Hz using standard silicon tapping-mode tips (force constant 40 N/m, resonant frequency 300 kHz), at room temperature in air.

Results

The core structure in byssal threads can be exposed by stripping away the protective outer cuticle. Images from the core of the proximal thread are typically superior to those from the distal portion in that the fractured surfaces tend to be flatter and less distorted by cleavage. The reason for this may be related to the greater proportion of matrix to fiber and lower cross-linking density in the proximal portion. One such cleaved plane is shown in Figure 2. Dimensions of the crystalline area are about 3 × 1.5 μm. Since the relaxed proximal thread exhibits a highly corrugated structure, the only clear view of the macromolecular arrays is revealed along the outer surface of the folds. Enlargements from the crystalline area of an outer fold (Figure 2) confirm a tightly layered "smectic" structure in which the layers are separated by about 200 nm and are perpendicular to the fiber axis (double headed arrow). More importantly, the bent-core mesogens are still readily apparent and resemble "lumpy bananas" with an overall angle of 130° (± 3; n=6) or a deflection of 50° from the long axis of the bundle. There is no evidence for the "flower" configuration modeled in Figure 1. Banana dimensions include an overall length of 200 nm (± 15; n = 6) and a center-to-center distance of 22 (nm ± 1; n = 7). The presence of two bumps (~ three bends) suggested these to be preCol-NG [12] since this is the only

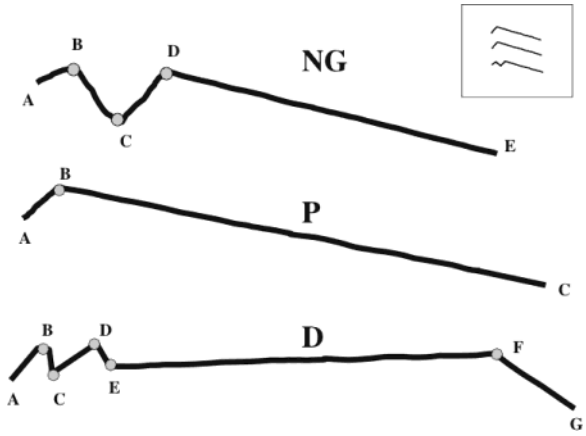

Figure 3. Stick models of the bend locations in the core collagen domains of the preCOLs of *M. galloprovincialis*. **NG** (top): A (res #225; begin collagen), B (# 256; G deletion), C (#318; G → D), D (#387; G → R), E (#662: end collagen**)**; **P** (middle): A (#221; begin collagen); B (#252: G deletion); C (#658; end); **D** (bottom): A (res #142; begin collagen), B (# 1173−177; G → S; X deletion), C (#184; G → D), D (#226; GX deletion), E (#255: G deletion**),** F (#601: G → R**)**, G (#672: end collagen**)**. Numbering in accordance with SwissProt files Q8MW53, Q8MW54, and Q8MW55. Inset upper right indicates bends for preCOLs NG, P, and D in *M. edulis* (top to bottom)

preCOL in *M. galloprovincialis* that encodes three distinct imperfections in its collagen domain. Figure 3 summarizes the locations of bends in the preCOLs of *M. galloprovincialis* using the SwissProt sequence numbers for P, D, and NG (Q8MW53; Q8MW54; Q8MW55; http://us.expasy.org). Although the bend angles cannot be predicted with certainty, the lengths of collagen between bends can be predicted with some accuracy using a translational rise of 0.3 nm per amino acid in collagen.[13] With B, C, and D as reference endpoints for the segments B−C and C−D, lengths of 19 (B−C) and 21 nm (C−D) were calculated. Again, only the thrice-bent preCOL-NG is consistent with the interbend distances (Figure 3). Measured distances for B−C and C−D in preCOL-NG were 20 (±3 nm; $n = 5$) and 23 nm (±2 nm; $n = 5$), respectively, which compare favorably with the calculated ones. The A−B and D−E distances can also be calculated from the sequence as 9 and 83 nm, respectively. These have been added to the bent yellow rods superimposed on imaged preCOL-NG in Figures 2 and 4. It can be seen that the bent rod extends almost to the globular ends of the smectic array of preCOL-NG suggesting that the HIS and flanking domains may both be present in the globular region (circled in Figure 2).

