Twisted Protein Aggregates and Disease: The Stability of Sickle Hemoglobin Fibers

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We describe how twist could play an essential role in stabilizing 20 nm diameter sickle hemoglobin fibers. Our theory successfully reproduces the observed variation of helical pitch length with fiber diameter. With no remaining adjustable parameters it also yields a prediction for the torsional rigidity of sickle hemoglobin fibers that is in good agreement with experiment and hence retains the striking feature that such fibers can be highly mechanically anisotropic, even with a ratio of bending to torsional rigidity of about 50. We discuss how our study might be relevant to the development of treatment strategies.

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Twisted protein aggregates such as amyloid fibrils and sickle hemoglobin fibers are now implicated in diseases from Alzheimer's to sickle cell disease. In this Letter we argue that the twisted nature of the fibrils is an important feature in their (mis)design. We demonstrate how twist effects may be fundamentally responsible for the metastability, and hence proliferation of, pathological sickle cell fibers.

Sickle cell hemoglobin (HbS) possesses a single mutation at the $\beta 6$ site (glu $\rightarrow$ val) leading to the polymerization of its deoxy form into long fibers. The rigidification of red blood cells that results is the primary cause of sickle cell disease. It is now known that double strands of hemoglobin molecules, thermodynamically stabilized by intermolecular bonding associated with the $\beta 6$ site, are the fundamental building blocks of all known higher sickle hemoglobin aggregates [1–5]. Fibers consisting of seven double strands twisted about a common axis are often observed, growing to indefinite lengths. These have an average radius of approximately 11 nm and a mean helical pitch length of approximately 270 nm, here defined to be the length over which the fiber twists through half a revolution, 180°. We refer to these structures alone as fibers in what follows.

Other sickle hemoglobin aggregates are also known to occur, including macrofibers [6–9]. These helical aggregates contain from 20 to 200 or more double strands. The narrowest macrofibers have pitch lengths that convincingly extrapolate down to the pitch length of a single (sevenfold) fiber while the thicker macrofibers have significantly longer pitch lengths, up to 2 μm or more. In this case the constituent double strands are significantly less twisted; see Fig. 1. A macrofiber with an infinite pitch length would be a bulk crystal made up of untwisted double strands. In what follows we refer to this simply as the crystal state. The crystal has been reported to be the thermodynamic equilibrium state [7,10–12].

We now proceed to construct a theoretical model for the thermodynamic stability (free energy) and equilibrium pitch length of fibers and macrofibers. A somewhat similar treatment has been successfully applied to chiral rodlike molecules [13–15]. We treat both fibers and macrofibers as being comprised of some number of double strands that are mechanically deformed in a well-defined (helical) manner. The following simplifying assumptions are aimed at preserving the essential physics: (i) The macrofiber is treated as if it had circular cross section, whereas in practice it is sometimes slightly elliptical. (ii) We assume that both fibers and macrofibers have a

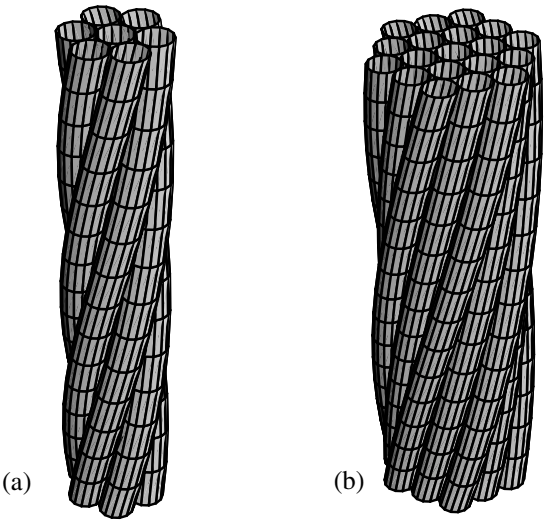

FIG. 1. Idealized sketch of a sickle hemoglobin fiber (a) containing 7 double strands of hemoglobin molecules and a macrofiber (b) of twice the radius assumed to contain 19 double strands. Both sketches are to scale for the radii and pitch lengths (twist) [1,8]. The macrofiber has unwound significantly with respect to the fiber; its pitch length has roughly doubled.

