Rapid and inefficient kinetics of sickle hemoglobin fiber growth

Brian T. Castle, David J. Odde*, David K. Wood*

In sickle cell disease, the aberrant assembly of hemoglobin fibers induces changes in red blood cell morphology and stiffness, which leads to downstream symptoms of the disease. Therefore, understanding of this assembly process will be important for the treatment of sickle cell disease. By performing the highest spatiotemporal resolution measurements (55 nm at 1 Hz) of single sickle hemoglobin fiber assembly to date and combining them with a model that accounts for the multistranded structure of the fibers, we show that the rates of sickle hemoglobin addition and loss have been underestimated in the literature by at least an order of magnitude. These results reveal that the sickle hemoglobin self-assembly process is very rapid and inefficient (4% efficient versus 96% efficient based on previous analyses), where net growth is the small difference between over a million addition-loss events occurring every second.

INTRODUCTION

Under deoxygenated conditions, sickle hemoglobin (HbS) self-assembles into long biopolymers that consist of 14 individual filaments interacting to form a fiber with a diameter of ~20 nm (1, 2). Substantial HbS fiber formation within red blood cells leads to an increase in cell stiffness and an increase in blood apparent viscosity, which can culminate in complete occlusion of the microvasculature and the downstream morbidities associated with the disease (3). Thus, the underlying mechanism of sickle cell disease is aberrant biopolymer assembly, and therefore, understanding the HbS fiber self-assembly process is critical to advancing our knowledge and treatment of the disease. A simple framework for the growth of biopolymers (4), used widely for HbS fiber elongation, states that the total flux of subunit addition and loss (J) from the polymer end is given by

$$J = k_{on}C - k_{off}$$  

The experimentally observed linearity of growth rate as a function of subunit concentration (C) is consistent with this one-dimensional (1D) model. From this relationship, linear fitting has yielded estimates of the kinetic on-rate ($k_{on}$) and off-rate ($k_{off}$) constants from the slope and intercept, respectively, of the observed trend for various biopolymers, including microtubules (5–8), F-actin (9, 10), and HbS fibers (11–13). While the 1D framework agrees well with experimental growth data, it was recently shown that assumptions inherent in the model break down for multistranded, 2D biopolymers such as microtubules (14), and consequently, the kinetic rates of monomer addition and loss for HbS fibers are potentially much higher (~10-fold) than previously estimated. Here, by performing the highest-resolution measurements of single HbS fiber dynamics to date (55 nm at 1 Hz) and using an analysis that accounts for the multistranded structure of HbS fibers, we show that the kinetic rates of HbS fiber assembly have previously been underestimated by at least an order of magnitude. Our results lead to an important revision of our understanding of HbS fiber self-assembly and of multistranded biopolymers in general, demonstrating that fiber growth is a rapid and highly inefficient process. As a result, our work suggests that HbS fiber growth, as opposed to nucleation (15), is a viable therapeutic target even at drug concentrations well below the total hemoglobin concentration. Furthermore, our approach provides a general framework for quantifying the kinetics of other self-assembling systems, such as amyloids, viruses, and supramolecular polymers.

