Mechanisms of Homogeneous Nucleation of Polymers of Sickle Cell Anemia Hemoglobin in Deoxy State

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The primary pathogenic event of sickle cell anemia is the polymerization of the mutant hemoglobin (Hb) S within the red blood cells, occurring when HbS is in deoxy state in the venous circulation. Polymerization is known to start with nucleation of individual polymer fibers, followed by growth and branching via secondary nucleation, yet the mechanisms of nucleation of the primary fibers have never been subjected to dedicated tests. We implement a technique for direct determination of rates and induction times of primary nucleation of HbS fibers, based on detection of emerging HbS polymers using optical differential interference contrast microscopy after laser photolysis of CO-HbS. We show that: (i) nucleation throughout these determinations occurs homogeneously and not on foreign substrates; (ii) individual nucleation events are independent of each other; (iii) the nucleation rates are of the order of $10^6 – 10^8$ cm$^{-3}$ s$^{-1}$; (iv) nucleation induction times agree with an a priori prediction based on Zeldovich's theory; (v) in the probed parameter space, the nucleus contains 11 or 12 molecules. The nucleation rate values are comparable to those leading to erythrocyte sickling in vivo and suggest that the mechanisms deduced from in vitro experiments might provide physiologically relevant insights. While the statistics and dynamics of nucleation suggest mechanisms akin to those for small-molecule and protein crystals, the nucleation rate values are nine to ten orders of magnitude higher than those known for protein crystals. These high values cannot be rationalized within the current understanding of the nucleation processes.

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Introduction

Sickle cell anemia, or homozygous sickle cell disease, is a genetic disorder, which affects about 250,000 children worldwide every year; many of them have multiple strokes and die before they are two years of age. The disease is caused by an A-to-T mutation within the sixth codon of the $\beta$-globin coding region. As a result, the glutamic acid residues at the sixth position of the two $\beta$-subchains are replaced by non-polar valine. Unlike the normal hemoglobin (Hb) A, the mutated hemoglobin has a high propensity to polymerize when in the tense (T) state in the venous circulation. The polymerization alters the shape and rigidity of the red blood cells, and triggers a sequence of pathogenic consequences.

Polymerization of HbS is one example of a number of disorders caused by phase transitions of abnormal proteins, which include the eye cataract, Alzheimer’s, Huntington’s, and the prion diseases, etc. Phase transitions are ubiquitous in healthy organisms as well; examples include the formation and modification of the cytoskeleton, the suspected microcompartmentation of the cytosol, etc. Data on the kinetics of phase transitions provide insights into their mechanisms and governing physical parameters and may help in the understanding of their normal and pathological effects in living systems.

As many other first-order phase transitions, the polymerization of deoxy-HbS starts with nucleation, in which a certain number of molecules assemble into an embryo of the new phase.
Although the progress of HbS polymerization has been monitored by a variety of techniques (see Eaton & Hofrichter\(^8\) for a review), to date there exists just one set of data on nucleation rates.\(^{28,31,32}\) These detailed determinations use an elegant theoretical model\(^33\) to extract nucleation rates from the evolution of the intensity scattered from growing polymer fibers.

Here, we implement a method for direct simultaneous determination of two independent characteristics of the nucleation kinetics of HbS polymer fibers: the homogeneous nucleation rates and the nucleation induction times. After polymerization is induced by laser photolysis of carbon monoxide-saturated HbS, nucleation of individual fibers is monitored by optical microscopy with differential interference contrast (DIC) in slides of uniform thickness containing supersaturated solution. The rate of homogeneous nucleation and the induction times are determined from the evolution of the statistics of appearance of HbS spherulites.

The results obtained using this technique shed light on the following questions: is nucleation of HbS fibers compatible with the general theories of nucleation of first-order phase transitions? What are the typical nucleation rates of the deoxy-HbS polymers? How do the determined values compare to those leading to red cell sickling \textit{in vivo}? How do the determined values compare to those in other, protein and non-protein, systems? How applicable are the mechanisms determined from \textit{in vitro} determinations to the nucleation of HbS polymers \textit{in vivo}? How many molecules are in the polymer nucleus? Are they arranged in disks of seven pairs as in grown HbS fibers, or do they have a different arrangement?

**Results**

**Polymerization, nucleation, and nucleation rate determination**

In brief, the determinations of the nucleation rate consist of the following experimental procedures. A CO-HbS sample is held in a glass slide of 5 or 10 \(\mu\)m uniform thickness. An area of \(\sim 90\ \mu\)m diameter is continuously illuminated with green laser or lamp to photolyze CO-HbS to deoxy-HbS and sustain the deoxy form. DIC microscopy is employed to monitor the number of HbS fibers and spherulites appearing in the illuminated area. Assuming that each spherulite is generated by a single nucleation event (see arguments supporting this assumption in this subsection) we determine the number of nuclei in the illuminated volume for the time elapsed after illumination starts. Then the illuminated spot is moved to another, randomly selected location on the slide and the time evolution of the number of nuclei is recorded again. In most cases, this is done 82 times at each temperature. In some cases 200 and more determinations are carried out. The nucleation rate is determined as the ratio of the mean number of nuclei that appear for a certain time to the volume occupied by deoxy-HbS. Exhaustive details are provided in Materials and Methods below.

If polymerization is driven by low-to-moderate supersaturations, the fibers do not branch and the solid phase consists of separate HbS fibers, such as those in Figure 1(a).\(^{34}\) At the later stages of polymerization, the solution turns into a gel consisting of straight fibers (Figure 1(b)). Since spherulites are easier to detect than single fibers, we work at slightly higher supersaturations, which ensure spherulitic morphology of the HbS polymer phase illustrated in Figure 1(c).

As in previous work (e.g. Briehl\(^30\)), a basic assumption in the nucleation rate determinations below is that each spherulite is generated by a single primary nucleation event. The relative error introduced by this assumption is equal to the probability of having two nucleation events within the slide area occupied by one spherulite. This probability can be evaluated as the ratio of the area occupied by all spherulites in the slide to the total area, in which nucleation can occur. As shown below, nucleation rate data are extracted from images of slides with fewer than ten spherulites, each occupying an area of \(< 10 \ \mu\)m \(\times \ 1\ \mu\)m. The probability of having a second spherulite hidden under or within one of the ten is about \((10/5000)^2 \times 10 = 4 \times 10^{-5}\).