Additional points of interest pertain to the orientation of the byssal mesogens. First, as they are not symmetric around the bend(s), the head is shorter than the tail. The head is defined as the N-terminus or the segment with the shorter arm. Second, these mesogens have a preferred head-to-head and tail-to-tail orientation. This is better illustrated in the stretched fiber given the deep undulations of the smectic mesophase in the relaxed state. Third, the bent mesogens are not upright or lying flat on their sides but are tilted. The tilt is detected by viewing the smectic arrays from the aligned carboxy termini (red arrow at left) and can be accurately measured at 24.6 ° (± 2 °; n=5) (Figure 2, inset). Jakli et al.[14] have identified two types of mesophase tilts: (1) tilt of the molecular plane in a head to tail orientation ("clinic") and (2) tilt in the direction of the bend ("leaning"). The tilt in preCols as revealed in AFM images (Figure 2) is neither of these. Instead, it is a tilt made by rotating the triangular plane of the bent-core mesogen around the axis connecting the ends (Figure 2 model upper right).

A point group symmetry corresponding to SmC_2 is evident in the stretched thread (Figure 4 and 5), but this is much less apparent in the relaxed arrays because the high degree of corrugation in the smectic sheets limits the amount of molecular detail available in any given focal plane.

An equivalent site to that shown in Figure 2 was located on another thread stretched to about 100% of its original length. This was dried in the stretched state, mounted and imaged by AFM. As above, this shows a very regular layered structure (Figure 4), but two features stand out. Viewed from the left (red arrow), the tilt has nearly vanished thereby drawing all structures upright. In addition, the globular C-terminal structures are no longer visible as such but rather as taut connecting strands. A stretch distance of 105 ± 8 nm ($n = 12$) was measured between the C-termini of a pair of connected preCOL-NGs (Figure 4; thin white line). It is difficult to ascertain whether the overall bend angle of preCOL-NG has increased with strain, since a trigonometric approach is frustrated by the fact that a clear differentiation between the rodlike and globular domains in stretched preCOL-NG is not well defined.

Discussion

We have shown that the protein mesogens of byssal threads are gigantic bent-core or banana shaped molecules. Given the typical dimensions of a synthetic banana mesogen (e.g. NOBOW 6 nm × 0.5 nm)[3], the preCOLs from mussel byssus are about 40 times larger (250 nm × 20 nm). The bent core of preCOLs is due to aberrations in the Gly-X−Y consensus repeat. PreCOL-NG has three aberrations: Gly deletion at # 256; Gly to Asp at #318, and Gly to Arg at #387. The overall deflection of the short arm from the main axis is by 50°, which is reminiscent of the ∼60° deflection due to sequence aberrations in the collagen domain of complement C1q.[13]

Earlier studies by Vitellaro-Zuccarello[4] and Waite et al.[7] proposed that each byssal mesogen consists of a hexagonal (6+1) bundle. This study has demonstrated that the dimensions of preCOL-NG as imaged by AFM are consistent with a 6+1 bundle. In contrast to the variety of available achiral synthetic mesogens, the preCOL bundles must be chiral given the many chiral attributes already present: each preCol consists of a sequence of L-amino acids that forms a left-handed polyproline type II helix, and a trimer of three helices is twisted into a right-handed super-helix.[15] The smectic liquid crystalline structure that is so well defined in byssal precursors persists in a frozen or locked state in the mature threads. The 25° tilt evident in the relaxed fibers was unexpected. Fiber extension appears to straighten the tilt. A model of the stretched assembly that summarizes the essential features is illustrated in Figure 5. Note that the assembly is essentially a sheet of preCOLs in which every axial pair is related by C_2 symmetry. The molecular mechanism of