RESULTS

Previous studies quantifying HbS fiber growth dynamics have relied on indirect measurements (11, 16) or have been limited by outdated microscope technology (13, 17). Recent advances in scientific cameras and illuminators, however, now enable unprecedented nanoscale accuracy and precision in biological measurements via light microscopy (18–20). To precisely quantify HbS fiber on-off kinetics here, we sought to develop a high spatial and temporal resolution technique to image and analyze single fiber assembly dynamics. Differential interference contrast (DIC) microscopy has previously been used to directly estimate fiber growth and observe the characteristic double nucleation process (17), whereby HbS fibers either nucleate spontaneously in solution (homogeneous nucleation) or nucleate off the side of existing fibers (heterogeneous nucleation) (21). We used a similar approach here, imaging HbS fiber assembly by DIC microscopy with contrast enhancement via serial averaging and background subtraction (6, 17). While kinetic rate constants have previously been extracted from features of bulk HbS assembly, the resulting numbers are highly variable (21, 22). Furthermore, these estimates from bulk assembly have more recently been disregarded in favor of those estimated from the direct observation of single fiber growth via DIC microscopy (23, 24) (see Supplementary Discussion for additional details on the previous literature estimates of HbS assembly kinetics). For these reasons, we focus here on single HbS fiber growth and have developed a robust methodology that uses both the mean rate and the variance in the rate of assembly for quantifying single fiber growth kinetics. Under our imaging conditions, we observed growth phenomena consistent with documented characteristics of HbS fiber assembly, including double nucleation (Fig. 1, A and B, and movie S1) and gelation. The observation of such phenomena was not limited to, or preferential to, a single imaging plane (i.e., along the coverslip) but rather occurred throughout the sample (fig. S1). In addition,
Fig. 1. Semi-automated tracking of HbS fiber assembly dynamics. (A) Groups of HbS fibers (spherulites) imaged by DIC microscopy. Groups of HbS fibers are called spherulites. Individual spherulites are the result of homogeneous nucleation in solution, followed by heterogeneous nucleation of new fibers off of existing ones. (B) Heterogeneous nucleation of new HbS fibers off the side of existing fibers. Arrows indicate the nucleation site of the new fiber. (C) Result of function fitting within the user-defined region of interest (left; green lines) for an example HbS fiber (right). Red dots are the individual fiber backbone positions estimated as in (D). The blue line is the determined fiber axis fit to the backbone positions. The cyan dot is the position of the fiber end estimated as in (E). (D) Pixel intensity values from a cross section of an HbS fiber imaged by DIC microscopy. The line is the best-fit Gaussian first derivative to the pixel values (Eq. 7). The red dot indicates the function mean, taken to be the backbone position of the HbS fiber. (E) DIC values along the fiber axis determined from fitting the HbS fiber backbone positions. The line is the best-fit Gaussian survival function to the contrast values. The cyan dot is the function mean, taken to be the position of the fiber end. a.u., arbitrary units. (F) Kymograph showing HbS fiber growth over time. Position is along the vertical axis, while time is along the horizontal axis. Scale bars, 30 s and 5 μm for the horizontal and vertical bars, respectively. (G) Semi-automated tracking results for the HbS fiber shown in (F). (H) Growth rates ($v_g$) as a function of HbS activity ($γC$). Activity coefficients were determined as described in (11). Circles are the values estimated from semi-automated tracking at 25°C (black) and 37°C (gray). Squares are reported values from (17), diamonds are reported values from (11), and the triangle is a reported value from (13). Lines are the projected trends from the data in (11). Vertical error bars are SD of the growth rate ($n = 126$ and 110 fibers at 25° and 37°C, respectively). Horizontal error bars are 10% uncertainty in the concentration due to pipetting. All experimental images are an average of 25 frames and were collected at 25°C and $C = 3.1$ mM.
nucleation and subsequent growth were highly dependent on concentration, as previously documented (25). Below the concentrations used in our assay, nucleation and fiber growth did not occur, while above the concentrations, nucleation proceeded too rapidly for reliable imaging of single HbS fibers. Because of this, imaging was constrained to HbS concentrations just above the solubility, comparable to that determined from centrifugation assays (25).

To precisely track the growing end of HbS fibers, we used a semi-automated algorithm previously developed for tracking the plus-end of microtubules labeled with fluorescent probes, which was modified slightly to accommodate the characteristics of DIC signal as compared to fluorescence (see Materials and Methods). Briefly, within the region of interest, points along the HbS fiber backbone were approximated by fitting the first derivative of a Gaussian to cross sections perpendicular to the direction of fiber growth (Fig. 1C), and then, the contrast values along the determined fiber axis were fit with a Gaussian survival function, where the function mean was taken to be the position of the fiber end (Fig. 1, D and E). Tracking results from our algorithm agreed well with the growth dynamics observed in kymographs of the same fiber (Fig. 1, F and G). While most fibers grow at an approximately constant rate, we note that there is notable variability around this rate (Fig. 1G, inset). It is this variability of assembly that is important for quantifying the underlying addition-loss kinetics of HbS subunits (14). We also found that the average net assembly rates at both 25° and 37°C were consistent with previous results from multiple assays (Fig. 1H). On the basis of these observations and those discussed above, we conclude that HbS fiber nucleation and growth in our assay are consistent with those documented in previous studies of both single fiber and bulk assembly.