**Homogeneous nucleation or nucleation on a substrate?**

For tests of whether the primary nucleation of the observed spherulites is facilitated by the upper and lower slide covers, or dust particles in the solution, we record the locations of appearance of the spherulites in four repetitive identical runs at the same area of a slide. The runs were separated in time by 20–30 minutes, allowing complete dissolution of the spherulites nucleated in the preceding run. With slide thickness of 5 \(\mu\)m or 10 \(\mu\)m, buoyancy-driven convection due to possible thermal gradients is suppressed.\(^{35,36}\) Thus, possible particles in the solution could only be moved by Brownian diffusion, in which the expected displacement \(\Delta t \approx \sqrt{4Dt} \approx 10 \ \mu\)m for a \(\sim 1\ \mu\)m particle with diffusivity \(D \sim 10^{-9}\) cm\(^2\) s\(^{-1}\) over \(\Delta t = 1200\) s. The predicted displacement is comparable to the observed sizes of the spherulitic domains, i.e. if such particles serve as substrates for heterogeneous nucleation, the locations of the spherulites in subsequent runs would overlap. Figure 2 shows that no correlation exists between the locations and orientations of the spherulites in the four runs. We conclude that the nucleation events that lead to the spherulites are not facilitated at particular spots on the slide covers or by particles larger than \(1\ \mu\)m in the solution.

For further evidence for the mechanism of primary nucleation, we carried out several determinations of the mean (over \(\sim 100\) identical runs)
number of nuclei that appear in solutions held in slides of 5 \( \mu \)m and 10 \( \mu \)m thickness after identical time periods and with special care to ensure equal temperatures of the two runs, for details, see below. We found that the mean number of nuclei in the illuminated area in 10 \( \mu \)m slides is double the number in 5 \( \mu \)m slides, i.e. the number of nuclei generated over a certain time is proportional to the solution volume. Furthermore, we observed that spherulites are never firmly attached to the surface of a glass slide. The latter two observations rule out heterogeneous nucleation on the glass surfaces.

Statistics of nucleation

The time required for complete dissolution of the spherulites is usually comparable or longer than the time of spherulite nucleation and growth. Hence, repetitive runs to determine the nucleation statistics are carried out by illuminating randomly selected different areas in the slide.

Figure 3(a) shows that as spherulites grow, they get close to one another, fill the illuminated area, and interfere with the processes of further nucleation. Hence, we only count spherulites before their regions overlap. Furthermore, as shown below, the nucleation rates are determined from the earliest part of the data on spherulite numbers, where the numbers increase linearly with time.

In Figure 3(b), we show determinations of the number of spherulites \( n \) in the illuminated area as

![Figure 1](image1.png)

**Figure 1.** Morphologies of polymer fibers observed during deoxy-HbS polymerization visualized in 10 \( \mu \)m slides at 22 °C using differential interference contrast microscopy. (a) Single fibers ~20 nm thick observed at HbS concentration 170 mg ml\(^{-1}\). (b) Gel composed of long single fibers, conditions the same as in (a), long observation times. (c) Spherulites observed at HbS concentrations 200 mg ml\(^{-1}\) and higher. (d) Thick gel with no individual fibers discernible at long polymerization times with HbS concentration of 250 mg ml\(^{-1}\).

![Figure 2](image2.png)

**Figure 2.** Locations and orientation of spherulites in four consecutive runs made at the same area on the slide, with full dissolution of fibers between the runs. \( T = 22 ^\circ C, C_{HbS} = 232 \text{ mg ml}^{-1} \).
a function of the time, \( t \), elapsed after CO-HbS photolysis. These determinations were performed at identical conditions, within the same slide. We see that even in the limited number of runs displayed, the delay times before the appearance of the first spherulites vary from 8 s to 13 s, the number of spherulites, for instance at 16 s, varies between one and five, and the shape of the \( n(t) \) dependence is unpredictable. These three facts illustrate the inherently stochastic nature of the processes of fiber nucleation.

In a stochastic process, the number of nuclei that appear in a certain volume is a random variable: repetitions of an experiment under identical conditions could give, for instance, one, ten, or no nuclei. Representative statistical distributions of the number of nuclei resulting from 200 experiments under identical conditions are presented in Figure 4.

To verify if the individual nucleation events are independent of each other, a prerequisite for the applicability of the general nucleation theories, we determine the nucleation statistics. We compare the distributions of the numbers of nuclei in Figure 4 with Poisson’s law:

\[
P(n) = \frac{N^n}{n!} \exp(-N)
\]

where \( n \) is the number of nuclei that appear in volume \( V \) during nucleation time \( t \), \( N \) is the mean number of nuclei in all runs with the same \( V \) and \( t \) reflected in the distribution.

For each distribution, we calculate \( N_{\text{fit}} \) from the best fit to equation (1) and the algebraic mean of the data in the distribution \( N \). The expression \( 1 - \sqrt{N/N_{\text{fit}}} \) was evaluated and its closeness to 0 was used as a criterion for the correspondence of the actual distribution of the data to Poisson’s law.\(^{37}\) In all cases, it was < 0.065. For further tests of the goodness of the fit to the Poisson’s law, equation (1), we calculate the parameter \( \chi^2 \):

\[
\chi^2 = \sum_n \frac{(F_n - P(n)N_{\text{fit}})^2}{P(n)N_{\text{fit}}}
\]

where \( F_n \) are the measured frequencies from Figure 4 and \( P(n) \) are Poissonian best-fit values. In most such determinations, for instance the data in Figure 4, the \( \chi^2 \) values correspond to confidence levels in the Poissonian character of the distributions of 90% or better; in a few cases the confidence level was as low as 80%. The relative error in determination of \( N \) is evaluated as \( \sqrt{N/(\text{number of runs})} \). Thus, for 100 runs yielding on the average one nucleus, it is 0.1.

Comparisons of the distributions of the spherulites in five series of 200 repetitive runs under identical conditions show (data not displayed) that although the number of spherulites is random, the statistical distributions corresponding to experiments performed under the same conditions are reproducible. All five distributions have the same shape, follow Poisson’s law, and have very similar mean values.