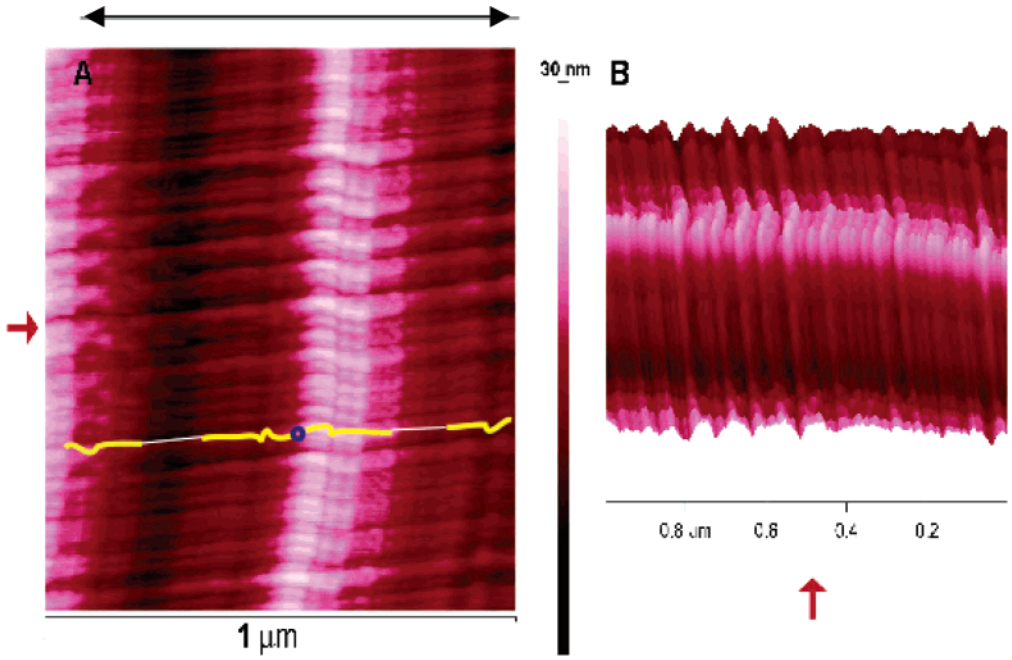

Figure 4. Image of a smectic array of preColD bundles in the same region of the thread (as in Figure 2B) stretched by at least 100% (left). Note reduction in tilt compared to Figure 2C for the relaxed thread that accompanies extension (right; red arrow). A pair of overlaid models related by a C_2 point symmetry is shown. The thin white line connecting them is the location of the globular stretch. Double headed arrow shows fiber axis.

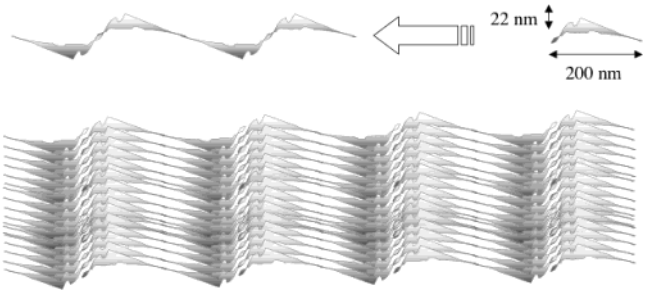

Figure 5. Model of a stretched array of preColD bundles as suggested by Figure 4. A single preColD (upper right), a linear assembly of four preCols (upper left); a smectic mesophase (bottom) with an unchanged bent-core angle.

mesophase locking has not been determined, but both covalent and metal-chelate type interactions have been postulated.[6] The chelate type interactions would involve the metal-mediated marriage of histidine-rich terminal domains, which could be triggered to bind metals by a pH jump from 5 (intracellular compartment) to 8 (seawater).

It is appropriate to ask how the observed preCOL structure might be correlated to the formation and function of the byssal threads. Bent-core liquid crystalline proteins are well adapted for the rapid formation of fibrous biological structures underwater. Mussels have only a few minutes to make each byssal thread. PreCOL and other protein liquid crystals have the virtue of being insoluble in water yet remain complex fluids; they have a high degree of 2- and/or 3-dimensional order, yet are malleable. In addition, bent-core mesogens exhibit very favorable drawing properties; the slenderness ratios (maximum length/minimum diameter) of fibers drawn from bent-core mesogens are among the highest reported.[3]

With regard to mechanical function, previous work on the collagen containing threads from both *M. edulis* and *M. galloprovincialis* has shown that ultimate or breaking strain is between 100 and 200% in the proximal portion and is an important feature of thread toughness.[10] This strain greatly exceeds the ultimate strain of ~10% for more typical collagenous structures such as tendon.[16] Toughness in a variety of biological materials appears to be linked to the reversible sacrificial unfolding of modular domains to provide extra length during deformation.[17] In titin, for example, such extra length is derived from one PEVK and many Ig domains.[18,19] Examination of preCOL-NG assemblies in the proximal thread has revealed many levels of hidden extra length that could, in principle, contribute to the extraordinary observed strain: the supramolecular corrugation of preCOL sheets, the 130° overall bend angle of the bent core, the 25° tilt, and the loosely globular terminal domains are all potential hidden lengths. AFM images of stretched byssal threads suggest that at least three of these contribute to the mechanical strain: extension of the corrugated sheets, unraveling of the flanking globular domains of the preCOL-NGs, and elimination of the tilt seen in unstrained thread.