Individual HbS fibers are known to interact laterally to form bundles upon assembly (26–28). Because HbS fiber diameter is below the diffraction-limited resolution of the microscope (20 nm versus ~200 nm), it is feasible that the DIC signal that we are tracking is not from a single fiber but rather from multiple fibers bundled together (11). Consistent with fiber bundling, we observed instances where multiple HbS fibers grew along the same trajectory, within the diffraction limit. As shown in Fig. 2 (A and B), what appears by eye to be a single HbS fiber is actually multiple fibers that have sequentially grown along the same trajectory (Fig. 2B and movie S2). In analyzing a cross section of the fiber, we observed a quantal increase of the magnitude of the DIC signal that coincided with the appearance of each sequential fiber (Fig. 2, B to E), suggesting that the DIC signal is directly proportional to the total number of HbS fibers. As an additional test, we compared the DIC signal intensity in multifiber

![Fig. 2. DIC signal is directly proportional to the number of HbS fibers.](image-url)
regions, defined by heterogeneous nucleation or the intersection of HbS fibers, to the signal in immediately adjacent regions (Fig. 2F). We found that the signal in multifiber regions had a median value that is 2.2-fold higher than the adjacent regions (Fig. 2G), which is consistent with the presence of two fibers. Thus, the amount of DIC signal can be used to directly quantify the number of fibers within a region of interest. For all fibers tracked under the same conditions as shown in Fig. 2, we found that the mean (± SEM) DIC signal was 46.0 ± 1.2, quantitatively consistent with the signal contributed by each individual fiber in Fig. 2E. Therefore, we conclude that we are tracking two fibers. Thus, the amount of DIC consistent with the presence of two fibers. Thus, the amount of DIC

...drift equation given by

\[\langle \Delta L^2 \rangle = v_g^2 \Delta t^2 + 2D_p \Delta t + \sigma^2\] (2)

where \(\Delta L\) is the length change over a given time step \(\Delta t\), \(v_g\) is the net assembly or growth rate, \(D_p\) is the effective diffusion coefficient of the dynamic end, and \(\sigma^2\) is the measurement noise. The two unknown values of \(k_{on}\) and \(k_{off}\) were then estimated from the two equations for \(v_g\) and \(D_p\)

\[v_g = a(k_{on}C - k_{off})\] (3)

\[D_p = \frac{\sigma^2}{2}(k_{on}C + k_{off})\] (4)

where \(a\) is the change in length contributed by a single monomer (0.45 nm for HbS fibers; 6.3 nm/14 individual filaments). This approach is valid when the net assembly rate is small (i.e., \(v_g < 10\) nm/s); however, under our experimental conditions, HbS fibers grow comparatively fast (Fig. 1H), and therefore, the growth rate, or quadratic term in Eq. 2, dominates the MSD fit and causes the fit to the other terms to be unreliable. To overcome this and get a more accurate estimate of the effective diffusion coefficient, we took advantage of the statistics of diffusive motion, where the mean and variance of diffusive movements are given by \(\mu = vt\) and \(\sigma^2 = 2D \Delta t\), respectively. Thus, the mean (\(\mu_{\Delta L}\)) and the variance (\(\sigma_{\Delta L}^2\)) of the distribution of length displacements (\(\Delta L\)) in time \(t\) are related to \(v_g\) and \(D_p\) according to

\[\mu_{\Delta L} = v_g t\] (5)

\[\sigma_{\Delta L}^2 = 2D_p t + \sigma^2\] (6)

where \(\sigma^2\) is the experimental noise in estimating \(\Delta L\), equal to twice the square of the single–time point precision of tracking the fiber end \((\sigma^2 = 2\sigma^2)\). Averaging sequential image frames in time such that \(t = n\Delta t\) in Eqs. 5 and 6, where \(n\) is the number of frames and \(\Delta t\) is the image acquisition time for a single frame, the experimental noise (i.e., due to photon counting) will decrease approximately as \(n^{-1}\) (Fig. 3A). Comparatively, combining Eqs. 4 and 6, we can see that the variance due to on-off kinetics will increase linearly with \(n\) and have a slope equal to \(2D_p\) (Fig. 3A). Therefore, a point exists where the two trends intersect, such that, below this point, the experimental noise dominates and, above this point, the dynamics of assembly dominate the variance of the \(\Delta L\) distribution (Fig. 3A, dashed line).