The good correspondence of the experimentally determined distributions to Poisson’s law shows that all individual nucleation events are independent of each other. Thus, the statistics of nucleation of the sickle cell hemoglobin polymers indicate that under the conditions probed here it follows the general laws of nucleation of first-order phase transitions.

**The homogeneous nucleation rates**

Figure 4 shows that with increasing nucleation time \( t \) the distribution of the number of spherulites shifts to the right, changing from near-exponential at short times to near-Gaussian at long times. Figure 5 shows that the time dependencies of mean number of spherulites found from 82 independent runs have a characteristic shape: after an initial delay period, when no nuclei are seen, the number increases linearly with time. These linear parts of the dependencies in Figure 5 correspond to steady-state nucleation, characterized by the
nucleation rate $J$. The last part of the dependencies reflects saturation due to growth of spherulites and overlapping of their supply fields. These data were not included in determinations of the nucleation rates. Note that all points in Figure 5 were taken from images similar to the first five frames in Figure 3(a), i.e. where the interaction between spherulites is not apparent. Thus, the time dependencies of the averaged numbers of nucleated spherulites offer a stricter criterion for non-interacting spherulites, from which nucleation rates can be extracted.

The nucleation rate $J$, i.e. the number of nuclei that appear in a unit solution volume within a unit time, is determined for each dependence in Figure 5 as the slope of the straight line characteristic of the steady regime of nucleation. Figure 6(a) shows the dependence of the homogeneous nucleation rate $J$ on temperature $T$ for a solution of deoxy-HbS with concentration $C = 232 \text{ mg ml}^{-1} = 3.32 \text{ mM}$ in $0.15 \text{ M}$ potassium phosphate buffer with $\text{pH} = 7.35$. The values in Figure 6(a) come from two independent experiments, starting from solution preparation, as discussed in Materials and Methods below. The good correspondence between the two data sets is evidence for the reproducibility of the determinations by the technique used here.

As expected, the nucleation rate $J$ in Figure 6(a) is a strong, exponential function of temperature. The values of nucleation rate from this and other runs at similar HbS concentrations and temperatures are in the range $10^5$–$10^8 \text{ cm}^{-3} \text{ s}^{-1}$. Converting to units mM s$^{-1}$ as others,$^{31,32}$ $J = 10^8 \text{ cm}^{-3} \text{ s}^{-1} = 1.66 \times 10^{-10} \text{ mM s}^{-1}$,
with \( \log J = -9.78 \). The published determinations\(^{31,32} \) were carried out at higher HbS concentrations and in the 25–35 °C temperature range. If one extrapolates the decrease of \( J \) (denoted with \( f_0 \))\(^{31,32} \) with decreasing HbS concentration from 4.26 mM = 275 mg ml\(^{-1} \) to 3.92 mM = 253 mg ml\(^{-1} \) at 25 °C reported by Cao & Ferrone\(^{32} \) to the concentration that we use, a value close to ours obtains.

### The driving force for polymerization

To evaluate the thermodynamic supersaturation

\[
\Delta \mu / k_B T = (\mu_{\text{polymer}} - \mu_{\text{solution}}) / k_B T
\]

(\( \mu \) is the chemical potential of the HbS in the respective phase, \( k_B \) is the Boltzmann constant and \( T \) is the absolute temperature) at the determination temperatures, we use the virial expansion\(^{39-46} \) limited to the sixth-order term as justified:\(^{42,43} \)

\[
\Delta \mu (C, T) / k_B T = \ln \frac{C}{C_{\text{eq}}(T)} + \sum_{i=1}^{6} \frac{i+1}{i} B_{i+1} [C]^i - C_{\text{eq}}(T)^i
\]

(3)

where \( C \) is the deoxy-HbS concentration, and \( C_{\text{eq}} \) is its value at equilibrium with the polymers. The values of the virial coefficients \( B_i \) are obtained using the expressions for non-interacting hard spheres from Hill\(^{39} \) for justification, see Minton\(^{42,43} \).

\[
B_2 = \frac{8V}{2M}, \quad B_3 = \frac{15V^2}{3/2M}, \quad B_4 = \frac{24.48V^3}{4/3M},
\]

\[
B_5 = \frac{35.3V^4}{5/4M}, \quad B_6 = \frac{47.4V^5}{6/5M}, \quad B_7 = \frac{65.9V^6}{7/6M}
\]

(4)

The specific volume \( V \) was determined from fits to re-plotted data on sedimentation equilibria\(^{41} \), best fits are obtained with \( V = 0.79 \text{ cm}^3 \text{ g}^{-1} \), close to the values for other proteins\(^{44,45} \) and to the Hb value reported.\(^{8} \) Since the virial coefficients for non-interacting spheres are temperature-independent, the only temperature dependence in equation (3) comes from \( C_{\text{eq}}(T) \):

\[
C_{\text{eq}}(T) = 0.319 - 0.000883(T - 273.15) + 0.000125(T - 273.15)^2
\]

(5)

We find that the supersaturation levels are moderate: for \( C = 232 \text{ mg ml}^{-1} \) as in Figure 6(a), \( \Delta \mu / k_B T \) does not exceed 1.7 even at the highest temperature probed.

### Nucleation induction time

An advantage of the method used here is that it allows independent determinations of the nucleation rate and of the nucleation delay time. As Figure 5 shows, we determine the nucleation delay time from the intersection point with the time axis of the dependence of the mean number of nuclei on time. Another possibility is to average the individual delay times in plots similar to those in Figure 3, which would yield a different characteristic of nucleation kinetics. As shown by Kashchiev,\(^{46} \) the delay time resulting from the former method contains the nucleation induction time \( \theta \), and hence, this is the method used here. The time \( \theta \) is a measure of the rate of transformation of the molecular distribution in a metastable single-phase solution to the distribution in a solution in which nucleation of the new phase proceeds at a steady rate.\(^{47,48} \)

The delay times are plotted as a function of temperature in Figure 6(b). The data in the two series diverge, while the reproducibility of the nucleation rates in Figure 6(a) is preserved. This discrepancy illustrates the independence of these two characteristics of the nucleation kinetics; an understanding of the physical phenomena underlying it is still missing at his point. The delay times are the sums of two components: the nucleation induction times \( \theta \) and the instrument detection times. The instrument detection times are determined by the resolution of the microscope of \( \sim 0.5 \mu m \) and the growth rate of the spherulites. We measure the spherulite growth rates as 1.15–2.5 \( \mu m \text{ s}^{-1} \) at 19 °C.
and \( C = 232 \text{mg ml}^{-1} \) and hence the detection times are < 1 s. Thus, the apparent delay times in Figure 6(b) are good approximations to the respective \( \theta \).