In summary, our results provide answers to the three specific questions posed in the Introduction. PreCOL-NG has a banana shape with an overall bend of 130°; PreCOL-NG exhibits a "monolithic" assembly in which it is the only detectable constituent. This assembly has a C_2 point group symmetry in terms of its axial interactions, and, at least over short distances, a smectic register. Deformation of preCOL-D assemblies to about 100% strain reveals three trends: a flattening of the folded sheets, loss of the initial 25° tilt, and a straightening of the globular terminal regions. The existence of liquid crystals as large as the byssal preCOLs was a prescient prediction by Vorländer[20] in 1924: "What happens to molecules as they become ever longer? Will the

liquid crystalline state eventually disappear? According to my studies, there is no boundary for this state with respect to polymer chain length. Rather, there is a point at which these substances no longer melt without decomposing nor are they accessible to microscopic examination." (translated from German by Waite). Giant banana-shaped mesogens are very accessible to closer scrutiny by AFM. This is a significant discovery to overcoming the technical limits recognized by Vorländer.[20] It is quite likely that nature has many other examples of fibers and films based on giant bent-core mesogens.[5,6] Fundamental studies of these could provide exciting bio-inspired design paradigms for a host of new materials properties and applications.

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References and Notes

(1) Achard, M. F.; Bedel, J. P.; Marcerou, J. P.; Nguyen, H. T.; Rouillon, J. C., *Eur. Phys. J. E* **2003**, *10*, 129−134.
(2) Jákli, A.; Toledano, P. *Phys. Rev. Lett.* **2002**, *89*, 2755041−2755044.
(3) Jákli, A.; Krüerke, D.; Nair, G. G. *Phys. Rev. E.* **2003**, *67*, 0517021−0517026.
(4) Coleman, D. A.; Fernsler, J.; Chatham, N.; Nakata, M.; Takanishi, Y.; Kórblova, E.; Link, D. R.; Shao, R. F.; Jang, W. G.; MacLennon, J. E.; Mondainn-Monval, O.; Boyer, C.; Weissflogg, W.; Pelzl, G.; Cien, L. C.; Zasadzinski, J.; Watanabe, J.; Walba, D. M.; Takezoe, H.; Clark, N. A. *Science* **2003**, *301*, 1204−1211.
(5) Knupp, C.; Chew, M.; Squire, J. *J. Struct. Biol.* **1998**, *122*, 101−110.
(6) Knight, D. P.; Feng, D. *Tissue Cell* **1994**, *26*, 155−167.
(7) Waite, J. H.; Vaccaro, E.; Sun, C. J.; Lucas, J. M. *Philos. Trans. R. Soc. London* **2002**, *B357*, 143−153
(8) Vitellaro-Zucarello, L. *J. Ultrastruct. Res.* **1980**, *73*, 135−147.
(9) Bairati, A. In *Form and Function in Zoology*; Lanzavecchia, G., Valvassori, R., Ed.; Mucchi: Modena, 1991; p 163.
(10) Waite, J. H.; Qin, X. X.; Coyne, K. J. *Matrix Biol.* **1998**, *17*, 93−106.
(11) Bairati, A.; Vitellaro-Zuccarello, L. *Cell Tissue Res.* **1976**, *166*, 219−234.
(12) Lucas, J. M., Vaccaro, E., and Waite, J. H. *J. Exp. Biol.* **2002**, *205*, 1807−1817.
(13) Kilchherr, E.; Hofmann, H.; Steigemann, W.; Engel, J. *J. Mol. Biol.* **1985**, *186*, 403−415.
(14) Jákli, A.; Krüerke, D.; Sawade, H.; Heppke, G. *Phys. Rev. Lett.* **2001**, *86*, 5715−5718.
(15) Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. *Science* **1994**, *266*, 75−81.
(16) Gosline, J.; Lillie, M.; Carrington, E.; Guerrette, P.; Ortlepp, C.; Savage, K. *Philos. Trans. R. Soc.* **2002**, *357B*, 121−132.
(17) Smith, B. L.; Schäffer, T. E.; Viani, M.; Thompson, J. B.; Frederick, N. A.; Kindt, J.; Belcher, A.; Stucky, G. D.; Morse, D. E.; Hansma, P. K. *Nature* **1999**, *399*, 761−763.
(18) Rief, M.; Gautel, M.; Oesterhelt, F.; Fernandez, J. M.; Gaub, H. *Science* **1997**, *276*, 1109−1112.
(19) Li, H.; Oberhauser, A. F.; Redick, S. D.; Carrion-Vasquez, M.; Erickson, H. P.; Fernandez, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10682−10686.
(20) Vorländer, D. *Chemische Kristallographie der Flüssigkeiten*; Akademische Verlags-gesellschaft: Leipzig, 1924; p 19.

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