To find the point in our experimental data where the two variance sources intersect, we tracked the fiber end position and then estimated the variance of the length displacements between sequential time points under varying extents of frame averaging. As shown in Fig. 3 (B to E), the displacement mean and variance follow the expected trends; the mean increases linearly throughout, while the variance initially drops with low levels of frame averaging (due to experimental noise reduction) and then begins to rise with further averaging (due to assembly dynamics). We find that the variance due to on-off kinetics at 25°C begins to dominate the displacement variance around \(n = 15\) frames (0.6 s) (Fig. 3C). By fitting the mean and variance trends with Eqs. 5 and 6, respectively, we independently obtained estimates of the growth rate (\(v_g\)) and effective diffusion coefficient (\(D_p\)), as well as the experimental noise (\(\sigma^2\)). Combining Eqs. 3 to 6, we see that we have three unknowns (\(k_{on}\), \(k_{off}\), and \(\sigma\)) that fit to two experimental trends (\(\mu_{\Delta L}\) and \(\sigma_{\Delta L}^2\)). This method yields more reliable estimates, relative to MSD fitting, when the growth rate is high.

Similar to Gardner et al.’s (14) approach, once estimates of \(v_g\) and \(D_p\) are obtained, we can solve for the two unknowns, \(k_{on}\) and \(k_{off}\), using Eqs. 3 and 4 above. Because of the high concentration of hemoglobin, we replaced \(C\) in Eqs. 3 and 4 with \(C_{eff} = \gamma C\), where \(C_{eff}\) is the effective concentration resulting from the reduction in the total volume due to hemoglobin self-crowding (29). Activity coefficients, \(\gamma\), were estimated as described in (11). From the length displacement analysis, we estimate \(k_{on} = 3.0 \pm 0.12\) \(\mu M^{-1} s^{-1}\) (mean ± SEM); \(n = 126\) HbS fibers) per HbS fiber end at 25°C (Fig. 3, B and C). This value is more than an order of magnitude higher than previous estimates obtained using the 1D framework (0.05 to 0.2 \(\mu M^{-1} s^{-1}\)) (11–13, 17). We note that the framework used here does not disregard the 1D concentration dependence of the net rate (compare Eqs. 1 and 3), but rather uses both the net rate (\(v_g\)) and the variability around that rate (\(D_p\)) to quantify the underlying addition-loss kinetics. Despite our comparatively high estimate in the on-rate constant, values from the 1D framework are consistent with the mean displacements observed here (Fig. 3D). With regard to the variance, however, we find that kinetic estimates from the 1D framework severely underpredict the experimentally observed values (Fig. 3E).

We performed the same analysis at physiological temperature (Fig. 3, F and G), where we estimate \(k_{on} = 4.6 \pm 0.26\) \(\mu M^{-1} s^{-1}\) (mean ± SEM; \(n = 110\) HbS fibers) per HbS fiber end. Similar values were obtained when performing an MSD analysis similar to that in (14) described above (Fig. S2). This higher value leads to a temperature dependence in the on-rate constant similar to that previously documented (11). However, the on-rate constants we estimate here are more than an order of magnitude higher than previous estimates (Fig. 4A and see Supplementary Discussion for additional details). As a result, we similarly estimate off-rates that are greater than those estimated using the 1D framework \((k_{off} = 5.2 \pm 0.2 \times 10^7\) and \(3.5 \pm 0.2 \times 10^7\) \(s^{-1}\) at 25°C and 37°C, respectively) (Fig. 4B). Further, the observed decrease in the off-rate from 25°C to 37°C, or equivalently from high to low HbS concentrations, shows that the off-rate is not constant but rather is concentration dependent. A concentration-dependent off-rate, such as we observe here and previously documented for microtubules (14), is in direct disagreement with the 1D
model, where the off-rate is assumed to be independent of concentration (Eq. 1). While previous estimates using the 1D framework adequately explain the net growth rate, they cannot explain the experimentally observed variability in HbS fiber growth. Thus, the actual rates must be closer to what we have estimated here. On the basis of these observations, we conclude that previous estimates using the 1D framework have systematically underestimated the addition-loss kinetics of HbS fiber assembly (discussed further in Supplementary Discussion).