pitch length Λ that is always much greater than its radius R, as is confirmed by experimental observations [8]. Thus we throughout neglect contributions to the energy density that are higher than leading order in R/Λ. (iii) We treat the deformation of the double strands making up the macrofiber interior within a continuum theory for R that does not explicitly restrict the number of double strands to an integer. Such an approach should be asymptotically exact for macrofibers comprised of many individual double strands and semiquantitative even when there are only a few. (iv) Motivated by the fact that axial strains dominate when the twist is small we model the lateral intercations between the double strands as a simple (average) attractive contact energy.

We propose that the distortion energy per unit volume of macrofiber of radius R and pitch length Λ is given by

$$F = F_{\text{surf}} + F_{\text{be}}. \tag{1}$$

Here F_{surf} represents the free energy cost associated with those hemoglobin surfaces that are on the exterior surface of the macrofiber and therefore benefit from fewer attractive (e.g., hydrophobic) interactions with neighboring molecules than do those in the macrofiber interior. A fair approximation to this may be obtained by employing an interfacial energy cost per unit area of external macrofiber surface γ, leading to a total interfacial energy $2\pi R\gamma$ per unit length of cylindrical macrofiber and a corresponding energy density

$$F_{\text{surf}} = 2\gamma/R, \tag{2}$$

which does not depend on the macrofiber pitch length Λ. The second term in Eq. (1), F_{be}, represents the mechanical bending and extensional energy of packing the double strands into the helical macrofiber. This can be estimated using simple geometry and continuum mechanics, as we now describe.

A double strand that is located some radial distance r from the central symmetry (z) axis of the macrofiber is subject to both bending strain, being helical, and either extensional or compressional strain. To see this note that the outermost double strands, which wrap around the external surface of the macrofiber, have a longer contour per unit length of macrofiber than do those near its core. That all of the strands have the same pitch is seen in experimental 3D reconstructions of fibers and macrofibers. Moreover electron density projections of fiber reconstructions made with the electron density of hemoglobin (from x-ray crystallography) and the position of the hemoglobin molecules (from the reconstructions) yield excellent correspondence between the micrographs and the projections [2,16–18]. Thus we assume that the fibers cannot slide freely against one another axially and so the outer fibers are extended and the core ones compressed.

The energy density F_{bend} due to bending of the constituent double strands can be derived by identifying the unit vector field $\hat{\mathbf{t}}$ that is locally tangential to the orientation of the double strands. To leading (first) order in r/Λ the double strand tangent vector is $\hat{\mathbf{t}} = \hat{\mathbf{z}} + \hat{\boldsymbol{\theta}}\pi r/\Lambda$, where $\hat{\mathbf{z}}$, $\hat{\boldsymbol{\theta}}$, and $\hat{\mathbf{r}}$ are the usual orthogonal unit vectors in cylindrical coordinates. The local squared bending curvature is then simply $C^2 = \frac{d\hat{\mathbf{t}}}{dz} \cdot \frac{d\hat{\mathbf{t}}}{dz}$ at the same order [19]. In order to determine the total bending energy density we need to take an average over the bending energy of all double strands located throughout the macrofiber interior. Noting that the magnitude of the curvature C does not depend on z this energy per unit volume of aggregate is given by

$$F_{\text{bend}} = \frac{1}{\pi a^2}\frac{\kappa_{\text{ds}}}{2}\int_{r<R} C^2 d^2r, \tag{3}$$

where $\int_{r<R} d^2r = \int_0^{2\pi} d\theta \int_0^R rdr$ and the factor $1/(\pi a^2)$ accounts for the area density of double strands within the macrofiber interior with a the effective radius of a double strand. The parameter κ_{ds} is the bending rigidity of a single double strand. Using the classical result for the rigidity of a circular cylinder of radius a made up of an isotropic elastic material with extensional modulus E [19] we have $\kappa_{\text{ds}} = \pi E a^4/4$. Thus we obtain

$$F_{\text{bend}} = E\alpha_1/\Lambda^4 \tag{4}$$

with $\alpha_1 = \pi^5 a^2 R^4/16$.