**The nucleus size**

The nucleation theorem has been used to determine the nucleus size from data on the dependence of the homogeneous nucleation rate \( J \) on supersaturation \( \Delta \mu \).\(^{46,49,50}\) For non-isothermal data sets, where supersaturation \( \Delta \mu \) is varied via temperature, a more recent form of the nucleation theorem applies. The resulting method employs two data sets: the homogeneous nucleation rate \( J \) and the nucleation induction time \( \theta \):

\[
n^* = k_B \frac{d}{d \Delta \mu} [T \ln(\theta)] + \frac{k_B}{\Delta \nu_e} \ln(C_0) \tag{6}
\]

where \( C_0 \) is the concentration of single HbS molecules in the solution, and \( \Delta \nu_e \) is the entropy of HbS polymerization.\(^{46}\) Equation (6) is derived assuming that \( J \) and \( \theta \) are in the same units, such as \( \text{m}^{-3} \cdot \text{s}^{-1} \).

Figure 7 displays the nucleation kinetics data from Figure 6 plotted in the coordinates of equation (6), i.e. the product \( T \ln(\theta) \) with \( J \) rescaled in \( \text{m}^{-3} \cdot \text{s}^{-1} \), as a function of the supersaturation \( \Delta \mu \) rescaled with \( k_B \). The theories related to the nucleation theorem do not treat the intercept of the dependence of \( T \ln(\theta) \) on \( \Delta \mu \). Thus, while it is likely that the different intercepts of the two lines in Figure 7 are linked to the deviations in induction times between the two series in Figure 6(a), the physical phenomena that underlie these differences are unclear to us. However, for the purposes of determination of the nucleus size, it is sufficient that the slopes of the linear fits are 6.9 for both data series in Figure 7.

To evaluate the second term on the right-hand side of equation (6), we use that

\[
T \Delta S = 7.4 \text{kcal mol}^{-1} \quad \text{at} \quad 25 ^\circ \text{C}, \tag{7}
\]

\( \ln(C_0) \equiv 4.5 \). Combining this value with the slope from Figure 7, we get for the nucleus size:

\[
n^* \approx 11.4
\]

i.e. sizes of 11 or 12 molecules are within the error limits of this determination.

**Discussion**

**Nucleation of HbS polymer fibers**

The polymerization of deoxy-HbS has been discussed in terms of a double nucleation model.\(^{29}\) The first step in polymerization is homogeneous nucleation, in which single fibers are randomly generated in the bulk of a supersaturated solution. As the fibers grow, they serve as substrates for the nucleation of new fibers.\(^{10}\) This leads to thickening and branching of the fibers.\(^{51,52,53}\) As with numerous other phase transformations proceeding via nucleation,\(^{51,52,53}\) the rate of polymerization was found to be a very strong, likely exponential function of the concentration of HbS. Depending on the range of concentrations probed, the strong dependence has been described as tenth to 20th power to 30th and 50th power.\(^{32}\)

The nucleation of secondary fibers on an existing fiber is often referred to as “heterogeneous nucleation”.\(^{8,28,29}\) Typically, the term heterogeneous nucleation is used for nucleation on a foreign substrate.\(^{46,56,57}\) Nucleation on a substrate is energetically different from that in bulk of the old phase; the wetting of the substrate by the new phase is expected to decrease the nucleation barrier and render heterogeneous nucleation faster by many orders of magnitude. In practice heterogeneous nucleation is widespread and includes nucleation on foreign molecules (impurities), microscopic particles (dust or bubbles), etc. It may occur concurrently with homogeneous nucleation and care must be taken in data interpretation to distinguish between homogeneous and heterogeneous nucleation. In the second stage of HbS polymerization, the substrate is the newly appearing phase, i.e. the substrate is not a foreign object, and it is available only after HbS polymerization has started. In another deviation from “classical” heterogeneous nucleation, it is not always faster than homogeneous nucleation; at low-to-moderate supersaturations single un-branched fibers, such as those in Figure 1(a), are often observed.\(^{9,10,58}\)

There have been some early claims that sickling is akin to gelation of a colloid suspension supported by the fact that completely polymerized samples behave rheologically as a gel.\(^{13}\) This interpretation was contradicted by the finding that unlike gels, the polymer has constant density and structure, independent of external conditions for polymerization,\(^{60,61}\) indicating that HbS
Homogeneous nucleation or nucleation on a substrate

The results in Figure 2 do not provide evidence against heterogeneous nucleation on particles or pieces of the cell membrane smaller than 1 μm, or on protein molecules larger than hemoglobin; each of these could serve as centers of heterogeneous nucleation. Indeed, if these entities have sizes of 100 nm or smaller, they could move about the solution volume driven by Brownian diffusion and in subsequent runs induce nucleation at different locations. If their concentrations were constant, they would not lead to dependence of the number of nucleated spherulites on the solution volume. However, dynamic light-scattering characterization of Hb solutions prepared following procedures identical with those used here showed a single species of a size between 5 nm and 6 nm, compatible with the 5.5 nm known for hemoglobin. The high limit of the detection range of these determinations is above 1 μm. In view of the high sensitivity of light-scattering to larger species (e.g. Eisenberg & Crothers), we conclude that the sensitivity of light-scattering to larger species (e.g. volume upon polymerization) has been determined. It has recently been emphasized that this and other phase transitions, in which an ordered solid (crystal, fibril, polymer) emerges for a dilute and disordered fluid (gas or solution) should be viewed as a transition along two order parameters, density and structure. Thus, the fibers and spherulites in Figure 1(a) and (c) have a higher concentration of HbS molecules than the surrounding solution, and the molecular arrangement is ordered.