Fig. 3. Rapid kinetics of HbS fiber self-assembly. (A) Projected trend in the variance of the length displacement ($\Delta L$) distribution as a function of frame averaging from the original acquisition rate (25 Hz; $t = 1/25$ s). Blue and red lines are the portion contributed by the experimental noise and assembly dynamics, respectively, while the black line is the sum of the two. The dashed line marks the edge of the regimes where either experimental noise or assembly dynamics dominate the experimentally measured variance, as determined by intersection between the projected trends. (B and C) Mean (B) and variance (C) of the $\Delta L$ distribution as a function of frame averaging for samples of 3.1 mM hemoglobin at 25°C ($n = 126$ fibers from three samples). (D and E) Mean and variance values for all fibers at 25°C are shown (gray dots). Solid lines are the same as in (B) and (C). Dashed lines show the expected trend using the values of $v_g$ and $D_p$ from the trends observed in (11). In (E), the experimental noise contribution is the same for both the solid and dashed lines. Values are shown on a log scale to facilitate comparison against the projected trend from the 1D model. (F and G) Mean (F) and variance (G) of the $\Delta L$ distribution as a function of frame averaging for samples of 2.3 mM hemoglobin at 37°C ($n = 110$ fibers from three samples). Black lines in (B) and (F) are the best fit of Eq. 5. Black lines in (C) and (G) are the best fit of Eq. 6 to the values estimated from all fibers. Blue and red lines in (C) and (G) show the portion of the fit contributed by the experimental noise and assembly dynamics, respectively. In all plots, the open circles are the median value calculated across all fibers. Error bars are 95% confidence intervals obtained from bootstrapping.

Fig. 4. 1D model underestimates the kinetics of assembly for multistranded polymers. Estimated on-rate (A) and off-rate (B) constant as a function of temperature. Note that the rates shown here are per HbS fiber end, rather than per individual protofilament. Circles denote the values from this study, while diamonds are reported values from (11), squares are reported values from (12), and triangles are reported values from (13). Solid and dashed lines are the best-fit exponential trend to the data from this study and from (11), respectively. *P < 0.001 by one-way analysis of variance (ANOVA). (C) Single-time point tracking precision as a function of sampling frequency when using our semi-automated approach. Values are those extracted from the fit to the experimental noise shown in Fig. 3C for the data collected at 25°C.
DISCUSSION
By combining high-sensitivity imaging with contrast enhancement and semi-automated tracking, we were able to track fiber assembly with 55-nm precision at 1 Hz (Fig. 4C). To our knowledge, these are the highest spatiotemporal resolution measurements of single HbS fiber assembly to date. Other studies estimating HbS fiber kinetics or imaging HbS fiber assembly have relied on indirect measurements (11, 21) or on manual tracking and imaging technology that has been improved substantially in recent years (12, 13, 17), namely, scientific cameras with higher speed and sensitivity. An added benefit of this approach is that it is label free. Therefore, it will have more general applicability compared to approaches reliant on fluorescent probes (18, 19, 30), specifically to those biopolymers where a convenient tag may not be readily available. While our high-resolution measurements aided us in demonstrating that the on-off kinetics had previously been underestimated, the resolution of previous measurements is not specifically responsible. For example, the discrepancy between the experimentally observed variance of growth and that predicted from the 1D model becomes increasingly apparent at longer time scales (Fig. 3E). Rather, it is the misapplication of the 1D model to multistranded polymers that resulted in previous underestimates, the consequences of which lead to several important updates to our understanding of HbS fiber self-assembly, as we discuss below. The misapplication of the 1D model becomes apparent by quantitatively analyzing not only the mean rate of fiber growth but the mean and the variance in fiber growth together.

Overall, our nanoscale analysis now shows that HbS fiber growth is a rapid and highly inefficient process, where the net rate of assembly is the comparatively small difference between a large number of monomer addition and loss events. As an example, our on-rate constant estimate predicts that under physiological conditions (5 mM at 37°C), only 30,000 subunits out of the nearly 1 million addition events occurring every second are stably incorporated into the polymer (~4% efficiency). Conversely, previous estimates from the 1D framework would suggest that assembly under the same conditions is >95% efficient (see Supplementary Discussion for complete calculations). From this inefficient self-assembly process, where the vast majority of subunits binding to the polymer end are rapidly lost, it follows that as the total number of addition events increases, so too does the total number of loss events. This means that the off-rate is dependent on the free monomer concentration [Fig. 4B and discussed in (14)], as a result of the on-rate’s concentration dependence (on-rate = \( k_{on} [Y] \)). Thus, a fundamental assumption of the 1D model, that the off-rate is concentration independent, is incorrect for HbS fibers and multistranded biopolymers in general. This same assumption was invoked in the derivation of the equations used to quantify the bulk HbS assembly process through double nucleation (21). Therefore, the equations and the resulting quantifications of bulk kinetics need to be revisited in light of our results and analysis. Although we did not directly quantify nucleation kinetics in this study, the updated on- and off-rates measured here likely explain the observed variability of nucleation (31) and will undoubtedly have direct implications for nucleation kinetics, such as the inefficiency of homogeneous nucleus formation.