We next calculate F_{ext}, the energy density of extending or compressing the double strands. With this aim in mind the length of a helical double strand located at a distance r from the macrofiber axis (per unit length of macrofiber) is $L = 1 + \frac{1}{2}r^2(\pi/\Lambda)^2$ by the Pythagorean theorem. Thus the average length of the double strands (per unit length of macrofiber) can be easily shown to be $\bar{L} = 1 + \frac{1}{4}(\pi/\Lambda)^2 R^2$ and the extensional energy follows from integrating the squared extensional strain [19], $\sigma_{\text{ext}} = L(r) - \bar{L}$ at leading order,

$$F_{\text{ext}} = \frac{E}{2}\int_{r<R} \sigma_{\text{ext}}(r)^2 d^2r. \tag{5}$$

Hence

$$F_{\text{ext}} = E\alpha_2/\Lambda^4 \tag{6}$$

with $\alpha_2 = \pi^5 R^6/96$. Thus F_{be} is merely the sum of the energies per unit macrofiber length Eqs. (4) and (6), divided by its area πR^2. Hence Eq. (1) becomes

$$F = 2\gamma/R + E\alpha_3/\Lambda^4, \tag{7}$$

where $\alpha_3 = \pi^4(a^2R^2/16 + R^4/96)$. The scaling $F_{\text{be}} \sim \Lambda^{-4}$ merely follows the average strain $\sim\Lambda^{-2}$, squared.

This result represents the distortion energy of a macrofiber of a specified pitch length Λ and radius R. However, in the absence of any propensity for the macrofibers to be twisted, the minimum of this energy is at zero twist $\Lambda^{-1} \to 0$. This reflects the fact that we have not yet included the molecular twist that leads to helical

macrofibers. Since the macrofiber prefers to twist slightly into long pitch length helices we approximate the free energy density as

$$G = F - \psi/\Lambda, \tag{8}$$

which can be thought of as a truncated expansion of the free energy in powers of the twist Λ^{-1}. Here ψ is a homogeneous, positive Lagrange multiplier that controls the pitch length of the helical macrofiber. We propose that ψ represents a material property of the HbS strands reflecting the orientation and positions of molecules. For sufficiently small twists this is a rigorous description of the resulting twisting forces (torques). The Lagrange multiplier ψ will appear as our single fit parameter. We first test the agreement of our theory with experimental data for the distribution of macrofiber pitch lengths. When we later examine how well the theory predicts the torsional rigidity of a single fiber we will *not* make any further adjustments to the fit parameter ψ. We believe that this represents a fairly stringent test on the accuracy of our theory.

The equilibrium pitch length, for a given macrofiber radius, corresponds to the minimum free energy state $\frac{\partial G}{\partial \Lambda} = 0$, which is satisfied at

$$\Lambda = \Lambda^\star = (4E\alpha_3/\psi)^{1/3}. \tag{9}$$

This expression can be fitted to the experimental data of [8], see Fig. 2, where the single best least squares fit parameter is found to be

$$\psi = 3.5 \times 10^{-4}\ \mathrm{J\,m^{-2}}. \tag{10}$$

While the agreement shown in Fig. 2 supports our theoretical description an additional test is that our model correctly reproduces the torsional rigidity obtained from measurements of thermal fluctuations in pitch length [20]. In the regime of small twist the free energy of a fiber per unit length is related to the torsional rigidity c and the overtwist $\tau(\Lambda) = \frac{\pi}{\Lambda} - \frac{\pi}{\Lambda^\star}$ according to [19]