The nucleation rates

The values of J in Figure 6(a) are comparable to those occurring in the cytosol of non-permanently sickled erythrocytes. Indeed, a red blood cell whose volume is ~94 femtoliters = 10^{-10} cm^{-3} spends from 10 s to 20 s in the venous circulation. During that time, one or a few nucleation events, followed by fast growth (as shown above, growth rates of the polymers are a few μm s^{-1}) and branching, may lead to sickling of the cell. Thus, the nucleation rates of sickling of non-permanently sickled cells are of the order of 10^{9} cm^{-3} s^{-1}.

Note that the conditions in the red cells are different from those in our experiments. Most importantly, the HbS concentrations in the sickle red cells are in the range 320–460 mg ml^{-1}, with a consistent mean of ~360 mg ml^{-1} and this should result in significantly faster nucleation rates. The high HbS concentration is likely compensated by the partial oxygenation of the HbS due to the residual oxygen vapor pressure of ~40 Torr, 70% of the Hb is in oxy-form. Furthermore, the red cell cytosol contains several compounds in concentrations comparable to that of HbS whose effects on polymerization and in particular on nucleation have only partly been studied. Despite these factors that may affect nucleation, the similarity of the nucleation rates suggests that the mechanisms of nucleation deduced from in vitro experiments are a relevant first step in the understanding of polymerization in the red cells of sickle cell anemia patients.

The values of J of sickle cell hemoglobin polymers are seven to ten orders of magnitude higher than those of formation of other ordered solid phases of proteins, summarized in Table 1. In fact, the HbS polymer nucleation rates are similar to the nucleation rates of water droplets at atmospheric pressure and room temperature; the phase-transition rates of small molecules are

<table>
<thead>
<tr>
<th>Protein</th>
<th>C (mg ml^{-1})</th>
<th>Δμ/k_BT</th>
<th>J (cm^{3} s^{-1})</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>30–80</td>
<td>3–3.7</td>
<td>0.01–1</td>
<td>50,83,99</td>
</tr>
<tr>
<td>Insulin</td>
<td>1–3</td>
<td>3.2–3.7</td>
<td>0.01–0.1</td>
<td>L. Filobelo, unpublished</td>
</tr>
<tr>
<td>Hemoglobin C</td>
<td>10–20</td>
<td>2.5–3</td>
<td>~0.01</td>
<td>66</td>
</tr>
<tr>
<td>Apoferritin (estimates)</td>
<td>0.1–1</td>
<td>2.2–3.9</td>
<td>0.1–1</td>
<td>100,101</td>
</tr>
</tbody>
</table>
expected to be significantly faster. While the protein concentrations in the determinations in Table 1 were significantly lower than the concentrations of HbS in the determinations discussed here, the supersaturation levels were higher. Since the nucleation rate is expected to be a linear function of concentration and exponential function of supersaturation,46,81,82 the huge discrepancy cannot be solely attributed to the action of protein concentration. One could argue that the nucleation rates for proteins crystals were determined under conditions optimized for crystal perfection and are not the maximum possible for a given protein; often, multiple protein crystals form in a container. Even if one allows for $10^3 \times$ faster nucleation of crystals under “unfavorable” conditions, this still leaves a discrepancy of four or five orders of magnitude between the two types of nucleation.

This discrepancy cannot be understood at this point. Still, we would like to offer two hypotheses, to be tested in further work. One can speculate that it may be linked to the lower, one-dimensional translational symmetry of the HbS polymers. Polymerization is a phase transition occurring along two order parameters, density and structure, and arguments have been presented suggesting that such transitions occur when a structure fluctuation is superimposed on a density fluctuation.83–86 Thus, it is feasible that a lower degree of symmetry of the new phase could lead to lower barriers for the second stage of the process, ordering, occurring within a density fluctuation.

Another possibility also linked to the superposition of density and structure fluctuations is that the high nucleation rates of HbS polymers stem from the high fluidity of the concentrated hemoglobin solution within the quasi-droplet of the density fluctuation. As expected for suspensions of non-interacting particles, the viscosity of hemoglobin solutions at concentrations similar to those in the red cell cytosol is $\sim 10$ cP ($1 \text{ P} = 10^{-1}$ Pa s),87,88 i.e. barely above that of the solvent, while solutions of other proteins with concentrations $\sim 200$ mg ml$^{-1}$ are very viscous or gel-like. While the high fluidity of the concentrated solutions should lead to linearly faster kinetics of ordering, it also leads to faster rate of probing of various structures, i.e. of density fluctuations within the quasi-droplet of the density fluctuation, and this may result in exponentially faster rate of finding of the “right” structure. In support of this hypothesis, we note that dense liquid phases of HbS enhance nucleation and growth of HbS polymers.34 This is in contrast to the viscous dense liquid phases of other proteins,44,90 which suppress nucleation83 and growth90 of protein crystals.

### The nucleation induction time

The delay time and the nucleation induction time $\theta$ determined here are inherently different from the nucleation time lag or the 1/10 time determined by others,28,29,31,32,90 the latter two represent expectancy times until a polymer reaches a point from which light is scattered, or at which the solution turbidity is monitored. As such, they are linked in a complex way to the mean time separating nucleation events in steady state.46 As discussed above, the induction time $\theta$ characterizes the transformation of the molecular distribution in the meta-stable single-phase solution to the distribution in a solution in which nucleation proceeds at a steady rate.47,48

Theoretically, the nucleation induction time $\theta$ can be evaluated as:

$$\theta = \frac{2}{3} \pi Z f^*$$  \hspace{1cm} (8)

The parameter $Z$, the Zeldovich factor, is also an important component of the pre-exponential factor of the nucleation rate expression.46,47 It is proportional to the radius of curvature of the free-energy barrier for nucleation around its maximum; its lack of dimension stems from the scaling of the free energy with $k_B T$ and using the number of molecules in the near-critical cluster as a spatial variable. The Zeldovich factor accounts for the deviations of the cluster size distribution from that in the state of forced equilibrium in a nucleating system, assumed in earlier nucleation theories. This equilibrium assumption requires the action of a mechanism of decay of supercritical clusters, while in fact supercritical clusters undergo uninhibited growth.91 Typical estimates are $Z \approx 0.1$ at low supersaturations typical of phase transitions with small molecules.82 For large biological macromolecules, for which the characteristic supersaturations during nucleation are several-fold the thermal energy, $Z \approx 0.01$ are likely.92 Note that evaluations of $Z$ using equation (6) are necessarily only approximate: the small size of the HbS polymer nuclei suggests that the addition or detachment of single molecules to near critical clusters leads to significant changes in its thermodynamic characteristics. Thus, continuous considerations, such as those behind equation (6), can only provide semi-quantitative understanding of the nucleation processes.