A perceived challenge to effective targeting of HbS assembly in the clinic is the high physiological concentration of hemoglobin requiring potential drugs to be administered at very high concentrations (15, 32, 33), beyond what is tolerable (34, 35). As a result, targeting intermolecular interactions with a small-molecule inhibitor has generally been rejected as a therapeutic treatment for sickle cell disease. This conclusion is expected when examining the self-assembly efficiency, as it effectively sets the minimum level of drug binding to hemoglobin required to perturb fiber growth. Previous estimates of kinetic rates suggest that self-assembly is >95% efficient, and therefore, drugs would have to be administered at saturating concentrations to substantially influence the self-assembly process (35). Our data, however, demonstrate that HbS fiber growth is highly inefficient (~4% efficiency), where addition and loss are nearly equal, suggesting that only a small perturbation is necessary to shift the overall reaction in favor of fiber disassembly. For example, a drug that produces a moderate effect (2 \( k_{off} T \) or 1.2 kcal/mol) on the intermolecular interactions would only need to bind to 5% of hemoglobin (62.5 \( \mu\)M total drug concentration) to inhibit fiber growth (see Supplementary Discussion for complete calculations). Therefore, it may be possible to treat affected individuals with compounds at concentrations that are only a fraction of the total amount of hemoglobin. This has been the basis for the therapeutic targeting of microtubules, which also exhibit rapid, inefficient growth (14) and are sensitive to drugs at concentrations 1000-fold lower than the concentration of tubulin (36, 37), including the most widely prescribed anticancer drug, paclitaxel, and other microtubule-targeting agents such as vinblastine and colchicine. We note that while this predicted effect to inhibit HbS fiber assembly is independent of nucleation, inhibiting fiber growth through targeting intermolecular interactions would, at the very least, reduce the number of sites available for heterogeneous nucleation. This would make the bulk assembly process more reliant on homogeneous nucleation, leading to longer delay times and slower exponential growth, thereby reducing the severity of the disease (38). Thus, our results indicate that targeting HbS on-off kinetics and the fiber growth process with drug treatment has the potential to be a viable approach for more effective treatment of sickle cell disease, and we are optimistic that this demonstration of feasibility may spur development of much needed antisickling therapies.

MATERIALS AND METHODS
Sample preparation
Sickle hemoglobin (H0392; Sigma-Aldrich, St. Louis, MO) samples were suspended in 0.1 M potassium phosphate buffer (pH 7.0). Before imaging, HbS samples were deoxygenated on ice under a vacuum for 1 hour, after which samples were then brought to the final imaging concentration of 0.1 M, which also exhibit rapid, inefficient growth (14) and are sensitive to drugs at concentrations 1000-fold lower than the concentration of tubulin (36, 37), including the most widely prescribed anticancer drug, paclitaxel, and other microtubule-targeting agents such as vinblastine and colchicine. We note that while this predicted effect to inhibit HbS fiber assembly is independent of nucleation, inhibiting fiber growth through targeting intermolecular interactions would, at the very least, reduce the number of sites available for heterogeneous nucleation. This would make the bulk assembly process more reliant on homogeneous nucleation, leading to longer delay times and slower exponential growth, thereby reducing the severity of the disease (38). Thus, our results indicate that targeting HbS on-off kinetics and the fiber growth process with drug treatment has the potential to be a viable approach for more effective treatment of sickle cell disease, and we are optimistic that this demonstration of feasibility may spur development of much needed antisickling therapies.

DIC microscopy
HbS fibers were imaged by DIC on a Nikon Ti2E stand (Nikon Instruments Inc., Melville, NY) equipped with a 1.4 NA (numerical aperture) condenser oil-immersion lens, 100× 1.49 NA Apo TIRF oil-immersion objective, and DIC N2 prisms (Nikon Instruments Inc.). Bias retardation of +5° (k/36) was achieved with a de Sénarmont compensator (Nikon Instruments Inc.) in the light path. Images were
collected and processed to subtract illumination background [similar to that described as part of the video enhancement process in (6)] at 25 Hz for up to 3 min with an Andor Zyla 5.5 sCMOS camera (Andor Technology Ltd., Belfast, UK) under control of NIS-Elements AR software (v. 5.02; Nikon Instruments Inc.). An additional 1.5x tube lens resulted in 150x total magnification during acquisition (42 nm per pixel).