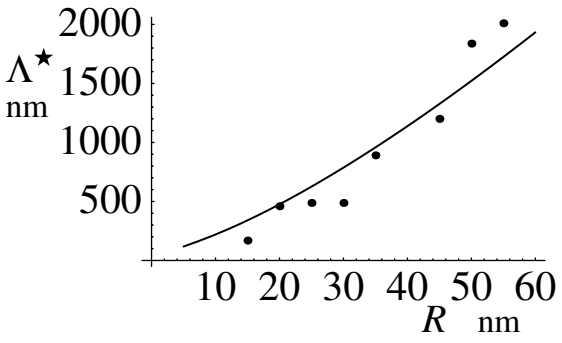

FIG. 2. Experimental data for the variation of the pitch length of sickle hemoglobin macrofibers Λ (nm) with their radius R (nm) (solid circles) [8] is to be compared with the theoretical curve given by Eq. (9) as shown. There is a single fit parameter $\psi = 3.5 \times 10^{-4}\ \mathrm{J\,m^{-2}}$.

$$G = G(\Lambda^\star) + \frac{c}{2}\tau(\Lambda)^2/(\pi R^2), \tag{11}$$

where the factor $1/\pi R^2$ converts the energy per unit fiber length to an energy density. Thus equating the derivative $\frac{\partial^2 G}{\partial \Lambda^2}$ of this with the corresponding derivative of Eq. (8) at $\Lambda = \Lambda^\star$, using Eqs. (7) and (9), we obtain the torsional rigidity of a macrofiber of radius R,

$$c = 3(4E\alpha_3)^{1/3}R^2\psi^{2/3}/\pi, \tag{12}$$

which defines the torsional persistence length according to $l_c = c/k_BT$.

Thus our theory yields a prediction for the torsional persistence length of a single fiber in terms of its extensional modulus E via Eq. (12). Using the parameter values for $R = 11$ nm, $a = 4$ nm, $E = 51$ MPa [20], and ψ Eq. (10) this gives

$$l_c = 2.5\ \mu\mathrm{m}, \tag{13}$$

which is in agreement with the experimental estimate reported elsewhere [20] to two significant figures. This level of agreement must be regarded as somewhat fortuitous given that our theory neglects corrections of order $R/\Lambda \lesssim 10\%$. Nonetheless, the fact that the theoretical estimate is close to the experimental estimate is a feature that strongly supports our model. It is particularly notable that our model reproduces the large observed material anisotropy so closely with no additional fit parameters. This anisotropy may be quantified by comparing the l_c with the corresponding *bending* persistence length $l_p = 130\ \mu$m [20]. For an isotropic material these should be comparable but for sickle hemoglobin fibers $l_c/l_p \approx 1/50$.

We now examine the free energy density G of a macrofiber of radius R by substitution of Eqs. (7) and (9) into Eq. (8),

$$G = 2\gamma/R + 5\psi^{4/3}/(2^{8/3}E^{1/3}\alpha_3^{1/3}), \tag{14}$$

where we recall that α_3 is itself a function of R.

The free energy G is shown in Fig. 3. Broadly speaking it exhibits the following properties: For large values of the interfacial energy $\gamma \gtrsim 7\ \mu\mathrm{J\,m^{-2}}$ the free energy density is monotonic decreasing for all radii of interest with a global minimum at $R \to \infty$, which indicates that the crystal is the most thermodynamically stable state. As the interfacial energy is reduced $\gamma \lesssim 6.5\ \mu\mathrm{J\,m^{-2}}$ a local minimum appears in the free energy. For values as small as $\gamma = 6\ \mu\mathrm{J\,m^{-2}}$ the global free energy minimum has shifted to very thin fibers where our continuum theory becomes less reliable. It is reasonable to expect all aggregation to be strongly suppressed at the smallest values of γ. Thus our theory is able to correctly reproduce the known global thermodynamic stability of the crystal and even predicts an energy barrier for transition from metastable fibers to the crystal. This would contribute to