The parameter $f^*$ is the frequency of attachment of molecules to the critical cluster (nucleus). It can be evaluated from the rate of growth of $\sim 1 \mu \text{m s}^{-1}$, see determination above, as $f^* = 180$ molecules $\text{s}^{-1}$. Using these values of $f^*$ and $Z$, we get $\theta \approx 12$ s, with shorter $\theta$ if higher attachment rates apply. Although the induction times in the two series of determinations in Figure 6(b) differ, especially at low $T$ values, for both series they are of the same order of magnitude as this estimate. In further agreement with the predictions of the Zeldovich theory, they decrease as $T$ is increased.

### The nucleus size

The most important characteristic of the nucleation process is the nucleus size $n^*$. The nucleus is
a cluster that has equal probabilities to grow and dissolve and is thus in unstable equilibrium at the peak of the free energy landscape along the nucleation reaction pathway. The size of the nucleus largely determines the value of the free energy at this peak, the free energy barrier for nucleation. The nucleation theorem examines how the nucleation barrier varies in response to supersaturation changes and shows that the respective derivative allows determination of the nucleus size. Recently, it has been pointed out that the nucleation theorem relies on very few confining assumptions about the nucleation process, and is thus a general, system-independent nucleation law.

The earliest estimates of the nucleus size for HbS polymerization were based on determinations of the delay time $t_d$ of polymerization monitored via the solution turbidity. In a quasi-chemical approach, the apparent reaction order $m$ was determined as a slope of the plot of $\log(t_d)$ versus $\log(C_C)$, obtaining a value around 32. In subsequent investigations, the necessity to account for the non-ideality of HbS solutions was acknowledged, bringing the reaction order to 9 or 6. Still, determinations of the nucleus size $n^*$ from the reaction order remained a difficult problem, with estimates ranging from $n^* = m + 1$ to $n^* = 2m$.

In another series of studies, the apparent reaction order was determined from the time evolution of the HbS polymerization monitored with light-scattering. Homogeneous nucleation rates were extracted from the distributions of the time to achieve one-tenth of the final reaction yield, assuming that the primary nucleation of HbS polymers is the only source of stochasticity of the data. The apparent reaction order was $47 \pm 5$. The authors found that no simple relation of the reaction order of the tenth-time to molecular parameters exists and employed the double nucleation model to determine the size of nuclei as $7.31$. Note that this result, obtained at higher supersaturation, does not contradict our finding: the nucleus size is expected to decrease at increasing supersaturation.

Thus, the determination of nucleus size using the nucleation theorem offers advantages: the nucleation theorem is a general nucleation law, which does not rely on assumptions about the kinetic pathway or the structure of critical nucleus. The data used in the evaluation of $n^*$ are the dependencies of $J$ and $\theta$ on temperature and the thermodynamic properties of the HbS solution.

The determination of the nucleus size of the HbS polymers calls attention to an interesting point: $n^*$ is smaller then 14, the number of HbS molecules in the cross-section of the fiber. Obviously, such small nuclei cannot contain one, or two, or several 14-member “disks”. The question of whether the nucleus contains 10–12 molecules from the 14 in the cross-section, or five or six pairs of molecules in one of the Wishner–Love double strands, or another ordered, or an altogether disordered configuration is an open one at this point.

**Perspectives for future work**

A line of intriguing and relevant questions arising from analogies to recent findings on the nucleation mechanisms of crystals and on the phase behavior of hemoglobin mutants have not been addressed here. Some of these are related to the fundamentals of the nucleation mechanisms: is the HbS polymer nucleus ordered or disordered? Does nucleation occur in one or two steps, i.e. is there a disordered precursor to an ordered HbS critical cluster? What is the exact shape and structure of the critical cluster? Others are related to the participation of the cell membrane and other red cell cytosol components in the nucleation process: is HbS polymer nucleation affected by any of the red cell cytosol components present in concentration comparable to those of HbS? Is the nucleation of HbS polymers enhanced at certain areas of the inside of the red cell membrane?

**Materials and Methods**

**Solution preparation**

Hemoglobin S was isolated from the blood of sickle cell anemia patients kindly provided by Dr R. E. Hirsch from Albert Einstein College of Medicine. The blood was washed in isotonic 0.9% (w/v) NaCl solution and centrifuged to remove white blood cells. The red blood cells were lysed with deionized water and the solution was centrifuged again to remove cell membranes. The remaining Hb solution was filtered through a 0.45 μm Sterivex-HV filter. To purify HbS, the solution was dialyzed into a large volume of 20 mM Tris buffer (pH 8.5), and loaded onto a Q-Sepharose FPLC column (XK 50, Amersham Biosciences). Separation of different Hb variants was achieved by elution using a gradient of NaCl. The purity of the fractions was checked by gel electrophoresis (PhastSystem, Amersham Biosciences).

After the purification procedure, the gels showed a single band of HbS. The sample was concentrated by centrifugation in Centricon Plus-20 filters (Millipore). For polymerization experiments, the solution was dialyzed into 0.15 M potassium phosphate buffer (pH 7.35), using Slide-A-Lyzer Cassettes (Pierce). The solution was finally concentrated to about 270 mg ml$^{-1}$.

After purification, Hb is in the oxy form. The quantum yield of O$_2$-Hb is 50 times lower than that of CO-Hb so CO-Hb is better suited for use in photolysis experiments. To prepare CO-Hb we used the following procedure: 1 ml of stock solution was placed for one hour in a ten liter glove box with an atmosphere of 100% CO saturated with water vapor at room temperature. While exposed to CO, the sample was carefully and mildly stirred four times. During this step, HbS molecules lose oxygen and bind CO. After this the atmosphere in the glove box was changed to 100% He, saturated with water. The sample was again mildly stirred four times. The second step reduces the concentration of free CO in the solution, allowing for full photolysis of Hb at lower laser power in the subsequent experiments. After this procedure, the concentration of the stock solution was determined, the
solution was stored as stock under liquid nitrogen and used as needed.