**Image analysis**

Additional DIC enhancement performed after collection included local background subtraction, followed by sequential image averaging and 2 x 2 binning. For local background subtraction, an average of the first 500 frames (20 s) was used as a reference and subtracted from all frames in the movie. An offset of 100 was added to avoid negative values. Performing local background subtraction increased the DIC signal contrast of growing fibers specifically, beyond that accomplished by subtracting the illumination background alone. Frames used as the local background reference were not included in all subsequent analyses. Image averaging and binning to reduce the experimental noise of tracking were performed within the semi-automated tracking program (described below). Kymographs and cross-sectional scans of individual HbS fibers were performed in ImageJ with a linewidth of 3 pixels. Cross-sectional pixel values were fit to Eq. 7 below using MATLAB R2015b (The MathWorks Inc., Natick, MA). Reported DIC signal values are the values of I_F obtained from fitting of Eq. 7. Unless otherwise stated, all analyzed images were an average of 25 frames.

**Semi-automated tracking of DIC signal**

DIC signal of HbS fibers was tracked using TipTracker software (18, 30), previously developed for tracking the plus-ends of fluorescently tagged microtubules. The following modifications were made to accommodate the characteristics of the DIC signal as opposed to the fluorescent tags for which the algorithm was originally developed.

1. Fiber backbone positions were estimated via fitting pixel values along the fiber cross section \((x' \text{ and } y')\) axes with the first derivative of a Gaussian according to

\[
I(y') = I_{BG} + I_F \frac{(y' - y'_m)^2}{2 \sigma^2} \exp \left(-\frac{(y' - y'_m)^2}{2 \sigma^2}\right) \tag{7}
\]

where \(I_{BG}\) and \(I_F\) are the intensity scalars of the background and HbS fiber, respectively, and \(y'_m\) is the mean value taken to be the backbone position of the fiber. The plus-or-minus is determined by the direction of bias retardation; minus for the bias of +5° (λ/36) used in the setup described above.

2. The Gaussian survival function used to determine the position of the fiber end was fit to the DIC signal contrast along the fiber backbone, estimated by taking the absolute value of the difference between pixel values 1 Airy unit to the left and right of the determined fiber backbone \([x' \text{ and } y']\) axis described in (18).

All tracking was performed in MATLAB R2015b. To control for image artifacts affecting the analysis, individual fibers were excluded if another fiber or piece of debris was within ~1 μm of the tracked fiber end, if a heterogeneous nucleation event occurred within 2 μm of the fiber end, or if the fiber could not be tracked for consecutive frames totaling a minimum of 30 s.

**Statistical analysis**

Unless otherwise noted, data are means ± SEM. One-way analysis of variance (ANOVA) statistical tests were performed in MATLAB (R2015b) using the anova1 function. Exact \(P\) values, \(F\) statistics, and degrees of freedom for each test can be found in table S1.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/3/eaau1086/DC1

**Supplementary Discussion**

Fig. S1. HbS fiber nucleation and growth occur throughout the sample. Fig. S2. MSD analysis confirms variance analysis. Table S1. Exact values from ANOVA statistical tests. Movie S1. HbS fiber assembly. Movie S2. Bundle of HbS fibers grown along the same trajectory.

Reference (39)

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Acknowledgments

Funding: The research was supported by NIH grants R01GM76177 (to D.J.O.) and R01HL132906 (to D.K.W.). Author contributions: D.J.O. and D.K.W. conceived and supervised the study. D.K.W. and B.T.C. designed the experiments in consultation with D.J.O. B.T.C. performed all experiments and analysis and wrote the manuscript. All authors commented on and contributed to the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Original image and analysis data, as well as computer code, are available upon reasonable request to the corresponding authors.

Submitted 7 May 2018
Accepted 29 January 2019
Published 13 March 2019
10.1126/sciadv.aau1086

Citation: B. T. Castle, D. J. Odde, D. K. Wood, Rapid and inefficient kinetics of sickle hemoglobin fiber growth. Sci. Adv. 5, eaau1086 (2019).