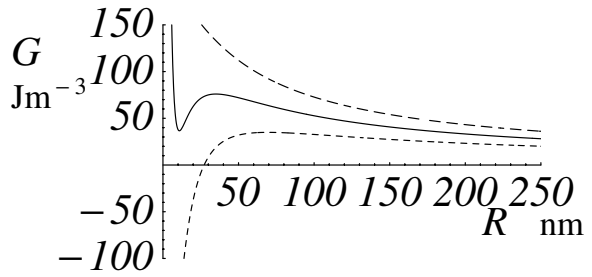

FIG. 3. Theoretical prediction for the free energy density G ($\mathrm{J\,m^{-3}}$) of a macrofiber of radius R (nm) according to Eq. (14) with $\psi = 3.5 \times 10^{-4}\ \mathrm{J\,m^{-2}}$, as determined earlier; see Fig. 2. Three curves are shown: the lower (small dashes), middle (solid), and upper (large dashes) lines correspond, respectively, to fiber-solution interfacial tensions of $\gamma = 5$, 6, and 7 $\mu\mathrm{J\,m^{-2}}$. The local minimum around $R = 11$ nm, corresponding to a single hemoglobin fiber, is clearly visible for $\gamma = 6\ \mu\mathrm{J\,m^{-2}}$. For small interfacial tensions thin macrofibers, with less internal strain, are stable, while larger interfacial tensions stabilize thicker macrofibers, even at the expense of larger internal strains.

the observed proliferation and persistence of fibers of this thickness.

A recent analysis of the attraction between sickle hemoglobin aggregates, which employs observations of how they "zipper" together, yields an interaction energy per unit length of the order of $\sigma = 7k_BT/\mu$m [21]. It was argued that this is similar to the scale of interactions that one would expect from van der Waals and colloidal depletion forces, although screened electrostatic repulsion may also play an important role. An interfacial energy $\gamma = 6.5\ \mu\mathrm{J\,m^{-2}}$ would yield an attraction of magnitude σ within a naive contact energy treatment provided the average width of the effective contact strip between the two fiber surfaces was of the order of $\sigma/2\gamma \approx 2$ nm. This scale seems quite plausible in view of the structure and dimensions of the fiber. Thus fiber interfacial energies for which our theory predicts metastability around $R \approx 10$ nm may indeed be physically reasonable.

Our results may give clues for potential therapeutic treatments given that (i) either a reduction *or* an increase in the effective interfacial tension will serve to destabilize fibers. In the case of a reduction in γ hemoglobin molecules become more soluble and destabilize large aggregates. In the case of an increase in γ hemoglobin molecules become less soluble and stabilize both large macrofibers and the crystal with respect to single fibers. Since networks of single fibers are thought to drive the rigidification of red blood cells a mitigation of the pathology might apear in either case. Solvent conditions including, e.g., salinity, pH, and the concentrations of both HbS and O_2 may be expected to affect the parameter γ. (ii) A reduction in the magnitude of the parameter ψ controlling the inherent propensity to twist will result in (macro)fibers that are less twisted. This stabilizes thick macrofibers and the crystal relative to thinner macrofibers and fibers. It also serves to reduce the twist rigidity $c \sim \psi^{2/3}$ of the macrofibers which would likely enhance the kinetic rate at which they grow thicker. It may be that this reduction in ψ can be acheived via the introduction of a suitable mutant hemoglobin, selected on the grounds of it having a crystal structure consistent with less twisted packings. Ultimately this might lead to gene therapy strategies using these mutants. It is encouraging that the destabilization of high aspect ratio fibers could lead to significant mitigation of the pathology of the disease, even if the total volume fraction of aggregated hemoglobin does not decrease appreciably; freely suspended crystals of hemoglobin contribute little to cellular rigidification.

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