For each experiment a solution sample of CO-HbS was prepared by mixing 20 µl of stock solution in 0.15 M potassium phosphate buffer at pH 7.35 with 1 µl of sodium dithionite in the same buffer to give final concentration of 0.055 M sodium dithionite. About 3 µl of this solution was placed on a standard 75 mm × 50 mm microscope glass slide (Corning), covered with a 22 mm × 22 mm cover glass, and sealed with Mount-Quick (Daido Sangyo, Japan). All of the above procedures were performed in a glove box under He atmosphere saturated with water. Tests showed that when handling volumes of several microliters, control of humidity during sample preparation is crucial. Thus, the rate of evaporation of a 20 µl droplet of the phosphate buffer used here was found to be 2.4 µl hour⁻¹ at relative humidity of 55% and was still 0.4 µl hour⁻¹ at 95% relative humidity.

After sealing, the spectrum of the solution in the slide in the range of 450–700 nm was determined to verify that it contains 100% CO-HbS and no met-HbS. All nucleation experiments were performed immediately after the sample preparation; samples older than 24 hours aged markedly, having higher nucleation rates and smaller spherulites sizes, consistent with the observations reported by Ferrone et al.²⁸

To determine the solution concentration, we used Drabkin’s reagent (Sigma). For concentrations of 260 mg ml⁻¹ the solution was diluted up to 500-fold. We measured the absorption at 540 nm using extinction coefficient ε = 0.6614 ml mg⁻¹ cm⁻¹. The procedure of concentration determination for HbS samples required particular attention because of the strong dependence of the nucleation rate on concentration. The smallest pipette volume used here was 1 µl with an accuracy of ± 1.5% (Eppendorf Research Series 2100 pipetter). Using 500 µl solution volumes with a 1 ml pipette with an accuracy of 0.5% and was independently experimentally verified), and using the accuracy of the photometric readout of 0.1%, we get 1.6% for the accuracy of the concentration determination. For a concentration of 260 mg ml⁻¹ it yields an error of ± 4.2 mg ml⁻¹.

**Experiment setup**

The experiment setup is built around a Leica DM R fluorescence microscope and is shown in Figure 8. Observations of HbS fiber nucleation are made in transmitted light with DIC optics, a 63x HCX APO L U-V-I water immersion objective and a 0.90 S1 achromatic condenser. Pictures are taken with VCC-151 color video camera (Hitachi), FlashPoint-3D frame grabber (Integral Technologies Inc.) and custom-made software. With the current configuration the shortest time between frames is 1 s.

Photolysis is achieved by a beam of a continuous-wave Nd:YAG laser (LCS-DTL-316 from Power Technology Inc., λ = 532 nm, I_{total} = 2–200 mW). Alternatively, we use a 100 W gas discharge Hg lamp (Leica). When laser is used, its beam is expanded by a spatial filter assembly consisting of two objective lenses (focus lengths F₁ = 16.5 mm and F₂ = 60 mm) and a 12 µm pinhole. The pinhole is positioned over the focus of the first lens. The position of the second lens is varied to control the beam expansion ratio and its divergence so that an illuminated spot of a desired size in the working plane of the microscope could be achieved. Besides expansion, this arrangement provides for filtering of laser beam non-uniformities.

The expanded and filtered beam is input through the microscope’s light path for fluorescence excitation illumination. The diameter of the photolyzed region typically is ~90 µm, adjusted by a variable aperture diaphragm. A beam splitter with a dichroic mirror and filter is used, respectively, to direct the photolyzing beam towards the solution sample through the specialized fluorescence objective and to reject the laser light

![Figure 8](image-url)
scattered in the direction of observation. Since the filter completely cuts off the photolysis wavelength from the viewing path, to measure and control the diameter of the photolyzing beam, we used a slide with Rhodamin 6G dye in ethanol. To achieve precise timing and to automate the data acquisition, a computer-controlled shutter (Melles Griot, opening time ~ few milliseconds) is used.

With slide thickness of 5 μm and diameter of the photolyzed region of ~90 μm, the total photolyzed volume is ~3 × 10⁻⁶ cm³. The shortest times between captured frames are 1 s, and since we can confidently resolve at most ten spherulites in the illuminated volume, these parameters determine the fastest nucleation rate that we can measure as 10⁷ cm⁻² s⁻¹. Faster nucleation rates can be measured by using a faster camera.

**Slide thickness uniformity**

The nucleation statistics is determined through independent runs in different locations on the slide containing HbS solution. This requires accurate determinations of the slide thickness and verification of the thickness uniformity throughout the slide. Furthermore, to prevent sample overheating, low optical density of the slide loaded with a relatively high concentration of HbS, ~200 mg ml⁻¹ and higher, is required. This can be achieved by using slides as thin as 5 μm or 10 μm. To determine the uniformity of the slide thickness, we measured the spatial distribution of slide optical density (OD) on spatial coordinates with a Beckmann DU-68 spectrophotometer (sample thickness h is proportional to the optical density because of Lambert–Beer law OD = εCh, where ε is extinction coefficient, C is sample concentration). For these measurements, the slide was placed in the focus of the spectrophotometer light beam with a spot size of 2 mm × 0.3 mm, and scanned in two perpendicular directions.

The determinations with slides without thickness control revealed that their thickness was extremely non-uniform (Figure 9(a)), with variations of as much as 50%. For thickness control, we used glass spheres (Duke Scientific) placed between the slide and the cover slip. These glass spheres are available in diameters of 2.0(±0.5) μm, 4.9(±0.5) μm, 10.0(±1.0) μm. Using these spheres as spacers, we achieve slide thickness uniformity of ±2% for 10 μm spheres and within ±5% for 5 μm spheres (Figure 9(b)). In most determinations, 5 μm thick slides were used that provided for lower overheating due to absorption of the photolysis light.

**Temperature of polymerization**

To control the temperature of the sample we use a 50 mm × 50 mm × 20 mm box assembled by gluing brass side walls to a glass slide. The top of this assembly is left open for microscope objective access. A RTE-100 waterbath (Neslab) circulates deionized water through the box over the slide and sets its temperature between 0 °C and 80 °C. The use of water immersion objectives provides for high quality DIC images in this arrangement. Temperature is measured with a HH 506R thermocouple thermometer (Omega Engineering Inc.) with 0.1 K accuracy and is input to a computer using a serial port. We checked the temperature inside the slide by placing a small thermocouple under the cover-slip; its temperature reading was equal to that in the circulating water.

Because our sample absorbs light at the wavelength of photolysis, the steady-state temperature rise in the photolyzed region is different from the temperature outside of the illuminated region. To measure this temperature we deposited in a slide a ~10 μm grain of OMEGASTICS crayon (Omega Engineering) that melts at 40.5 °C. To determine the temperature near the central axis of the beam we masked half of it and positioned the crayon grain near its center (Figure 11(a)). Then we varied the temperature of the water circulating over the slide and recorded the temperatures at which crayon melted with laser power on, as in Figure 11(b), and off. With the laser off, the crayon melted at the manufacturer-specified 40.5 °C. The overheating due to laser light increases roughly linearly with laser power, slide thickness and HbS concentration, and is about 4.5 deg. C with HbS concentration of 232 mg ml⁻¹, slide thickness of 15 μm and laser power of 10 mW. Our observations are consistent with those reported by Ferrone et al., thus, we rely on their conclusion that the typical times to reach steady temperature are of the order of several milliseconds, significantly shorter than our observation times of ~1 s.
The Gaussian profile of the laser beam (see below) gives rise to a non-uniform temperature field. To limit this non-uniformity, we expanded the beam and used a variable aperture diaphragm to cut out the beam periphery, where the intensity drops off. In the remaining central part of the beam, the intensity non-uniformity was < 35%. With the above determination of the overheating due to light absorption, the temperature non-uniformity at laser power 10 mW in a 5 μm slide, the most common arrangement used here, is < 0.5 °C.

**Photolysis illumination intensity and uniformity**

The total intensity of the photolysis illumination and its spatial distribution determine the extent of HbS conversion to deoxy-state, its spatial distribution, and the temperature regime in a sample. We measured the intensity profile by scanning either a 12 μm diaphragm placed at the sample plane or a 0.5 mm diaphragm placed near the expanding lens of the telescope. Figure 10(a) shows the intensity profile with laser illumination; it is adequately described by a Gaussian function. Tests showed that the beam width does not depend on the laser intensity and can be adjusted in a wide range from several μm to 200 μm. When the gas discharge lamp was used (Figure 10(b)), an essentially flat profile of intensity obtained.

We determined the attenuation of the light by the diaphragms and lenses in the optical pathway by placing the detector of the light power meter in front of the laser, and in the slide focal plane. We found that the attenuation ratio is 0.52.

To determine the laser power needed to achieve full photolysis of the CO-HbS, we carried out the following tests. We use the fact that CO-Hb has higher extinction coefficients at λ = 532 nm than deoxy-Hb. At low laser power all Hb is in CO form and the optical density is highest. When laser power is increased, more and more Hb molecules lose CO and the optical density drops until, at a certain intensity setting, it reaches the level of deoxy-Hb. To determine the degree of photolysis, we determined the optical density of a slide loaded with CO-HbA (HbA was used to avoid interference from fiber formation with the optical density measurements). We used the illuminating pathway of the microscope, in which a narrow band-pass interference filter (Edmund Industrial Optics, λ = 436 nm, halfwidth 10 nm) was mounted between the illumination lamp and the sample.

![Figure 10](image1.png)

**Figure 10.** Intensity profiles of the photolyzing beams in the plane of slide with HbS solution: (a) illumination with a laser beam, a 90 μm diaphragm around the center of the beam leaves intensity non-uniformity of < 35%; (b) lamp illumination, 90 μm diaphragm around the center of the beam leaves near-uniformly illuminated area.

![Figure 11](image2.png)

**Figure 11.** Measurement of overheating of the slide in steady state. (a) A ~10 μm grain of OMEGASTICK crayon with Tmelt = 40.5°C is placed near the center of half-blocked laser beam, not visible because of the filter that absorbs photolyzing light reflected in the direction of observation. (b) The grain is intact at 35°C, at 36.1°C a crescent-shaped molten area is detectable in the direction of the beam; at higher temperature the molten area increases. Upon reversal of temperature changes, molten crescent disappears at 36°C.
Figure 12. Dependence of transmittance T at 436 nm of a 230 mg ml⁻¹ solution of CO-HbA on the power density F of the photolyzing beam at 22 °C at different times ∆t after initiation of photolysis. Horizontal broken lines mark transmittance of CO-HbA (upper line) and deoxy-HbA (lower line) calculated from published spectra. Right ordinate axis is scaled as a gauge for the fraction of deoxy-HbA in the solution.

The intensity of the light transmitted through the sample was measured with 1830-C power meter (Newport) whose sensor was placed in the image plane of the microscope, instead of the video camera in Figure 8. To reject the reflected photolyzing laser beam, we used three short-pass filters (Melles Griot, λ = 500 nm) and a polarizer. A diaphragm set to an area of 16 μm diameter was placed in front of the power meter sensor, and adjusted so that only the light transmitted through the central part of the illuminating sample reaches the power meter. The laser power was varied in the range 5–200 mW and was additionally attenuated by calibrated filters with optical densities 1, 2 and 3. Two consecutive determinations, 1 and 2, were done with and without the solution slide in the optical pathway (Figure 11).

Figure 12 shows that power density of 0.3 kW cm⁻², corresponding to a laser power of about 10 mW the photolysis reaches ~100% in the whole illuminated area for less than 1 s. At power densities >0.8 kW cm⁻², the transmittance increases. We tentatively assign this effect to photochemical “bleaching” of the sample.

Thus, we chose laser power of 10 mW corresponding to power density of 0.3 kW cm⁻², at which the dissociation of the CO-HbS into deoxy-HbS is completed within a time period sufficiently short to only insignificantly bias the determinations of the induction times and nucleation rates.

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